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Identification par CGHarray de gènes candidats impliqués dans la tumorigénèse et le pronostic des tumeurs hypophysaires

Hélène Lasolle

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Identification par CGHarray de gènes candidats impliqués dans la tumorigénèse et le pronostic des tumeurs hypophysaires

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Résumé:

L'objectif de ce travail était d'analyser le lien entre variations du nombre de copies (CNV) et pronostic des tumeurs hypophysaires.

Nous avons réalisé une analyse par CGHarray de 195 tumeurs hypophysaires (56 gonadotropes, 11 immunonégatives, 56 somatotropes, 39 lactotropes et 33 corticotropes) dont le suivi 5 ans après la chirurgie était disponible.

La première partie de ce travail a consisté à mettre en place une méthode de centralisation basée sur l'hybridation in situ en fluorescence (FISH) suite à l'identification d'erreurs de centralisation. Les $\log_2(\text{ratios})$ bruts et les résultats de FISH différaient significativement pour 11 tumeurs.

Dans la seconde partie, le lien entre les altérations et la récurrence a été évalué à l'aide de modèles de régression logistique. La quantité d'altérations n'était pas associée au pronostic dans la cohorte complète, mais était associée au type tumoral : élevée dans les tumeurs lactotropes, elle était plus variable dans les tumeurs corticotropes et somatotropes, et très faible dans les tumeurs gonadotropes et immunonégatives. La quantité d'altérations était associée à plus de récurrences dans les tumeurs lactotropes, après ajustement sur l'invasion et la prolifération. Aucun CNV pronostique n'a été mis en évidence. Dans les tumeurs somatotropes et corticotropes, les mutations de *GNAS* et *USP8* n'étaient pas associées à la récurrence ni à la quantité de CNV.

En conclusion, l'instabilité génomique était associée au type tumoral, et à un pronostic défavorable dans les tumeurs lactotropes. Les mécanismes à l'origine de ces altérations ne sont pas entièrement compris et l'étude du microenvironnement pourrait être une piste de réflexion.

Mots-clés :

Tumeurs hypophysaires ; pronostic ; instabilité chromosomique ; aneuploïdie ; CGHarray

Title:

Genomic alterations in pituitary tumors: identification of candidate genes associated with tumorigenesis and prognosis

Abstract:

The purpose of this work was to analyze the impact of copy number variations (CNV) on sporadic pituitary tumor prognosis.

CGH array analysis was performed on 195 fresh-frozen pituitary tumors (56 gonadotroph, 11 immunonegative, 56 somatotroph, 39 lactotroph and 33 corticotroph), with 5 years post-surgery follow-up.

Firstly, we evaluated the reliability of aCGH centralization by comparing aCGH results with classical centralization vs. Fluorescent In Situ Hybridization results (FISH). We identified centralization errors, as FISH results and raw CGH $\log_2(\text{ratios})$ differed significantly in 11 tumors, and proposed a method able to correct this centralization bias.

Then, the effect of alterations on recurrence was studied using logistic regression models. The quantity of CNV was dependent on tumor type: higher in lactotroph compared to corticotroph and somatotroph, and lower in gonadotroph and immunonegative tumors. It was not predictive of recurrence in the whole cohort. In lactotroph tumors, genome instability, especially quantity of gains, significantly predicted recurrence independently of invasion and proliferation. However, no specific CNV was found as a prognostic marker. In somatotroph and corticotroph tumors, *USP8* and *GNAS* mutations were not associated with quantity of CNV or recurrence respectively.

CGH array analysis showed genome instability was dependent on pituitary tumor type. The quantity of altered genome was associated with poorer prognosis in lactotroph tumors whereas gonadotroph and immunonegative tumors showed a 'quiet' profile. The mechanism is unclear and analysis of microenvironment could be helpful.

Key words:

Pituitary tumors; prognosis; chromosomal instability; aneuploidy; CGHarray

Intitulé du laboratoire :

Inserm U1052, CNRS UMR5286, Lyon I University: Signaling, metabolism and tumor progression

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ABREVIATIONS

AD	Agonistes dopaminergiques
AHR	Aryl hydrocarbon receptor
AIP	AHR interacting protein
CABLES1	CDK5 and ABL1 enzyme substrate
CDH23	Cadherin-related 23
CDK4	Cyclin-dependent kinase 4
CDKN1A/p21	Cyclin-dependent kinase inhibitor 1
CGH	Comparative genomic hybridization
CNV	Copy number variations
DRD2	Dopamine receptor D2
EGFR	Epidermal growth factor receptor
ESR	Estrogen receptor 1
FIPA	Familial isolated pituitary adenoma
HEPN1	Hepatocellular carcinoma downregulated 1
HMGA	High mobility group AT-hook
IHC	Immunohistochimie
MDM2	Mouse double minute 2 homolog
PAM16	Resequene translocase-associated motor 16
PRKAR1A	Protein kinase A regulatory subunit-1-alpha
RB1	Retinoblastoma protein
REXO4	RNA exonuclease 4
RXRG	Retinoid X receptor gamma
SFRP4	Secreted frizzled-related protein 4
SMAD3(P)]	Mothers against decapentaplegic homolog 3
SSTR1	Somatostatin receptor 1
TBX19	T-box transcription factor
TH	Tyrosine hydroxylase
TIMP	Tissue inhibitor of metalloproteinases
VEGF	Vascular endothelial growth factor
XLAG	X-linked acrogigantism

1 INTRODUCTION

1.1 Aspects cliniques

1.1.1 Épidémiologie

Les tumeurs hypophysaires représentent le 3^{ème} type de tumeurs cérébrales chez l'adulte en termes de fréquence, après les méningiomes et les gliomes. La prévalence des formes symptomatiques est estimée environ à 1/1 000 individu (1–4).

Les découvertes fortuites sont fréquentes, autour de 20% (14% dans les études sur autopsie, 22% dans les études sur imagerie, variant selon la technique utilisée)(1).

Les tumeurs lactotropes sont les plus fréquentes, représentant 50% des tumeurs environ, avec une incidence annuelle de 2/100 000 personnes, suivies des tumeurs non fonctionnelles (35% environ avec une incidence annuelle de 1/100 000 personnes), des tumeurs somatotropes (10% environ 0.4/100 000 personnes), des tumeurs corticotropes (3% environ, 0.2/100 000 personnes) et thyrotropes (0.03/100 000 personnes)(3).

1.1.2 Symptomatologie

La symptomatologie est extrêmement variable selon les tumeurs et l'on distingue habituellement :

- le syndrome tumoral incluant les conséquences de la croissance tumorale locale, à savoir, les troubles visuels liés à une compression chiasmatique, les céphalées, les atteintes neurologiques à type de syndrome du sinus caverneux.
- l'insuffisance antéhypophysaire
- le syndrome sécrétoire concernant 2/3 des tumeurs hypophysaires, et résultant de la sécrétion hormonale des cellules tumorales. Il comprend, par ordre de fréquence, l'hyperprolactinémie entraînant hypogonadisme et galactorrhée, le syndrome de Cushing, l'acromégalie/ gigantisme, les rares thyrotoxicoses et exceptionnels syndromes d'hyperstimulation ovarienne.

1.1.3 Pronostic

Le pronostic des tumeurs hypophysaires, de croissance lente, est globalement excellent. Le taux de récurrence après exérèse chirurgicale est estimé à 30% à 5 ans (5–7). Le

contrôle est la plupart du temps obtenu grâce à d'autres thérapeutiques classiquement utilisées, telles que les agonistes dopaminergiques, les analogues de la somatostatine et la radiothérapie. Une étude sur des tumeurs non fonctionnelles a montré qu'en cas de progression après une chirurgie, le risque de seconde progression était de 35%, et parmi ces patients, le risque de 3^{ème} progression était de 26%, correspondant à un risque global de 3% environ (5). Le risque de seconde progression après une radiothérapie était réduit à 12.5%.

Certaines tumeurs vont cependant présenter une agressivité accrue et entrent dans le cas de tumeurs agressives qui seront détaillées ci-après. Leur épidémiologie est mal connue. L'identification de métastases à distance fait poser le diagnostic de carcinome hypophysaire, ce qui complique 0.2% des tumeurs hypophysaires (8).

1.2 Tumorigénèse hypophysaire

Les mécanismes de la tumorigénèse hypophysaire ne sont à l'heure actuelle encore pas complètement élucidés.

1.2.1 Embryogénèse hypophysaire

Les tumeurs hypophysaires sont développées aux dépens de l'antéhypophyse qui est dérivée de la poche de Rathke, issue du revêtement ectodermique du plafond du stomadéum (9). La différenciation des progéniteurs de la poche de Rathke en cellules endocrines dotées de capacités de sécrétion est guidée par différents facteurs de transcription, à l'origine de la classification histologique des tumeurs hypophysaires (10) :

- PIT1 encodé par *POU1F1* est indispensable à la différenciation des cellules somatotropes, thyrotropes et lactotropes, en association avec le récepteur aux œstrogènes (ER α) pour ces dernières, ainsi que GATA2 et TEF pour les cellules thyrotropes.
- TPIT encodé par *TBX19* est indispensable à la différenciation des cellules corticotropes.
- SF1 ou *NR5A1*, en association avec ER α et GATA2 induit la différenciation des cellules gonadotropes

Les tumeurs hypophysaires sont classées, d'après la dernière version de la classification de l'OMS (11,12), selon la lignée cellulaire dont sont issues les cellules tumorales (Table 1). Alors qu'en cas de sécrétion et expression hormonale à l'analyse immuno-histochimique la classification est évidente, les tumeurs immuno-négatives requièrent la recherche d'expression de facteurs de transcription spécifiques aux différentes lignées (13). Ainsi, les tumeurs immunonégatives concernent jusqu'à 20% des tumeurs hypophysaires (14) alors que les études récentes utilisant les facteurs de transcription ont réduit la proportion de tumeurs « null-cell » à quelques pourcents. La caractérisation génétique a montré que la grande majorité se rapprochait des tumeurs de lignée gonadotrope (15).

Table 1 : Classification histologique des tumeurs hypophysaires

Type histologique	IHC	Facteurs de transcription	Spécificités
PIT1			
Adénome somatotrope			
Richement granulaire*	GH	PIT1	Expression périnucléaire de LMWK*
Pauci-granulaire	GH	PIT1	« corps fibreux»
Adénome mixte GH-PRL			
	GH, PRL	PIT1, ER α	
	GH, PRL	PIT1, ER α	
Adénome lactotrope			
Richement granulaire	PRL	PIT1, ER α	Immunopositivité PRL cytoplasmique diffuse
Pauci-granulaire	PRL	PIT1, ER α	Immunopositivity PRL juxta-golgienne
Adénome thyrotrope			
	β -TSH, α -SU	PIT1, GATA2 \ddagger	
TPIT			
Adénome corticotrope			
Richement granulaire	ACTH	TPIT	Expression intense et diffuse de LMWKs
Pauci-granulaire	ACTH (faible)	TPIT, GATA3	Expression intense et diffuse de LMWKs
Adénome à cellules de Crooke			
	ACTH	TPIT	« Ring-like » expression de LMWK similaire aux cellules Crooke
SF1			
Adénome gonadotrope			
	β -FSH, β -LH, α -SU	SF1, ER α , GATA2 \ddagger	
Immunonégatives			
« Null cell »	Aucun	Aucun	
Adénomes plurihormonaux avec combinaisons inhabituelles (rare)	Multiple	Multiple	

*kératines de bas poids moléculaire

1.2.2 Clonalité

L'hypothèse d'une origine monoclonale, dans laquelle un évènement génétique ou épigénétique conférerait un avantage sélectif à une cellule qui serait à l'initiation du développement tumoral, est à l'heure actuelle privilégiée. Ces données sont principalement issues des études d'inactivation du chromosome X (16–18). Cependant, des études associant des analyses de perte d'hétérozygotie ont montré l'existence de plusieurs populations tumorales dans les formes agressives, en comparant les tumeurs initiales vs récurrences et métastases (19,20). Ces résultats discordants peuvent être la conséquence de variations d'échantillonnage mais ne peuvent pas faire exclure la possibilité d'une sélection de différentes populations au cours du développement tumoral.

Le degré de différenciation de la cellule initiatrice n'est pas connu et le rôle des cellules souches hypophysaires est depuis peu évoqué. Ainsi, la présence de cellules rassemblant des caractéristiques de cellules souches, notamment l'expression de SOX2, OCT4, CD133 et NESTIN, a été identifiée au niveau de tumeurs hypophysaires humaines (21–25). Une fois mises en culture dans des milieux spécifiques à la croissance des cellules souches, ces cellules présentaient une croissance sous forme de colonies sphériques et, dans certains cas, continuaient de croître sous la forme d'un développement tumoral après xénogreffe (21,22).

L'absence d'hyperplasie des cellules environnant la tumeur corrobore l'hypothèse de l'origine monoclonale.

1.2.3 Aspects moléculaires

1.2.3.1 *Les mutations germinales*

Les formes héréditaires représentent environ 5% des tumeurs hypophysaires et leur exploration a permis de découvrir plusieurs gènes prédisposant, dont certains sont responsables d'atteintes hypophysaires uniquement (Familial Isolated Pituitary Adenoma ou FIPA) alors que d'autres sont impliqués dans des manifestations syndromiques.

1.2.3.1.1 Non syndromiques

1.2.3.1.1.1 AIP

L'implication d'*AIP* dans les cas de FIPA a pour la première fois été décrite par Vierimaa *et al.* en 2006 (26), et concerne 3.6% de tumeurs hypophysaires non sélectionnées (27). De transmission autosomique dominante avec pénétrance incomplète (15 à 30%)(26,28,29), les manifestations cliniques sont dominées par la survenue de tumeurs hypophysaires somatotropes et lactotropes. Ces tumeurs sont de plus grande taille que les tumeurs sporadiques, plus souvent invasives, pauci-granulaires, résistantes aux analogues de la somatostatine de 1^{ère} génération et surviennent à un âge plus jeune (25 ans en moyenne)(28,30–32).

Le gène *AIP* est un gène suppresseur de tumeur localisé au niveau du chromosome 11q13. Comme les autres gènes suppresseurs de tumeurs, son inactivation homozygote est létale dans les modèles animaux (33,34). Il code pour la protéine co-chaperonne aryl hydrocarbon receptor (AHR) interacting protein (AIP). Le rôle d'*AIP* et de AHR dans la tumorigénèse hypophysaire n'est pas encore complètement compris. Il est démontré qu'une surexpression d'*AIP* a un effet anti-prolifératif (35) avec diminution de l'activité de la voie AMPc/ protéine kinase A (PKA)/ PDE4A (35,36), alors qu'une inactivation d'*AIP* entraîne une augmentation de la prolifération et de l'activité de PKA (37). *AIP* interagirait aussi avec le récepteur ryanodine, canal calcique du réticulum endoplasmique (38,39).

1.2.3.1.1.2 GPR101

De découverte récente, le syndrome XLAG (X-linked acro-gigantism) est lié à une micro-duplication de la région Xq26.3 contenant notamment le gène *GPR101* (40). La majorité des cas décrits sont liés à des mutations *de novo* survenues chez des femmes. En cas de transmission, une pénétrance complète est observée. XLAG est responsable de macro-adénomes somato-prolactiniques survenant à un âge jeune et responsables d'un gigantisme dans les premières années de vie (40–43).

Le gène *GPR101* code pour un récepteur couplé à une protéine G dont la surexpression entraîne une activation de la voie cAMP, mais la physiopathologie reste incomplètement comprise (31,40,44).

1.2.3.1.1.3 *CABLES1*

Des variants germinaux du gène *CABLES1* ont été identifiés chez 4/182 patients avec tumeurs corticotropes, et une diminution d'expression de *CABLES1* a été observée dans de nombreuses tumeurs corticotropes (45,46). Ce gène code pour une protéine impliquée dans la régulation du cycle cellulaire via l'inhibition de protéines cyclines dépendantes. Aucune transmission familiale n'a été identifiée à ce jour.

1.2.3.1.1.4 Autres

L'étude de 12 familles FIPA en a identifiées 4 présentant des variants germinaux du gène *CDH23* prédits comme pathogènes (47). L'étude de 125 patients avec tumeurs sporadiques a montré des variants germinaux chez 15 vs 2 des 260 contrôles.

L'étude d'une famille avec tumeurs lactotropes isolées a montré des variants potentiellement pathogènes des gènes *RXRG*, *REXO4* et *TH* (48). Ces gènes nécessitent des explorations complémentaires.

Une étude par GWAS (genome wide association study) grâce à une puce à SNP (single nucleotide polymorphism) a identifié 3 locus associés aux tumeurs hypophysaires sporadiques 10p12.31, 10q21.1 et 13q12.13, contenant les gènes *NEBL*, *PCDH15* et *CDK8* respectivement (49).

1.2.3.1.2 *Syndromiques :*

1.2.3.1.2.1 Néoplasie endocrinienne multiple de type 1 (NEM1)

La néoplasie endocrinienne multiple de type 1, de transmission autosomique dominante, est liée à une mutation du gène de la Ménine, gène suppresseur de tumeurs situé en 11q13. L'hyperparathyroïdie primaire en est la manifestation la plus fréquente. La NEM1 est aussi responsable de tumeurs neuroendocrines digestives, de lésions cutanées (lipomes, angiofibromes...), de tumeurs carcinoïdes bronchiques, de lésions thymiques, surrénaliennes, d'une augmentation de risque de cancer du sein (50,51) Les tumeurs hypophysaires sont observées dans 40% des cas environ. Il s'agit majoritairement de macro-adénomes lactotropes, suivis par les tumeurs non fonctionnelles et somatotropes. Elles surviennent à un âge plus jeune que les tumeurs sporadiques, sont plus volontiers invasives, mais la résistance aux traitements ne semble pas plus fréquente.

Les mutations hétérozygotes du gène de la ménine, très fréquemment associées à un mécanisme d'inactivation du second allèle au niveau de la tumeur (via perte d'hétérozygotie ou miRNA par exemple), entraînent une perte d'expression (52). La ménine est impliquée dans la régulation transcriptionnelle, la division cellulaire, la stabilité génomique, et la prolifération, notamment via la stimulation de l'expression d'inhibiteur de kinases cycline dépendantes p27^{Kip1} et p18^{ink4c} codés par les gènes *CDKN1B* et *CDKN2C* respectivement (53,54).

1.2.3.1.2.2 Néoplasie endocrinienne multiple de type 4

Plus récemment, des mutations germinales de protéines inhibitrices de kinases cycline dépendantes (*CDKN1B*, *CDKN1A*, *CDKN2B*, *CDKN2C*) impliquées dans la régulation du cycle cellulaire ont été identifiées dans des syndromes NEM1-like (55).

1.2.3.1.2.3 *PRKAR1A*

Le complexe de Carney associe la présence de lésions cutanées pigmentées, d'une hyperplasie bilatérale micronodulaire pigmentée des surrénales, de myxomes cutanés, cardiaques et d'une hyperactivité endocrine. Des tumeurs somatotropes ou lactotropes surviennent chez 10% des patients environ, et sont souvent associées à une hyperplasie (56). Il s'agit d'un syndrome de transmission autosomique dominante, lié dans 70% des cas à une mutation inactivatrice de *PRKAR1A*, sous-unité régulatrice de la protéine Kinase A situé en 17q24.2, entraînant alors une activation de la voie AMPc. Une mutation gain de fonction de *PRKACB* a été décrite chez un patient qui présentait notamment une acromégalie (57), alors que des mutations de *PRKACA* avaient été décrites chez des patients avec hyperplasie micronodulaire des surrénales (58). Par ailleurs, le locus 2p16 est associé à certains complexes de Carney sans mutation de *PRKAR1A* (59).

1.2.3.1.2.4 *GNAS*

Le syndrome de McCune Albright est lié à une mutation gain de fonction de *GNAS* survenue en post-zygotique et présente en mosaïque. La symptomatologie dépend du degré de mosaïcisme et associe taches café au lait, puberté précoce et dysplasie fibreuse. Dans 10 à 25% des cas, une sécrétion pathologique de GH est observée entraînant un

gigantisme ou une acromégalie (60). Une hyperplasie hypophysaire ou un développement tumoral peuvent s'observer.

1.2.3.1.2.5 *DICER1*

Les mutations germinales de *DICER1* sont responsables d'un syndrome de prédisposition tumoral, avec survenue de blastomes pleuropulmonaires, néphromes, tumeurs à cellules de Sertoli-Leydig et blastomes hypophysaires (61). *DICER1* encoderait une endoribonucléase impliquée dans la maturation des miRNA(62).

1.2.3.1.2.6 *SDH, MAX*

La survenue de tumeurs hypophysaires chez des patients présentant des paragangliomes et phéochromocytomes est de plus en plus décrite. A ce jour, environ 80 cas sont rapportés dans la littérature, parmi lesquels on retrouve majoritairement des tumeurs lactotropes, suivies de tumeurs non fonctionnelles et somatotropes (63). Ces associations sont fréquemment retrouvées chez des patients présentant une mutation germinale des gènes *SDHx* codant pour des sous-unités de la succinate déshydrogénase, dont le défaut d'action entraîne une stimulation de la voie de l'hypoxie via hypoxia-inducible factor-1 α (HIF-1 α)(64). Cliniquement, ces tumeurs sont majoritairement des macroadénomes, volontiers invasifs. Une mutation de *SDHB* a été décrite chez un patient avec un carcinome hypophysaire (65). Histologiquement, on peut noter des vacuoles intracytoplasmiques. L'association avec des mutations du gène *MAX* a aussi été décrite chez quelques patients (66).

1.2.3.2 *Les mutations somatiques*

L'étude des tumeurs sporadiques, notamment en séquençage, a montré un faible taux global de mutations ponctuelles et de rares mutations récurrentes. Par ailleurs, des mutations des gènes identifiés au niveau germinale (cités précédemment) sont exceptionnellement retrouvées en analyse somatique.

1.2.3.2.1 *GNAS*

Des mutations gain de fonction du gène *GNAS* sont observées dans 40 à 60% des tumeurs somatotropes (15,67–69). Le gène *GNAS* code pour la sous-unité α de la protéine G

stimulatrice dont l'activation entraîne une augmentation de l'activité adénylate cyclase et de la voie AMPc-PKA (70). Les analyses moléculaires montrent que les tumeurs mutées *GNAS* semblent présenter moins de variations du nombre de copies (67,71,72) et avoir un profil hypométhylé (15). L'expression de D2R (dopamine receptor 2) a été rapportée augmentée dans 1 étude pouvant faire supposer une meilleure réponse aux agonistes dopaminergiques (15). Les tumeurs somatotropes mutées *GNAS* auraient un phénotype plus favorable avec des tumeurs de plus petite taille, moins souvent invasives (73–77). La présence d'une meilleure réponse aux analogues de la somatostatine de 1^{ère} génération est par contre débattue(76,78).

1.2.3.2.2 *USP8, USP48, BRAF, NR3C1*

Le séquençage de tumeurs corticotropes a montré des mutations ponctuelles gain-de-fonction du gène *USP8* dans 20 à 60% des cas (15,68,69,79–81). *USP8* code pour une désubiquitinase inhibant la dégradation lysosomale d'EGFR. *USP8* mutée entraîne une diminution de la dégradation d'EGFR, avec un effet pro-prolifératif, une augmentation de la synthèse de POMC et d'ACTH. Les tumeurs mutées *USP8* semblent plus fréquentes chez les femmes, de plus petite taille, avec des taux d'ACTH supérieurs (81–83).

Beaucoup plus rarement, des mutations des gène *USP48* (84,85), autre désubiquitinase ont été décrites. De manière plus anecdotique, des mutations de *BRAF* (84,85) et *NR3C1*(84,86) sont rapportées dans des tumeurs corticotropes, ce dernier codant pour le récepteur aux glucocorticoïdes dont l'inactivation entraîne une perte du rétrocontrôle du cortisol sur la sécrétion d'ACTH.

1.2.3.2.3 *PIK3CA*

L'activation du gène *PI3KCA* entraîne une augmentation d'activité de la voie PI3K/AKT. Des mutations pathogènes du gène *PI3KCA* ont été décrites dans une étude chez 2.3% des tumeurs de tous type, et des amplifications de plus de 4 copies chez 30% environ, s'associant à l'invasion (87,88).

1.2.3.2.4 Autres

D'autres gènes ont été plus rarement rapportés comme *IDH1*(89,90), *KIF5A*(91), *TP53*(84), *HMGGA2*(92–94).

1.2.3.3 Epigénétique : régulation transcriptionnelle et post-transcriptionnelle

L'implication des mécanismes de régulation d'expression génique dans la tumorigénèse hypophysaire est de plus en plus étudiée.

Ainsi, l'étude des profils de méthylation a montré de nettes différences en fonction du type histologique. Les tumeurs gonadotropes et non fonctionnelles apparaissent comme hyperméthylées alors que les tumeurs de la lignée PIT1 apparaissent hypométhylées (15,68,95,96). La méthylation bloque l'accès de la machine transcriptionnelle aux gènes, ainsi la méthylation de gènes suppresseurs de tumeurs comme *CDKN2A*(97–99), *RB1*(100,101), *GADD45γ*, *CASP-8*, *PTAG* ou *FGFR2*(102) est observée et pourrait être impliquée dans la tumorigénèse. Le rôle des DNA-méthyltransférases *DNMT3A*, *DNMT3B* est suspecté (103,104).

Des modifications d'acétylation, méthylation, citrullination des histones ont été évoquées parallèlement(105–107)

Le rôle des ARN non codants dans la régulation post-transcriptionnelle de l'expression génique est maintenant connu. On citera notamment les micro-RNAs (miRNA) correspondant à de petits ARN non codants qui agissent via la liaison aux ARNm des gènes cibles, entraînant leur inactivation (108). Les long non-coding RNA (lncRNA) correspondent à de petits ARN de plus de 200 pb qui ne sont pas traduits en protéines, et peuvent aussi modifier l'expression de gènes, et sont de plus impliqués dans certains processus cellulaires.

Une modification d'expression de nombreux miRNA dans les tumeurs hypophysaires a été décrite (109–111). Selon le gène cible, les miRNA peuvent avoir un rôle prooncogène ou suppresseur de tumeur. La dérégulation de miRNA ciblant des gènes impliqués dans la tumorigénèse hypophysaire, comme *AIP*, a été décrite. L'étude du miRNome par Neou *et al.* a montré 4 profils d'expression de miRNA associés au type tumoral et à la

sécrétion(15). Dans une revue récente, 80 miRNA étaient dérégulés dans les tumeurs hypophysaires et seuls 2 (miR196a-2 and miR-212) étaient communs à différents types tumoraux. Parmi les lncRNA, l'augmentation d'expression de CCAT2 (112), AFAP1-AS1 (113,114), ISNG-AS1 (115) et la diminution d'expression de H19 (116,117) ont été observés dans les tumeurs hypophysaires. Certains lncRNA sont spécifiques aux types tumoraux, ainsi l'expression de RPSAP52 est augmentée dans les tumeurs gonadotropes et lactotropes où elle entrainerait une augmentation de l'expression de HMGA2 (118). L'expression de MEG3 est diminuée dans les tumeurs non fonctionnelles (119–122), alors que celle de HOTAIR y est augmentée (121).

1.2.3.4 Les altérations chromosomiques

Plusieurs études se sont intéressées aux altérations chromosomiques dans les tumeurs hypophysaires, initialement par analyse par hybridation génomique comparative (CGH) sur chromosomes en métaphase (123–127), puis sur puce (CGH array) (71) et enfin en séquençage NGS (15,68,69,72,128–130). Les variations du nombre de copie (CNV) sont fréquentes dans les tumeurs hypophysaires, avec une grande hétérogénéité inter-tumorale. Certaines tumeurs présentent très peu d'altérations alors que d'autres sont très altérées. Des génomes complètement aneuploïdes et des phénomènes de chromothripsis sont par ailleurs observés (71). Deux groupes de tumeurs se distinguent selon la quantité d'altérations, qui semblent s'associer au caractère fonctionnel dans la plupart des études (15,123–125,128). Au sein des tumeurs somatotropes, il semblerait que les tumeurs avec mutation de *GNAS* soient peu altérées comparativement aux tumeurs non mutées (71,72,130). Les tumeurs avec mutation de *GNAS* mises à part, Neou *et al* ont rapporté une corrélation négative entre méthylation, variations du nombre de copies et surexpression d'éléments transposables dans les tumeurs de lignée POU1F1/PIT1, pouvant laisser supposer que l'hypométhylation induit une instabilité chromosomique via l'activation d'éléments transposables (15). Aucun CNV ne semble significativement récurrent au travers des différentes études.

1.2.3.5 Microenvironnement

Le microenvironnement éveille de plus en plus d'intérêts en cancérologie. On définit par microenvironnement toutes les cellules non tumorales qui composent la tumeur, soit les

cellules immunitaires, les cellules endothéliales, les fibroblastes ainsi que les molécules et cytokines sécrétées.

L'infiltrat immunitaire des tumeurs hypophysaires a été étudié dans quelques études basées sur de l'immunohistochimie et cytométrie de flux. Elles rapportent un infiltrat lymphocytaire et macrophagique, dont la constitution et la polarisation pourraient s'associer à des caractéristiques comme l'invasion, le type tumoral et la sécrétion (131–137).

1.3 Mécanismes et prédiction de l'agressivité

1.3.1 Définition de l'agressivité

En 2018, l'European Society of Endocrinology a publié des recommandations concernant la prise en charge des tumeurs hypophysaires agressives et carcinomes hypophysaires (8). L'agressivité y est définie par la croissance inhabituellement rapide d'un adénome invasif, et/ou une croissance avec retentissement clinique, malgré une prise en charge optimisée basée sur les traitements conventionnels. En pratique, il s'agit habituellement de tumeurs de grande taille, invasives, présentant une croissance tumorale rapide malgré un traitement multimodal combinant chirurgie, radiothérapie et traitements médicaux conventionnels (agonistes dopaminergiques et analogues de la somatostatine). Cette définition manque de critères précis, notamment concernant la définition des traitements conventionnels et de la croissance. Une croissance tumorale rapide pourrait être définie par une augmentation de taille de plus de 20% dans les 6 mois en utilisant les critères RECIST (138–140). L'épidémiologie de ces formes est difficilement évaluée à l'heure actuelle. En l'absence de marqueurs permettant de prédire précocement l'évolution des tumeurs, il faut attendre plusieurs années après le diagnostic avant de définir le caractère agressif. Dans une enquête rétrospective européenne portant sur 125 tumeurs agressives traitées par chimiothérapie vs 40 carcinomes (141), 45% des tumeurs agressives étaient d'origine corticotrope, 20% d'origine lactotrope et 17% immuno-négatives. 58% de ces tumeurs étaient fonctionnelles.

1.3.2 Nomenclature

Pour souligner ce potentiel évolutif, l'International Pituitary Pathology Group a proposé en 2017 une modification du terme « adénome hypophysaire » pour « tumeur neuroendocrine hypophysaire ou pituitary neuroendocrine tumor » (PitNET) (142). En effet, le terme adénome renvoie habituellement à des tumeurs bénignes et ne paraît pas adapté aux tumeurs hypophysaires agressives. Ainsi, le terme PitNET, traduirait de manière plus pertinente le spectre des différentes tumeurs hypophysaires. Cependant, cette nouvelle nomenclature fait actuellement débat (143). Il est notamment reproché au terme « tumeur » d'introduire une connotation trop négative, alors que l'évolution des tumeurs hypophysaires est dans la grande majorité des cas favorable. De plus, les tumeurs anté-hypophysaires n'étaient jusqu'alors pas incluses dans les tumeurs neuroendocrines, mais endocrines, ne complétant pas les caractéristiques de cellules nerveuses.

1.3.3 Le carcinome hypophysaire

En l'absence d'existence de critère histologique de malignité, les carcinomes sont définis par la présence de métastases à distance apparaissant au cours du suivi (144). Exceptionnels, ils concernent 0.1 à 0.4% des tumeurs hypophysaires (14). Le diagnostic de carcinome n'est jamais contemporain de celui de la tumeur hypophysaire et le délai moyen avant l'apparition de lésions à distance se situe entre 5 et 9 ans dans les études rétrospectives dont nous disposons (145,146). Les localisations cérébrales et méningées concernent plus de la moitié des carcinomes, suivies par les localisations hépatiques et osseuses. Dans l'enquête rétrospective européenne, 47.5% des carcinomes présentaient une différenciation corticotrope, 37.5% lactotrope et 7.5% étaient immuno-négatifs (141). La redifférenciation de tumeurs silencieuses vers un phénotype sécrétant au moment de l'apparition des métastases est décrite.

La question de savoir si carcinomes et tumeurs agressives font partie de la même entité se pose, le carcinome étant l'évolution de la tumeur localement agressive, ou si les deux entités associent une physiopathologie distincte. Les seules caractéristiques qui diffèrent entre tumeurs agressives et carcinomes sont le taux de mortalité et le compte mitotique (141).

1.3.4 Prédiction de l'agressivité à l'heure actuelle

1.3.4.1 Invasion

Les tumeurs hypophysaires peuvent envahir les structures adjacentes, notamment les sinus caverneux et le sinus sphénoïdal, et sont alors définies comme invasives.

L'invasion peut être évaluée par l'imagerie, en peropératoire ou plus rarement, à l'examen anatomo-pathologique. La prévalence des tumeurs invasives dépend de la définition et de la technique d'évaluation utilisée mais se situe aux alentours de 40% (30 à 65%) (147–149)

Les classifications basées sur l'IRM en coupes coronales, de Knosp en 5 catégories (grade 0 à grade 4) (150), et Knosp modifiée (séparant le grade 3 en grade 3A et 3B)(147), sont largement utilisées afin d'évaluer la probabilité d'un envahissement parasellaire en peropératoire. Ainsi, les grades 1, 2, 3a, 3b et 4 sont associés à une invasion peropératoire en chirurgie endoscopique dans 1.5 à 8.3%, 9.9 à 23.5%, 26.5 à 61.5%, 70.6 à 78.6% et 100% des cas respectivement (147,148). A noter que ces prévalences diffèrent légèrement en cas de chirurgie microscopique et que ces données ne prennent pas en compte l'invasion sphénoïdale.

L'invasion est associée à un taux de résection incomplète supérieur et à un taux de récurrence accru (149). Ainsi le taux de résection complète décroît selon le grade de la classification de Knosp avec un taux de résection complète de 30% environ dans les grades 3B et proche de 0% dans les grades 4 (147,148,151). Dans une étude rétrospective lyonnaise portant sur 410 patients suivis dans 9 centres pendant 8 ans, 80% des tumeurs invasives avaient montré une progression ou récurrence, vs 32% des tumeurs non invasives (149). Dans l'enquête rétrospective européenne portant sur les tumeurs agressives, 109/125 tumeurs agressives montraient une invasion (141).

Donc l'invasion évaluée par l'IRM et l'examen peropératoire est un facteur de risque de récurrence et d'agressivité mais n'est pas une condition suffisante pour définir l'agressivité d'une tumeur hypophysaire. De plus, l'évaluation de l'invasion par IRM et par utilisation de la classification de Knosp présente des valeurs prédictives positives et négatives estimées à 88 et 86% respectivement (148) et expose donc à des faux positifs et faux négatifs. Certains auteurs évoquent un impact différent de l'invasion selon les structures concernées (sinus caverneux vs sinus sphénoïdal), mais cela reste à démontrer.

1.3.4.2 Histologie

La classification de l'OMS avait introduit en 2004 le terme d'adénome atypique, défini par la présence de caractéristiques atypiques témoignant d'un risque d'agressivité accru, soit un index mitotique élevé, et un ki67 > 3% ou un immunomarquage positif pour p53. Cependant, en l'absence de plus de standardisation, ces critères ne permettent pas une reproductibilité suffisante et la proportion de tumeurs atypiques varie de 3 à 18% des tumeurs hypophysaires en fonction des études(152). De plus, en l'absence d'étude démontrant l'impact pronostique, ce terme a été supprimé de la nouvelle classification de 2017. Celle-ci définit des classes histologiques à haut risque de récurrence : les tumeurs corticotropes silencieuses, corticotropes à cellules de Crooke, somatotropes sparsely granulated, plurihormonales exprimant PIT1 et lactotropes de l'homme. Les tumeurs somatotropes sparsely granulated sont volontiers de plus grande taille, dans certaines études plus invasives et surviennent à un âge plus jeune (153). Elles expriment moins souvent et de manière moins intense les récepteurs à la somatostatine de type 2, expliquant une moins bonne sensibilité aux analogues de la somatostatine de première génération (154). Cependant, l'impact pronostique de ces tumeurs somatotropes sparsely granulated, de même que les corticotropes silencieuses fait encore débat (155,156). Par ailleurs, les tumeurs immunonégatives semblent avoir un pronostic moins favorable que les tumeurs gonadotropes, même si cela n'est pas rapporté dans la dernière classification de l'OMS (157,158).

1.3.4.3 Classification française en 5 grades

L'équipe du Pr. Trouillas a proposé à Lyon une classification pronostique basée sur une combinaison de critères histologiques, radiologiques et cliniques. Cette classification a d'abord été évaluée sur une cohorte de 410 patients opérés et suivis au moins 8 ans (149). Elle est basée sur l'évaluation de l'invasion des sinus caverneux ou du sinus sphénoïdal à l'IRM selon la classification de Knosp, en peropératoire, ou plus rarement, à l'analyse histologique. S'y associent les critères histologiques de prolifération : un indice mitotique supérieur à 2 mitoses pour 10 champs à fort grossissement, un ki67 supérieur ou égal à 3% et une expression de P53 avec 10 noyaux positifs pour 10 champs à fort grossissement.

La présence de 2 critères de prolifération définit la tumeur comme proliférative. Ainsi, la classification différencie les tumeurs de grade 1a (sans critère d'invasion ni de prolifération), 1b (sans critère d'invasion mais prolifératives), 2a (invasives non prolifératives), 2b (invasives et prolifératives). Les tumeurs de grade 3 représentent les carcinomes. Dans l'étude princeps, le risque de persistance était multiplié environ par 3, 8 et 25 pour les grade 1b, 2a, 2b respectivement, comparativement au grade 1a (149). Cette classification a été validée sur une cohorte indépendante prospective de 213 patients avec un suivi de 3.5+/-1.9 ans (7). Le grade 2b était associé la récurrence/ progression de manière significative avec un odds-ratio de 3.7 comparativement au grade 1a. De plus, trois études rétrospectives belges et italiennes ont validé le caractère pronostique de la classification (159–161).

1.3.5 Aspects moléculaires de l'agressivité

De nombreuses études tentent d'explorer les origines moléculaires du comportement des tumeurs hypophysaires. En effet, une meilleure compréhension pourrait permettre l'identification de facteurs pronostiques, ainsi que la mise en évidence de cibles pour le développement de nouvelles thérapeutiques. Cependant, plusieurs facteurs rendent difficile la réalisation d'analyses robustes, parmi lesquels l'absence de chirurgie systématique des formes non agressives entraînant une impossibilité de réaliser des analyses moléculaires somatiques, la croissance lente de ces tumeurs nécessitant un suivi de plusieurs années pour évaluer le caractère agressif, l'absence de définition précise et standardisée de l'agressivité, la rareté des carcinomes. La plupart des études sont basées sur des cohortes rétrospectives de tumeurs hypophysaires opérées, dont le suivi est souvent limité et hétérogène. Ainsi, le critère majoritairement analysé est l'invasion qui, comme expliqué précédemment, n'est pas suffisant pour identifier une tumeur comme agressive ou récidivante, et dépend de la définition utilisée. Lorsque la récurrence est utilisée, la définition et le délai ne sont souvent pas explicités, et pas homogénéisés.

Table 2 : Liste des gènes ayant été associés à la récurrence ou la progression dans la littérature (1/4)

Classe	Gène	Technique	Association avec récurrence/ progression	Type (N tumeurs)	Carcinomes (N tumeurs)	Références
Régulation du cycle cellulaire	<i>CCND1</i> (cycline D1)	IHC	Augmentation	Plusieurs types (74) Silencieuses (127) Fonctionnelles (174)		(162–164)
	pRB	IHC	Augmentation	Silencieuses (127) Fonctionnelles (174)	LOH locus Rb (7)	(163–165)
	<i>CDKN1B</i> (p27)	IHC Western-blot Méthylation RT-qPCR	Diminution	Lactotropes (48) Somatotropes (46)	Diminution (5)	(166–168)
	<i>CDK2</i>	IHC,	Diminution	Non fonctionnelles (103)		(169)(166)
	<i>CDKN1A</i> (P21)	IHC, Western blot Méthylation RT-qPCR	Diminution (analyse multivariée)	Lactotropes (48) Non fonctionnelles (295)	Non détecté (4 GH)	(168,170,171)
	<i>CDKN2A</i> (p16)	IHC méthylation	Diminution	Silencieuse (127) Fonctionnelles (174)		(163,164)
Inhibiteurs WNT	<i>WIF1</i>	IHC RT-qPCR méthylation	Diminution (analyse multivariée)	Non fonctionnelles (295) Non fonctionnelles (165, même centre)		(171,172)
	<i>SFRP4</i>	IHC RT-qPCR	Diminution	Non fonctionnelles (165)		(172)
Facteurs de croissance	<i>EGF/EGFR</i>	IHC Western blot RT-qPCR	Augmentation	Corticotropes (52)	Augmentation (7/9)	(173–175)
	<i>VEGF</i>	RT-qPCR	Augmentation	Plusieurs types (46)	Augmentation (6/6)	(176,177)
	<i>TGF</i>	IHC	Diminution (analyse multivariée)	Non fonctionnelles (295)		(171)

Table 2 : Liste des gènes ayant été associés à la récurrence ou la progression dans la littérature (2/4)

Classe	Gène	Technique	Association avec récurrence/ progression	Type (N tumeurs)	Carcinomes (N tumeurs)	Références
EMT et adhésion	<i>LGALS3</i> (Galectin 3)	IHC RT-qPCR	Augmentation	Lactotropes (59) Lactotropes (220) Corticotropes (33)		(178,179)
	<i>SPAG9, SKIL, MTDH, HOOK1, CNOT6L, PRKACB</i>	Séquençage RNA	Association vitesse de croissance	Gonadotropes (8)		(180)
	<i>CHL1</i>	RNA Microarray	Association	Immuno-négatives (11)		(181)
Autres		Méta analyse	Augmentation	Tous types		(182)
	<i>PTTG</i>	IHC RT-qPCR	Augmentation	Tous types (74) Tous types (45) Lactotropes (44) Non fonctionnelles (35)		(162,183–185)
	<i>ERα</i>	IHC	Diminution	Lactotropes (89)		(186)
	<i>SF3B1</i>	PCR digitale	Mutation R625H	Lactotropes (227)		(187)
	<i>TERT</i>	Méthylation MS-HRM	Méthylation et Augmentation	Tous type, majoritairement non fonctionnelles (70)		(188)
	<i>RAS</i>	mutations			Mutations (4/12)	(189–191)
	<i>RIZ1 (PRDM2)</i>	WES RT-qPCR Methylation IHC	Diminution Résistance aux AD (12)	Lactotropes (12) NFPA et lactotropes (12 WS, 28 RT PCR) Lactotropes, somatotropes et NFPA (103)		(107,192,193)
	<i>TP53</i>	Séquençage			Mutations (2/6)	(194)
	<i>CHST12</i>	RNA microarray	Augmentation	Non fonctionnelles (66)		(195)
	Mitochondrial DNA variant	Séquençage AND mitochondrial	Variant T16189C dans tumeurs non récurrentes	Somatotropes, gonadotropes, immunonegatives (44)		(196)

Table 2 : Liste des gènes ayant été associés à la récurrence ou la progression dans la littérature (3/4)

Classe	Gène	Technique	Association avec récurrence/ progression	Type (N tumeurs)	Carcinomes (N tumeurs)	Références
Analyse pangénomique	PTTG, ADAMTS6, CRMP1, ASK, AURKB, CENPE, CCNB1	RT-qPCR RNA microarray	Augmentation	Lactotropes (25 + 44)		(183,197)
	DGKZ, CD44, TSG101, GTF2H1, HTATIP2	SNP array RNA microarray RTqPCR	Diminution	Lactotropes (13)		(198)
	CHL1, P2RY12, ERAP2, LPAR5, CHAD	RT-qPCR	Association	Immunonégatives (11)		(181)
	PCDH18, UNC5D, EMCN, MYO1B, GPM6A	Séquençage RNA	Association avec la vitesse de croissance	Gonadotropes (8)		(180)
	Reactome pathway chemokine receptors bind chemokines : IL8, CXCR1 and CXCR2	Séquençage RNA	Association avec la récurrence	Non fonctionnelles, somatotropes et corticotropes (30)		(68)
	FAM90A1 ING2	RNA microarray méthylation	Diminution Augmentation (multivariée)	Non fonctionnelles (71)		(199)

Table 2 : Liste des gènes ayant été associés à la récurrence ou la progression dans la littérature (4/4)

Classe	Gène	Technique	Association avec récurrence/ progression	Type (N tumeurs)	Carcinomes (N tumeurs)	Références
miRNA	miR122 miR493	miRNA microarray			Augmentation d'expression (2)	(200)
	miR508-5p miR130a-3p, miR146a- 5p	RT-qPCR	Diminution Augmentation	Plusieurs types (23)		(201)
	miR-183 miR-148a miR-375 miR-342-3p miR-23b miR-744 miR-98 miR-340 miR-574-3p miR-331-3p miR-1280	miRNA microarray	Diminution	Lactotropes (26)		(202)
lncRNA	COA6-AS1 RP11-23N2.4	RNA microarray	Augmentation	Non fonctionnelles (66)		(195)

1.3.5.1 La régulation du cycle cellulaire

L'implication de gènes régulant le cycle cellulaire a été décrite. La transition de la phase G1 à la phase S impliquant l'inactivation de Rb1 par phosphorylation grâce à des cyclines kinase dépendantes (CDK) est particulièrement étudiée. Rb1 phosphorylée est inactivée et entraîne une activation du cycle cellulaire. Deux familles de protéines, INK4 (p15^{Ink4b}, p16^{Ink4a}, p18^{Ink4c} et p19^{Ink4d}) et CIP/KIP (p21^{Cip1}, p27^{Kip1} and p57^{Kip2}), ont pour rôle l'inhibition des cyclines kinases dépendantes.

Des études de déséquilibre allélique ont montré une association entre le gène *CCND1* et l'invasion radiologique (203,204). *CCND1* code pour la cycline D1 qui active CDK4 qui phosphoryle la protéine Rb1. Certaines études en immunohistochimie ont montré une surexpression de cycline D1 dans les tumeurs hypophysaires comparativement à l'hypophyse saine, avec une association entre l'intensité de l'expression et la récurrence, l'invasion et le Ki67 (162–164,205,206).

Des pertes d'hétérozygoties ont été décrites au niveau du locus du gène *RB1* dans les tumeurs invasives, sans être associées à des modifications d'expression de pRb en IHC, suggérant la possible existence d'un gène suppresseur de tumeur au même locus (165,207). Inversement, une augmentation de l'expression de pRb était associée à plus de récurrence après chirurgie dans les tumeurs silencieuses (163) et fonctionnelles (164).

Parallèlement, une diminution de l'expression en IHC de la protéine inhibitrice de cyclines kinase dépendante p27^{Kip1}, encodée par *CDKN1B*, a été retrouvée dans les carcinomes et tumeurs corticotropes comparativement aux autres tumeurs hypophysaires (167), de même que dans les tumeurs lactotropes invasives comparativement aux non invasives (168). Seulement l'absence de différence significative dans d'autres études (208), notamment en analyse transcriptomique (209), et l'absence de seuil dans ces études ne permettent pas d'utiliser l'étude de la Cycline D1 et de p27^{Kip1} par IHC comme facteur prédictif.

Dans une étude récente sur des tumeurs somatotropes, l'invasion s'associait à une augmentation de l'expression en IHC et RT-PCR de CDK2, cycline E et une diminution de celle de p21^{Cip1} (encodée par *CDKN1A*) et p27^{Kip1}. De plus, des taux diminués de p27^{Kip1} s'associaient à une survie sans progression diminuée (166). Une absence de détection de p21^{Cip1} a déjà été mise en évidence dans des carcinomes somatotropes alors qu'elle était

détectée dans les adénomes somatotropes (170). Il est noté qu'il n'a pas été décrit de mutation ponctuelle récurrente dans ces gènes en analyse somatique. Le rôle de la méthylation des promoteurs de *CDKN1A* et *CDKN1B* dans la diminution d'expression est débattu (100,168).

Une diminution de l'expression de p16^{Ink4a} a été associée à une plus faible survie sans progression à 24 mois dans les tumeurs silencieuses (163) et fonctionnelles (164). Une hyperméthylation du gène *CDKN2A* semble être le mécanisme, cependant, le lien entre méthylation et comportement des tumeurs n'est pas retrouvé dans toutes les études (97,101,210,211).

Une étude rétrospective intéressante a analysé les facteurs pronostiques de la récurrence de 295 tumeurs hypophysaires non fonctionnelles avec un suivi de 3 ans post opératoire. L'expression en IHC de PAM16, TGF- β , WIF1, SMAD3(P), DRD2, CDK4, CDKN2A/p16, SFRP4, TBX19, SSTR1, HMGA1, RB1, E2F3, CDKN1A/p21, ESR1, MDM2, l'âge, le sexe, le volume tumoral (macroadénome ou tumeur géante), l'invasion selon la classification de Knosp, la qualité de la résection étaient évalués. En analyse multivariée, une expression faible de CDKN1A, WIF1 et TGF- β , un âge jeune et le volume tumoral important étaient associés à plus de récurrences (171). A noter que la résection incomplète n'était pas associée à la récurrence de manière indépendante. Une étude précédente du même centre sur 165 tumeurs non fonctionnelles avait retrouvé une association faible expression de WIF1 et SFRP4 (IHC et RT-PCR), deux inhibiteurs des protéines WNT, et invasion et survie sans récurrence, et (172). La méthylation du promoteur de WIF1 était significativement plus importante dans les tumeurs invasives.

Par ailleurs, l'activité apoptotique, évaluée via l'activité apoptotique et l'expression de BCL2, facteur anti-apoptotique, semble être augmentée dans les tumeurs agressives et carcinomes (212), mais ce résultat n'est pas retrouvé dans toutes les études (213,214).

1.3.5.2 Les facteurs de croissance

Les facteurs de croissance (Epidermal Growth Factor, Vascular Endothelial Growth Factor, Fibroblast Growth Factor) et leurs récepteurs semblent jouer un rôle dans la tumorigénèse et l'agressivité hypophysaire.

L'expression d'EGFR, appartenant à la famille des récepteurs ErbB (EGFR, HER2, HER3, HER4), semble être augmentée dans les carcinomes hypophysaires, en IHC et RT-

PCR sur un faible nombre de tumeurs de tous types (173). Dans les tumeurs corticotropes, une augmentation de l'expression d'EGF et EGFR était associée à la récurrence ainsi qu'à l'invasion (174,175). Ainsi, les mutations somatiques d'USP8 mises en évidence dans les tumeurs corticotropes sporadiques entraînent une activation d'EGFR. Le lien entre mutations d'USP8 et pronostic des tumeurs corticotropes n'est pas univoque. Alors que les tumeurs avec mutation d'USP8 sont de plus petite taille, moins souvent invasives et plus souvent en rémission post opératoire immédiate que les tumeurs non mutées (15,215,216). certaines études ont rapporté plus de récurrences à long terme parmi les tumeurs avec mutation d'USP8 (83,217). Ces résultats ne sont pas retrouvés dans toutes les études (82,216). Récemment, Neou *et al.* ont rapporté plus de tumeurs envahissant le sinus sphénoïdal et plus de tumeurs agressives (nécessitant des chirurgies rapprochées, une radiothérapie ou une chimiothérapie) parmi les tumeurs corticotropes non mutées USP8, sous réserve d'un suivi hétérogène (15). Aussi, les tumeurs avec mutation d'USP8 sont associée à une plus forte expression des récepteurs SSTR5 pouvant faire évoquer une meilleure réponse aux analogues de la somatostatine de 2^{nde} génération (pasiréotide), mais cela reste à démontrer en pratique (15,215,218) . Des traitements par inhibiteurs de Tyrosine kinase ciblant EGFR, notamment le lapatinib, ont été testés chez quelques patients présentant des tumeurs lactotropes agressives et carcinomes (219,220). Sur 7 patients traités, seul 1 a présenté une réponse tumorale significative.

Une augmentation de l'expression de VEGF, facteur clé dans l'angiogénèse de nombreuses tumeurs, a été retrouvée dans les tumeurs hypophysaires présentant des récurrences. Ainsi, une étude en RT-PCR portant sur 46 échantillons tumoraux a montré une association entre invasion et expression de VEGF (176). Sur 12 patients dont le suivi post opératoire était disponible, il existait une tendance vers une association entre récurrence et expression de VEGF. D'autres résultats dans la littérature sur la base d'études en IHC sont par ailleurs discordants. Certaines études retrouvent une association entre expression de VEGF et invasion ainsi qu'une augmentation d'expression dans les carcinomes (177,221,222), d'autres ne retrouvant une association ni avec la récurrence (223,224), ni avec l'invasion (225–227). Ces données pourraient être particulièrement intéressantes d'un point de vue thérapeutique. Ainsi, nous pouvons noter que l'octreotide pourrait avoir une action anti-angiogénique via une inhibition de VEGF (228). Dans ce

contexte, quelques carcinomes corticotropes traitées par anticorps monoclonal anti-VEGF, le bevacizumab, ont montré une stabilisation prolongée (229,230).

Le rôle des FGFs a aussi été étudiée. Ainsi, dans quelques études, une augmentation de l'expression de FGFR1 en RT-PCR (231), de FGF4 en IHC(232) étaient associées à l'invasion. L'augmentation de l'expression de FGF4-R par western blot (233) était associée avec un Ki67 élevée et tendait à s'associer avec l'invasion.

Une diminution de l'expression de TGF- β et ses effecteurs (Smad3) a été associée à l'invasion et la récurrence dans les tumeurs non fonctionnelles (171,234,235) mais d'autres études retrouvent des résultats contradictoires, notamment par analyse de méthylation (236).

1.3.5.3 Les molécules d'adhésion et la voie de transition épithélio-mésenchymateuse

La transition épithélio-mésenchymateuse est impliquée dans le développement de nombreuses tumeurs. Elle correspond à la transformation de cellules perdant leurs marqueurs épithéliaux (E-cadhérines, cytokératine...) et acquérant des caractéristiques de cellules mésenchymateuses (N-cadhérines, vimentine, métallo-protéases, fibronectine, TWIST1, certains facteurs de transcription comme SNAI1, SNAI2...). Ces cellules perdent alors leurs capacités d'adhésion et développent des capacités de mobilité et d'invasion.

Il a été montré dans plusieurs études que l'expression d'E-cadhérine (codée par *CDH1*) était réduite dans les tumeurs invasives. Ainsi, l'expression d'E-cadhérine et de beta-caténine étaient réduites en IHC dans des tumeurs invasives lactotropes (237), en IHC et RT-PCR dans des tumeurs non fonctionnelles, somato et corticotropes (238), ainsi qu'en RT PCR dans une cohorte de tumeurs de tous types (239). Dans une étude sur 45 tumeurs corticotropes, une diminution de l'expression de *CDH1* en RT-PCR et E-cadhérine en IHC étaient retrouvées dans les macroadénomes et syndromes de Nelson comparativement aux microadénomes (240). Neou *et al.* ont par ailleurs montré que les tumeurs corticotropes *USP8* wild type, de plus petites tailles et moins invasives que les tumeurs mutées *USP8*, étaient associée à une expression faible de la voie EMT en analyse de séquençage RNA (15). Dans leur étude portant sur 83 tumeurs somatotropes, Fougner *et al* ont montré que l'expression membranaire de E-cadhérine était associée à une

meilleure réponse préopératoire aux analogues de la somatostatine, alors qu'une perte d'expression était associée à l'invasion et une plus grande taille tumorale (241). L'hyperméthylation du promoteur de *CDH1*, retrouvée préférentiellement dans les tumeurs invasives, pourrait en être un mécanisme (103). Il existe cependant 2 études discordantes, sur 30 tumeurs de tous types (242) et 127 tumeurs de tous types, avec peu de précisions notamment concernant la définition de l'invasion (243).

Dans une étude récente, 8 adénomes gonadotropes classés comme lentement ou rapidement progressifs ont bénéficié d'un séquençage d'ARN. Cette étude a permis d'identifier 11 gènes associés avec la vitesse de croissance dont 6 en lien avec la voie de l'EMT (*SPAG9*, *SKIL*, *MTDH*, *HOOK1*, *CNOT6L* et *PRKACB*) (180).

La régulation de la transition épithélio-mésenchymateuse est étroitement liée à l'activité d'autres enzymes et protéines comme les métalloprotéases matricielles (MMP) dégradant la matrice extracellulaire, et leurs inhibiteurs (TIMPS). Ainsi, une surexpression de certaines MMP comme MMP-9 a été associée à l'invasion dans plusieurs études en IHC, microarray et RT-PCR portant sur de multiples types tumoraux (244–247), de même que MMP-2 (248) ou MMP-14 (249,250). Inversement, l'inhibiteur TIMP-2 semble significativement plus exprimé dans les tumeurs non-invasives (251) Un polymorphisme de MMP-1 a été détecté dans 90% des tumeurs invasives dans une petite cohorte (252). Une étude sur des tumeurs somatotropes a retrouvé une diminution de l'expression de *HEPN1* par RT-PCR dans les tumeurs invasives. L'inactivation de ce potentiel gène suppresseur de tumeurs induirait une augmentation de l'expression de MMP-2 et MMP-9 (253). A noter qu'une étude sur des tumeurs gonadotropes n'a pas montré de surexpression de MMP-9 en IHC dans les tumeurs invasives (254).

Par ailleurs, la surexpression d'ADAM12, protéine ayant des propriétés de métalloprotéase ainsi que désintégrine, a été retrouvée dans les tumeurs invasives. Elle aurait un rôle d'activation des voies EGFR/ERK et EMT (250,255).

D'autres molécules d'adhésion ont été étudiées, comme Neural Cell Adhesion Molecule (NCAM), dont l'expression a été retrouvée associée à l'invasion dans une étude sur 82 tumeurs hypophysaires de tous types (256). Cette association n'a cependant pas été retrouvée dans toutes les études (257,258). Une diminution de l'expression de *LAMA2*, codant pour une Laminine composant la matrice extracellulaire, en RT-PCR et IHC, a été retrouvée dans les tumeurs non fonctionnelles invasives et serait liée à une

méthylation de son promoteur (259). Le rôle de *LGALS3*, qui code pour une protéine impliquée dans l'adhésion, la Galectine 3, a été suggéré avec une augmentation de l'expression associée à la récurrence des tumeurs lactotropes et corticotropes (178,179).

1.3.5.4 Les gènes impliqués dans la régulation hormonale

Dans les tumeurs lactotropes, une faible expression en IHC des récepteurs à œstrogènes $ER\alpha$ a été associée à l'invasion, la prolifération et la progression; ce qui pourrait notamment expliquer le caractère plus fréquemment agressif des tumeurs lactotropes chez l'homme (186). L'analyse transcriptomique de 30 tumeurs lactotropes a montré une expression différentielle de nombreux gènes appartenant à la voie de signalisation de $ER\alpha$ entre hommes et femmes (260). Aussi, la corrélation entre expression en IHC d' $ER\alpha$ et analyse transcriptomique de *ESR1* était excellente.

1.3.5.5 Autres gènes

Le gène *PTTG1* (Pituitary Tumor Transforming Gene) est probablement le gène dont le lien avec l'invasion a été le mieux étudié. Son rôle est de faciliter la séparation des chromatides sœurs durant la mitose. Sa modification d'expression induit ainsi une instabilité chromosomique (261), et sa surexpression est associée à une stimulation de l'angiogénèse via une augmentation de l'expression de VEGF et de bFGF (231,262). Sa surexpression était associée à l'invasion de manière significative dans une méta-analyse incluant 24 études et 1,464 tumeurs hypophysaires (182). L'association avec la récurrence est cependant moins évidente (183,263). Filippella *et al.* ont rapporté qu'un cut-off de 3.3% en IHC prédisait la persistance/récurrence dans les tumeurs de plusieurs types avec une sensibilité de 60% et une spécificité de 76% (185), mais ce seuil n'a pas été validé ultérieurement.

La protéine EZR a été retrouvée surexprimée dans les tumeurs invasives dans une étude portant sur des 50 tumeurs non fonctionnelles mélangeant oncocytomes, tumeurs immuno-négatives et gonadotropes, avec augmentation de l'expression protéique en analyse protéomique et du mRNA en analyse transcriptomique (264). Une étude sur

lignée cellulaire a confirmé son rôle pro-invasif. De plus, il semblerait que le miRNA 183 cible *EZR* dans une étude sur des ostéosarcomes (265). Ce miRNA avait été retrouvé diminué dans les tumeurs lactotropes invasives (202).

Le rôle des télomérases, codées par le gène *TERT*, dans l'immortalisation de la cellule et la carcinogénèse a été évoqué dans plusieurs cancers et a été étudié dans les tumeurs hypophysaires. L'activité des télomérases a été étudiée par PCR dans 30 tumeurs hypophysaires : elle a été détectée chez 3 patients et était associée à des critères de prolifération (266), de même que l'expression de *TERT* qui a été retrouvée en immunohistochimie dans 28.6% des 49 tumeurs analysées (267). Cependant, il semblerait que l'expression de *TERT* soit similaire dans l'hypophyse saine (268) et ne soit pas associée à un allongement des télomères (269). L'étude du promoteur de *TERT* a montré l'absence de mutation ponctuelle dans les tumeurs hypophysaires à l'inverse de plusieurs tumeurs malignes (188,270). Alors que la méthylation du promoteur de *TERT*, étudiée par PCR dans 85 tumeurs de tous types, n'était pas associée aux récurrence et à l'invasion (271), une seconde étude sur 70 tumeurs majoritairement non fonctionnelles en MS-HRM (methylation sensitive high resolution melting analysis) a montré que la méthylation du promoteur était associée à une surexpression de *TERT*, plus de progression et une survie sans progression plus courte (34 vs 109 mois)(188).

L'impact de la voie PI3K/AKT a été évoqué, notamment par Lin *et al* dans une étude sur 353 tumeurs hypophysaires. Dans cette étude, une mutation activatrice de *PIK3CA* était retrouvée chez 8.8% des tumeurs invasives vs aucune tumeur non invasive, et s'associait à la récurrence post-opératoire(87). Les mutations de *RAS* sont un autre mécanisme possible d'activation de la voie PI3K/AKT et étaient retrouvées dans 7% des tumeurs invasives. Antérieurement, des mutations de *HRAS* avaient été décrites dans les lésions secondaires de carcinomes hypophysaires (190), mais non dans les tumeurs primitives et non métastatiques (191).

Le rôle d'autres gènes a été évoqué. Il semblerait qu'une baisse de l'expression d'*AIP* dans les tumeurs somatotropes sporadiques soit associée à l'invasion (272,273). Des mutations de *TP53* ont été identifiées dans des carcinomes hypophysaires (194). Dans les tumeurs

lactotropes, des études sur tumeurs humaines, modèles animaux et lignées cellulaires, montrent qu'une expression faible de D2R (codé par *DRD2*) est associée à une résistance aux agonistes dopaminergiques (274,275). Dans une étude sur tous types tumoraux, il n'existe par contre pas de lien entre invasion et expression de D2R (276).

1.3.5.6 Les analyses « pangénomiques »

Le développement des techniques d'analyse pangénomique (puces, séquençages de nouvelle génération d'ARN, d'ADN en exomes ou de génomes) ont permis une recherche plus large de gènes candidats.

Plusieurs études de séquençage d'ADN par NGS d'exomes de tumeurs hypophysaires existent. Certaines incluent plusieurs types tumoraux (15,68,69,72,128,192) alors que d'autres se sont focalisées sur les tumeurs lactotropes (91,193), corticotropes (84,85), non fonctionnelles (277), somatotropes (67,130,278) ou thyroïdotropes (279). Parmi ces études, peu ont précisément étudié le lien entre pronostic et altérations génomiques. Song *et al.* ont rapporté une association négative entre invasion (évaluée selon la classification de Hardy, Knosp et lors de l'analyse anatomopathologique) et mutations de *GNAS* dans les tumeurs somatotropes (40% des tumeurs mutées *GNAS* invasives vs 60% des tumeurs *GNAS*-wt), de même qu'avec la résistance aux analogues de la somatostatine (15.4% des tumeurs mutées *GNAS* résistantes vs 84.6% des tumeurs *GNAS*-wt)(69). Ces données ne sont pas retrouvées dans toutes les études, le lien entre mutation de *GNAS* et pronostic des tumeurs somatotropes est donc encore incertain (280). Comme cité ci-avant, Neou *et al* ont rapporté une association entre absence de mutation *USP8* et agressivité des tumeurs corticotropes (15). Les mutations de *BRAF*, *TP53* et *USP48* identifiées dans les tumeurs corticotropes n'étaient a priori pas associées à l'invasion ni à la récurrence (84,85). Les mutations de *USP48* s'associaient par contre à une plus petite taille tumorale. Lan *et al*, via le séquençage de 12 tumeurs lactotropes et non fonctionnelles, ont mis en évidence 15 variants associés à l'invasion, dont 3 dans le gène *RIZ1*. L'expression des gènes en question était significativement diminuée (*DPCR1*, *KIAA0226*, *MX2*, *PRB3*, *PRDM2*, *PRDM8*, *SPANXN2*, *TRIOBP*, *ZNF71*) ou augmentée (*DSPP*, *EGFL7*, *LRP1B*, *LRRC50*) dans les tumeurs invasives (192). Une

diminution de l'expression de PRDM2 (*RIZ1*) était notamment associée à la récurrence. La même équipe suggère via le séquençage de 12 tumeurs lactotropes qu'une diminution de l'expression de PRDM2 serait associée à plus de résistance aux agonistes dopaminergiques (193). Aucun variant de ce gène n'était rapporté dans l'étude de De Sousa *et al.* portant sur 12 tumeurs lactotropes (91). Les 2 études portant sur tumeurs non fonctionnelles et thyroïdiques n'ont pas notifié de variant qui soit associé au pronostic tumoral, à la réponse thérapeutique ou à l'invasion.

Une étude a analysé le séquençage d'ADN mitochondrial de tumeurs somatotropes, gonadotropes et immuno-négatives (196). Il n'y avait pas de lien entre le taux d'hétéroplasmie, le Ki67 et la taille tumorale. Cependant, un nombre plus important de variants a été identifié parmi les tumeurs à Ki67 élevé. Le variant T16189C a été retrouvé dans 40% des tumeurs n'ayant pas récidivé vs 0% de celles ayant récidivé. A noter, que le délai de suivi et la définition de la récurrence n'étaient pas précisés.

Parallèlement, les études transcriptomiques (principalement sur puces, en RT-PCR, et plus récemment en séquençage d'ARN) ont mis en évidence de très nombreux gènes et voies de signalisation sur ou sous-exprimés dans les tumeurs invasives ou récidivantes, mais aucun gène n'est mis en évidence de manière univoque.

Salomon *et al.* ont identifié en séquençage d'ARN chez 30 tumeurs dont le suivi était disponible, une association entre récurrence et expression de la voie des chimiokines, notamment des gènes *IL8*, *CXCR1* et *CXCR2* (68).

L'analyse transcriptomique en RT-PCR sur 44 tumeurs lactotropes de 9 gènes identifiés dans une première cohorte (197), a retrouvé 7 gènes surexprimés dans les tumeurs ayant présenté une récurrence vs tumeurs non récidivantes : *PTTG*, *ADAMTS6*, *CRMP1*, *ASK*, *AURKB*, *CENPE* et *CCNB1* (183).

Une analyse transcriptomique par puce de 40 tumeurs non fonctionnelles (majoritairement gonadotropes) a retrouvé 346 gènes différentiellement exprimés dans les tumeurs invasives vs non-invasives (254), parmi lesquels 2 ont été confirmés surexprimés par RT-PCR dans une autre cohorte de 19 tumeurs (*IGFBP5*, *MYO5A*), et 1 par IHC (*MYO5A*). Le gène *MYO5A* pourrait avoir un rôle dans la migration cellulaire.

Une étude transcriptomique portant sur 11 tumeurs immunonégatives dont 4 avaient récidivé après résection complète a retrouvé 5 gènes dont l'expression est associée à la récurrence (*CHL1*, *P2RY12*, *ERAP2*, *LPAR5*, *CHAD*). Le gène *CHL1* code pour une protéine d'adhésion et de matrice extracellulaire exprimée au niveau du système nerveux (181). Une autre étude transcriptomique et de méthylation sur 68 tumeurs non fonctionnelles a retrouvé 4582 gènes différentiellement méthylés, et 661 différentiellement exprimés entre tumeurs invasives et non invasives (281). Elle a permis de sélectionner 10 gènes hyperméthylés avec expression diminuée (*PHYHD1*, *LTBR*, *C22orf42*, *PRR5*, *ANKDD1A*, *RAB13*, *CAMKV*, *KIFC3*, *WNT4* et *STAT6*), et 1 gène hypométhylé avec augmentation d'expression (*MYBPHL*) dans les tumeurs invasives. La même équipe a identifié sur 71 tumeurs non fonctionnelles, 2 gènes (*FAM90A1* et *ING2*) dont l'expression et la méthylation s'associaient à la progression en analyse multivariée (199). Dans une autre étude portant sur 63 tumeurs de tous types, l'expression de *WNT4* était étroitement liée à celle de Beta-caténine dont l'expression était inversement corrélée à l'invasion (282).

1.3.5.7 Epigénétique

La méthylation

Il est maintenant démontré que l'épigénétique joue un rôle important dans l'expression des gènes. La méthylation des îlots CpG des promoteurs est notamment souvent associée à une diminution de l'expression des gènes concernés. Alors que deux études sur 24 tumeurs hypophysaires de tous types (95) et 34 tumeurs non fonctionnelles (283), n'avaient pas mis en évidence d'association entre invasion et taux de méthylation global, une étude sur 12 tumeurs non fonctionnelles (immunonégatives et corticotropes silencieuses) a retrouvé un lien entre profil hypométhylé et invasion (selon la classification de Hardy). Les gènes différentiellement méthylés étaient enrichis en gènes impliqués dans l'adhésion cellulaire (284).

L'étude des DNA méthyltransférases dans 63 tumeurs hypophysaires de tous types a montré une expression accrue de *DNMT1* et *DNMT3A* dans les tumeurs invasives selon la classification de Hardy. L'expression de *DNMT1* et *DNMT3A* était associée à une hyperméthylation de *RASSF1A*, *CDH13*, *CDH1*, et *CDKN2A* dont la réduction d'expression est associée à un rôle procarcinogène (103). Dans une seconde étude ayant analysé le

lien entre la méthylation de 35 gènes suppresseurs de tumeurs et l'invasion chez 105 tumeurs gonado, somato et corticotropes, la méthylation de *RASSF1* était inversement corrélée à l'invasion (285). Une méta-analyse a confirmé l'association entre invasion et méthylation de *CDKN2A*, *DAPK* et *Rb1* (102).

La régulation des histones

L'acétylation, la méthylation et la citrullination des histones participent à l'accessibilité de l'ADN à la machinerie de transcription via les histones acétyltransférases, déacétylases, méthyltransférases et enzymes de citrullination.

RIZ1, jouant un rôle d'histone méthyltransférase, était associé à une réduction de la méthylation de H3K4/H3K9 et une augmentation de la méthylation de H3K27, à moins d'invasion et une survie sans progression plus longue chez 103 tumeurs (non fonctionnelles, lactotropes et somatotropes)(107). Une diminution d'expression avait déjà été associée à la récurrence dans une étude sur tumeurs lactotropes et non fonctionnelles (192). L'augmentation de l'acétylation de H3K9 était, elle, associée aux critères de prolifération (106). L'expression des histones desacétylases SIRT1 et SIRT3 était associées à la taille des tumeurs somatotropes et non fonctionnelles, mais n'était pas associée à l'invasion ni au Ki67 (286). Aussi, il est supposé que l'histone acétyltransferase p300 participe à l'augmentation d'expression du gène *PTTG1* dont l'expression a été associée à l'invasion (105).

Autres régulateurs transcriptionnels

D'autres régulateurs transcriptionnels, tels que les protéines HMGA et notamment HMGA2 modifiant la conformation de la chromatine, ont été étudiés. HMGA2 augmente l'activité du facteur de transcription E2F1 et participe ainsi à des modifications de la régulation du cycle cellulaire et de l'apoptose. L'analyse de l'expression d'HMGA1 et HMGA2 (issues d'un épissage alternatif) a montré une expression nucléaire en IHC chez 40 à 100% des tumeurs alors qu'aucune expression n'est retrouvée sur hypophyse saine (287–291). Certaines de ces études retrouvent un lien entre surexpression d'HMGA1/2 et invasion en IHC (287,288,291), non retrouvé en RT-PCR (291). Quelques études ont analysé le lien entre pronostic et expression d'HMGA dans les tumeurs non fonctionnelles. Steno *et al.* ne retrouvaient pas de lien entre expression d'HMGA1 par IHC et présence

et/ ou croissance d'un résidu post-opératoire (289). Yao *et al* ont comparé les tumeurs issues d'une primo-chirurgie et d'une récurrence et n'ont pas trouvé de modification significative de l'expression d'HMGA1 (290). Liu *et al* ont étudié l'expression d'HMGA2 par IHC dans 55 tumeurs corticotropes et n'ont pas retrouvé de lien avec la récurrence(263).

1.3.5.8 La régulation post-transcriptionnelle : miRNA et lncRNA

De nombreux miRNA ont été retrouvés dérégulés dans les tumeurs hypophysaires, et semblent impliqués dans le comportement tumoral (109). La plupart des études citées ci-dessous reposent sur un faible de nombre de tumeurs et aucun des miRNA n'a réellement été validé de manière indépendante.

Il a été décrit une diminution de 11 miRNA (183, 148a, 375, 342-3p, 23b, 744, 98, 340, 574-3p, 331-3p, 1280) dans les tumeurs lactotropes invasives récidivantes (202). Le miRNA-183 cible le gène *KIAA0101* qui était surexprimé et est impliqué dans la régulation du cycle cellulaire via l'expression de p53 et p21. Les miRNA 122 et 493, ciblant *RUNX3* et *LGALS3* (cité précédemment), ont été retrouvés surexprimés chez 2 carcinomes corticotropes comparativement aux adénomes (200). Des modifications de régulation de nombreux miRNA ont été associés à l'invasion (292), parmi lesquels : miRNA 181b, 181d, 191 et 598 (293) étaient surexprimés dans les tumeurs non fonctionnelles invasives, miR-106b dans les tumeurs corticotropes invasives (294). MiR-3676-5p, miR-383, miR-137, miR-374a, miR-374b (ces trois derniers ciblant le gène *WIF1* cité précédemment) étaient sous-exprimés dans les tumeurs non fonctionnelles invasives(172,293). Une étude récente réalisée sur 23 tumeurs de tous type a retrouvé une relation inverse entre expression de miR-30a-5p, miR516b-3p, miR-23b-5p et invasion, expression de miR26b-5p et Ki67, expression de miR508-5p et agressivité, et une relation positive entre expression de miR130a-3p, miR146a-5p et traitement multimodal (201). Parmi les gènes cibles de ces miRNAs, on notera des gènes impliqués dans la voie PI3/AKT/mTOR, Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) impliqué dans la voie d'*AIP*.

Plusieurs miRNA dont l'impact sur le pronostic n'a pas été testé directement, ciblent plus ou moins directement des gènes dont le rôle pronostique est suspecté, comme miR-let-7(288,295) et *HMGA2*, miR186 et p21 /p27, ou encore les miRNA ciblant *PTTG 1*(109).

Aussi, certains pourraient avoir un lien avec la réponse thérapeutique. Ainsi une étude sur un faible nombre de tumeur somatotrope a retrouvé des miRNA différenciellement

exprimés entre répondeurs et non répondeurs aux analogues de la somatostatine de 1^{ère} génération (111).

Parmi les lncRNA, MEG3 et HOTAIR sont probablement les mieux étudiés dans les tumeurs hypophysaires et autres cancers. MEG3 semble impliqué dans la régulation du cycle cellulaire, via la stabilisation de p53 en inhibant son ubiquitination par MDM2 (296–298) avoir. Plusieurs études dans les tumeurs hypophysaires ont retrouvé une expression significativement diminuée dans les tumeurs non fonctionnelles, et plus spécifiquement gonadotropes (119,299,300). La méthylation du promoteur semble être en cause dans la diminution de l'expression de MEG3 (119,301). Une étude par RT-PCR sur 52 tumeurs non fonctionnelles a retrouvé un lien entre invasion et absence d'expression de MEG3, de même que invasion et augmentation d'expression d'HOTAIR (121). HOTAIR est responsable d'une modification de la conformation de la chromatine, et a été associé au pronostic de différents cancers dont les cancers du sein (302).

Récemment, une étude sur 68 tumeurs non fonctionnelles a trouvé un lien entre expression de 2 lncRNA (COA6-AS1 and RP11-23N2.4) et la progression tumorale (195).

Une augmentation de l'expression de lnc-SNHG1 a été retrouvée dans des tumeurs invasives dans une étude par RT-PCR sur 58 tumeurs dont le type n'est pas précisé. Après des études sur lignées cellulaires, les auteurs évoquent un rôle dans la migration cellulaire et l'activation de l'EMT et de la voie WNT/ β caténine, via l'inhibition de miR 302/372/373/520 (303).

Quelques études ont cité le lien entre invasion et certains lnc-RNA qui méritent d'être explorés, comme C5orf66-AS1 dont l'expression a été étudiée chez 11 tumeurs immuno-négatives (304) ou H19 (116). CCAT2 a été étudié chez 74 tumeurs où son expression semblait s'associer au pronostic, et sur lignées cellulaires où il semblait réguler l'expression de *PTTG1* (112). Il est à noter que dans cette dernière étude, de manière étonnante, la survie globale était étudiée. Il n'y avait cependant pas de précisions sur l'agressivité des tumeurs étudiées.

Enfin, d'autres RNA non codants tels que les circRNA, piRNA, siRNA commencent à être étudiés.

1.3.5.9 Le microenvironnement tumoral

Les études récentes et l'essor de l'immunothérapie en cancérologie ont mis en exergue le rôle du microenvironnement dans le comportement des cellules tumorales. Plusieurs études en IHC ont identifié un lien entre infiltrat immunitaire et caractéristiques cliniques dans les tumeurs hypophysaires, sans mettre en évidence de marqueur robuste validé. Ainsi, un infiltrat macrophagique CD68+ (305,306) et CD163+ s'associaient à l'invasion, de même que les cellules exprimant PD-L1 (136). Un infiltrat lymphocytaire PD-1 (133), macrophagique CD45+ (133) ou de cellules exprimant PD-L1 (134) s'associaient à des marqueurs de prolifération et notamment à un Ki67 élevé. Enfin, un infiltrat macrophagique CD45+ s'associait à plus de persistance/ récurrences après un suivi de 34+/-2 mois chez 72 patients (132). L'influence des lymphocytes T régulateurs (FOXP3) est aussi suspectée (136,307). Les tumeurs liées à des mutations germinales d'*AIP*, plus volontiers invasives, sont associées à un infiltrat macrophagique important qui pourrait participer à ce phénotype invasif (135). Une analyse récente en cytométrie de flux sur 11 tumeurs non fonctionnelles a montré que les tumeurs avec une quantité importante de cellules myéloïde CD11b+ avaient un Ki67 plus volontiers supérieur à 3%, et que les tumeurs invasives avaient un phénotype fonctionnel de macrophage M2 plutôt que M1 (137).

L'infiltrat immunitaire est très probablement influencé par la sécrétion de cytokines et chimiokines (307). Salomon *et al.* ont identifié, par séquençage de d'ARN, une surexpression des chimiokines et de leurs récepteurs dans les tumeurs récurrentes (évaluées après un suivi de 2 à 76 mois), en particulier *IL8*, *CXCR1*, *CXCR2* (68). Xing *et al.* ont retrouvé sur 35 tumeurs une association entre expression de *CXCR4* et *CXCL12*, et invasion (308).

1.3.5.10 Les altérations chromosomiques

Le lien entre altérations du nombre de copies et pronostic des tumeurs hypophysaires a été analysé dans quelques études.

Simpson *et al.* ont analysé la perte d'hétérozygotie au niveau de 122 marqueurs. Ils ont rapporté un nombre de pertes d'hétérozygotie globalement plus élevé dans les tumeurs invasives, concernant 11 bras chromosomiques pour les tumeurs somatotropes et 4 pour les tumeurs non fonctionnelles (1q, 3q, 9q et 12q)(309). Bates *et al.* ont rapporté plus de

pertes d'hétérozygotie dans les tumeurs invasives, notamment au niveau des loci 11q13, 13q12–14, 10q et 1p(310). Très récemment, Ben-Shlomo *et al* ont observé que le nombre de CNV corrélait négativement avec l'invasion et l'extension supra-sellaire (72). Salomon *et al* ont identifié plusieurs CNV liés à l'invasion; celui associé avec le plus de significativité correspondait à une délétion située en 12q21.31. La proportion de tumeurs invasive vs non invasives avec cette délétion ne sont pas précisées, de même que le log2ratio.

Buch *et al.* ont réalisé une analyse de perte d'hétérozygotie sur 96 tumeurs non fonctionnelles disposant d'un suivi minimum de 5 ans. Le nombre de pertes d'hétérozygotie était globalement plus important parmi les tumeurs récurrentes (21 vs 9%). Deux marqueurs microsatellites au niveau du chromosome 1q étaient associés avec la récurrence (311).

Tatsi *et al.* ont trouvé une association entre quantité d'altérations et invasion, en l'absence de mutation *USP8*, parmi une cohorte de 27 tumeurs corticotropes pédiatriques (129). Seulement, les tumeurs *USP8*wt représentaient 21 tumeurs, dont 2 invasives. Le faible nombre de tumeurs rend l'interprétation des résultats difficile. Par ailleurs, les tumeurs fortement altérées présentaient une plus grande taille que les tumeurs peu altérées, indépendamment de la présence d'une mutation *USP8* (18 vs 5.5 mm).

A Lyon, une analyse par CGH-array de 13 tumeurs lactotropes avait permis de mettre en évidence une association entre agressivité (tumeurs invasives ayant récidivé à court terme et carcinomes) et perte du bras court du chromosome 11. En effet, 5 des 6 tumeurs agressives présentaient une délétion concernant le bras court du chromosome 11 vs aucune des 7 tumeurs non agressives. La région de délétion commune comprenait 139 gènes ; 29 de ces gènes (122 analysés) étaient sous exprimés dans les tumeurs agressives, parmi lesquels *DGKZ*, *CD44*, *TSG101*, *GTF2H1*, *HTATIP2* pourraient avoir un impact sur l'agressivité, et 3 étaient surexprimés(198).

1.4 Analyse par CGH-array des tumeurs

L'objectif de l'analyse en CGHarray est de mettre en évidence des variations ou altérations du nombre de copies (CNV ou CNA selon leur caractère pathogène).

1.4.1 Les variations du nombre de copies et leurs conséquences

Les altérations du nombre de copies ont été identifiées très tôt dans le génome humain. Ainsi, les variations du nombre de copies existent à l'état physiologique et pathologique au niveau germinale. Elles sont la conséquence de non disjonctions lors d'une division cellulaire lorsqu'elle concernent un chromosome entier (aneuploïdie), ou la conséquence de cassures des brins d'ADN suivie d'une recombinaison anormale lorsqu'elle concernent des portions de chromosomes.

Les mécanismes à l'origine de la survenue des CNV ne sont pas complètement élucidés mais impliquent les processus de réplication de l'ADN (division cellulaire, méiose, réparation de l'ADN). Ainsi, le mécanisme de recombinaison homologue non allélique est le plus classiquement décrit. Il consiste en un mésappariement au niveau de duplications génomiques segmentaires (duplicons), correspondant à des segments de plus de 1 kb avec plus 95% d'homologie présents en 2 copies. Des recombinaisons sur des séquences répétées de petite taille présentant un haut degré d'homologie (les éléments Alu et LINE) existeraient. D'autres modèles sont décrits (modèle de jonction d'extrémités non homologues ou non homologous end joining, modèle d'interruption de la fourche de réplication et commutation de la matrice ou Fork Stalling and Template Switching = FoSTeS)). Le rôle de protéine recombinases (Rad51) est évoqué.

Dans l'aneuploïdie, il existe une anomalie de ségrégation chromosomique, au moment de la disjonction des deux chromatides sœurs. Des anomalies d'attachement entre les microtubules permettant la migration des chromatides vers les centrosomes et le kinétochore sont probablement en cause, impliquant les gènes du point de contrôle du fuseau tels que *MAD1*, *MAD2*, *BUB1B*... ainsi que du cyclosome APC/C. Ces gènes mutés sont notamment utilisés pour l'établissement de modèles animaux aneuploïdes.

Alors que l'aneuploïdie est rare dans les tissus sains (moins de 5% des cellules, potentiellement un peu plus au niveau des hépatocytes), elle peut concerner jusqu'à 90% des cellules tumorales (312).

Les analyses en cancérologie ont montré la survenue de très nombreux CNV de grande taille dans les cellules tumorales, entrant dans le cadre d'une instabilité chromosomique. Il existe en général une forte hétérogénéité faisant supposer l'accumulation d'altérations. Des modifications des mécanismes de réparation de l'ADN ainsi que de la régulation du cycle cellulaire sont très probablement impliqués dans la survie de ces cellules avec génome altéré qui induit normalement un arrêt du cycle cellulaire (312). Ainsi, les cancers avec mutation du gène *P53* sont associés à des caryotypes très modifiés. Le mécanisme de chromothripsis a été récemment décrit dans les cancers (exceptionnellement en génétique germinale) et correspond à la fragmentation brutale d'un chromosome en de multiples fragments qui se réarrangent ensuite de manière aléatoire (313).

Les conséquences de ces CNV peuvent être multiples avec :

- Une augmentation ou diminution de l'expression de gènes inclus ou au voisinage d'un CNV, notamment ceux conférant un avantage sélectif. En cas de délétion ou perte d'hétérozygotie, on note une haplo-insuffisance alors qu'en cas de gains de copie, les modifications de l'expression sont extrêmement variables. En effet, les copies gagnées peuvent être modifiées, situées à d'autres endroits du génome et soumises à d'autres régulateurs transcriptionnels.
- Une modification de la régulation de l'expression des gènes avec réorganisation de la chromatine, des promoteurs et régulateurs des gènes
- Des modifications globales du transcriptome en réponse au stress (312,314)
- La formation de gènes/ protéines chimériques par la combinaison de différents exons est aussi possible. Elle s'observe notamment la leucémie myéloïde chronique exprimant le transcrit de fusion BCR/ABL.

En pratique, les conséquences des CNV dans les cellules tumorales sont extrêmement difficiles à prédire, les altérations étant souvent multiples et diverses, s'associant à d'autres types de mutations. De plus, les mêmes altérations présentent des conséquences très différentes dans une cellule cancéreuse comparativement à une cellule normale. Il

semble que les gains de chromosomes entiers (aneuploïdie) soient associés dans les études *in vitro* à une diminution de la prolifération dans les cellules non cancéreuses alors qu'ils sont associés à une augmentation dans les cellules tumorales. Il existe aussi une augmentation du stress protéotoxique, une augmentation de la réponse interféron, une modification globale du transcriptome, y compris des gènes non concernés par l'aneuploïdie, proche de celle observée en cas de stress environnemental(312). De plus, les cellules avec des gains de chromosomes montrent une tendance à plus d'instabilité chromosomique sans que le mécanisme ne soit complètement élucidé (312). Cela implique probablement les différentes voies de réparation de l'ADN (315). Enfin, il est maintenant fortement suspecté que l'organisation tridimensionnelle de la chromatine ait un rôle sur la régulation transcriptionnelle via la formation de domaines topologiques associés (TADs). Ainsi il est probable que les anomalies chromosomiques structurelles et CNV modifient cette organisation, et que ceci participe aux modifications transcriptionnelles observées.

Dans beaucoup de types tumoraux, l'instabilité chromosomique a été associée à un pronostic péjoratif, notamment dans le cancer colorectal, mammaire, dans les gliomes, neuroblastomes, leucémies...(316). Certaines altérations ont été associées au pronostic et à la réponse tumorale, comme les délétions 1p/19q associées à un meilleur pronostic et une meilleure réponse thérapeutique dans les gliomes de bas grade (317), les gains du chromosome 1q associés à une survie plus courte dans les adénocarcinomes pulmonaires ; de même que la délétion 9p dans le carcinome à cellules claires(316).

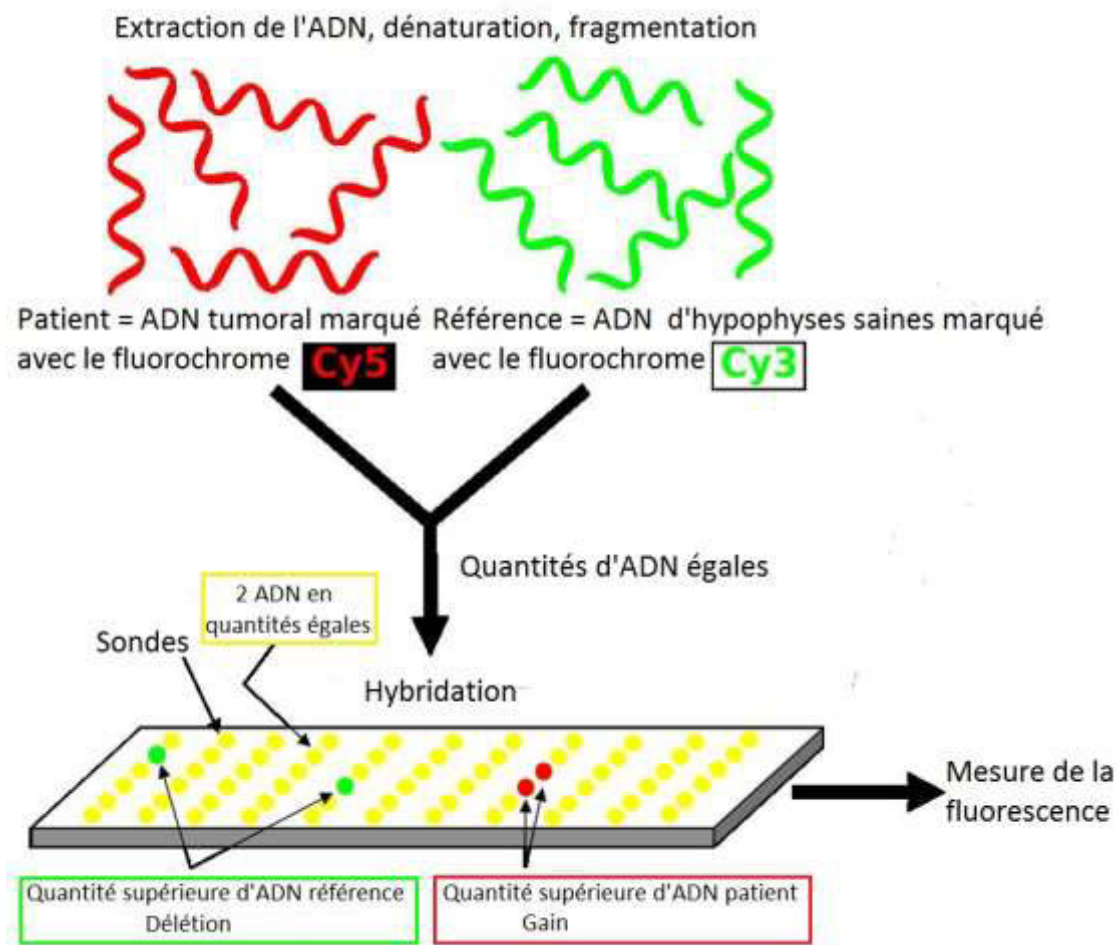
1.4.2 La technique de CGHarray

La CGH-array (comparative genomic hybridization) est une technique cytogénétique d'analyse chromosomique par puce à ADN. Elle consiste en une hybridation génomique comparativement à un ADN témoin, permettant la recherche de variations de nombre de copies (CNV). Elle ne permet pas la recherche de réarrangements structurels équilibrés. L'ADN à explorer et une quantité identique d'ADN d'un pool de témoins sont dénaturés, fragmentés, puis chacun d'eux est marqué par un fluorochrome différent (figure 1). Cet ADN marqué est hybridé sur une puce contenant des sondes couvrant l'ensemble du

génomique. Ces sondes sont des oligonucléotides synthétiques ou des Bacterial artificial chromosomes (BACs), et leur espacement définit la résolution de l'analyse. La fluorescence est ensuite mesurée par scanner. Le rapport entre la fluorescence du patient et celle du témoin est analysé pour chaque sonde et exprimé en \log_2 du ratio de la fluorescence du patient sur celle du témoin (\log_2 ratio). L'estimation pour chaque sonde de la quantité d'ADN par rapport à l'ADN témoin permet de définir la présence de fragments amplifiés ou délétés. Le \log_2 ratio pouvant prendre toute valeur, c'est un signal continu qui a pour but de modéliser des événements discrets (perte/ gain de fragments). Par ailleurs, l'analyse de SNP (single nucleotide polymorphism) permet d'étudier des zones de variations portant sur une seule paire de base, distribuées uniformément dans le génome humain, toutes les 100 à 1 000 paires de bases. Cela permet de détecter les pertes d'hétérozygotie (LOH).

Plusieurs plateformes sont à disposition parmi lesquelles Agilent, Affymetrix et Illumina sont les principales utilisées.

Figure 1 : La technique de CGHarray



1.4.3 Importance du prétraitement des données et particularités en cancérologie

Le prétraitement des données brutes de CGHarray est effectué indépendamment pour chaque patient. Il comprend trois grandes étapes, la normalisation/centralisation, la segmentation et le calling, qui peuvent éventuellement être réalisées lors de procédures les associant.

Les premières étapes de centralisation et normalisation sont généralement indispensables à l'analyse. La centralisation correspond à l'alignement des sondes diploïdes, correspondant à un $\log_2(2/2)$, sur le zéro. Plusieurs méthodes existent. La méthode classique et historique correspond à la centralisation sur la moyenne ou la médiane des sondes. Cependant, ces méthodes sont possibles uniquement si la quantité d'altérations attendue est très faible, comme en analyse constitutionnelle, et répartie de manière homogène entre gains et pertes. Elles ne sont donc pas possible en cancérologie.

La centralisation sur le mode permet de corriger un éventuel déséquilibre entre gains et délétions mais fait toujours l'hypothèse d'une majorité de sondes diploïdes. C'est aussi le cas du logiciel Cytogenomics® fourni par Agilent qui utilise la centralisation permettant la minimisation du nombre de sondes anormales.

La normalisation permet de corriger le biais d'intensité (différence d'intensité des 2 fluorochromes Cy5 et Cy3), accentué dans les intensités extrêmes, et de centraliser les données sur 0. Elle se fait généralement par l'ajustement d'une régression loess sur un MA plot. Le MA plot est un graphique représentant pour chaque sonde le \log_2 ratio en fonction de la moyenne du \log_2 des fluorescences (figure 2). L'ajustement d'une régression loess, correspondant à une régression polynomiale locale, permet pour chaque fluorescence moyenne de prédire un \log_2 ratio. Il existe un risque d'introduction de biais en cas d'estimation de la régression sur l'ensemble des sondes, surtout pour les \log_2 ratios (M) extrêmes pour lesquels il existe un risque de surcorrection(318). La méthode PopLowess réalise un clustering de type k-means avant d'estimer la régression loess et centraliser sur le cluster majoritaire(319). Van Houte *et al* ont proposé une méthode de centralisation et normalisation dans laquelle les étapes de normalisation-segmentation-calling sont réalisées de manière itérative jusqu'à stabilisation des valeurs (figure 3). A chaque cycle l'estimation de la régression loess est réalisée sur les sondes diploïdes. Elles sont sélectionnées par l'ajustement d'un mélange de lois normales, en fixant comme paramètre initial que la classe avec une moyenne de 0 représente 80% des sondes. Par ce biais, la classe majoritaire est sélectionnée comme étant diploïde (320) .

Figure 2: MA plot d'un patient avant (à gauche) et après centralisation-normalisation (à droite) PAR CGHNormaliter

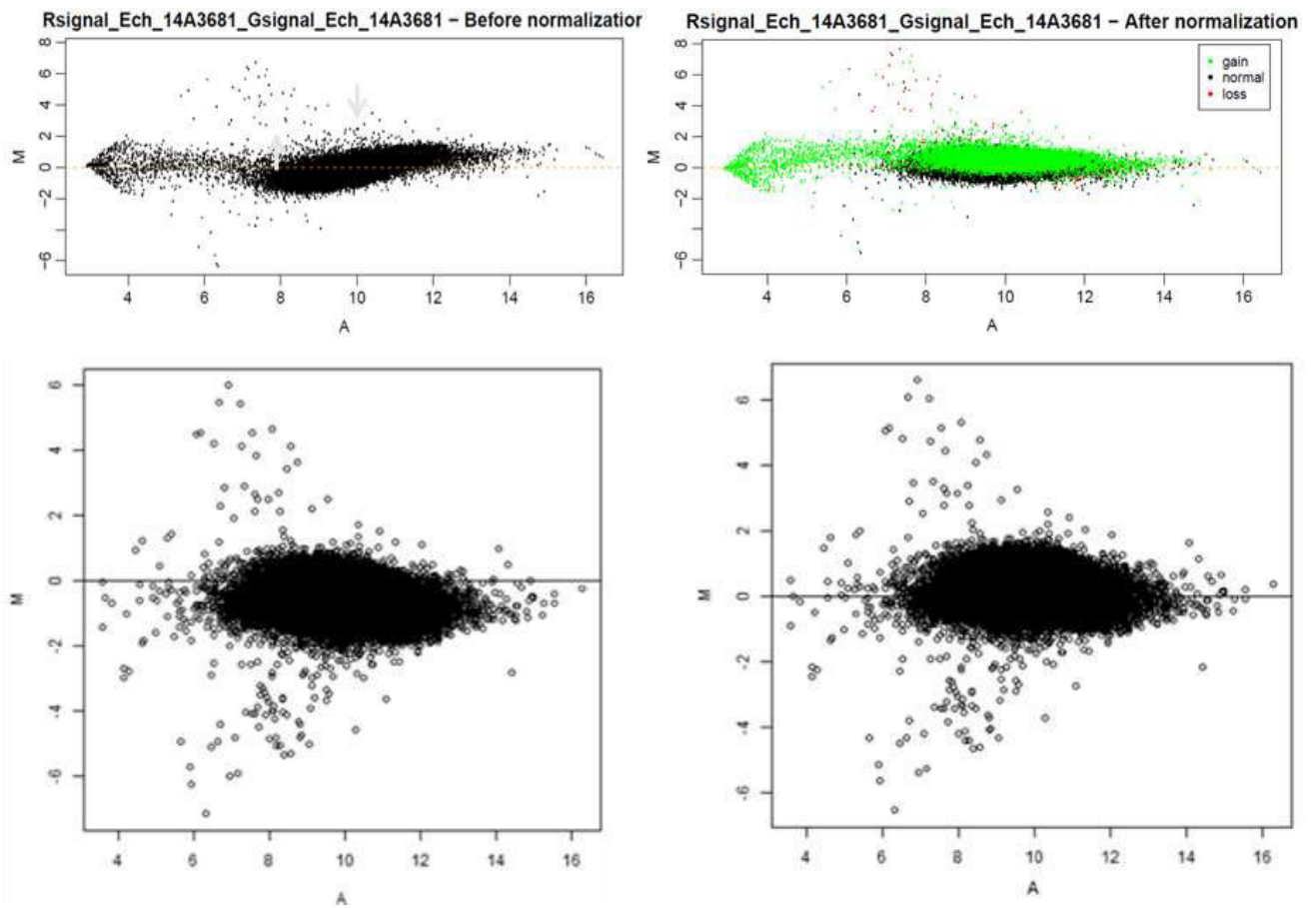
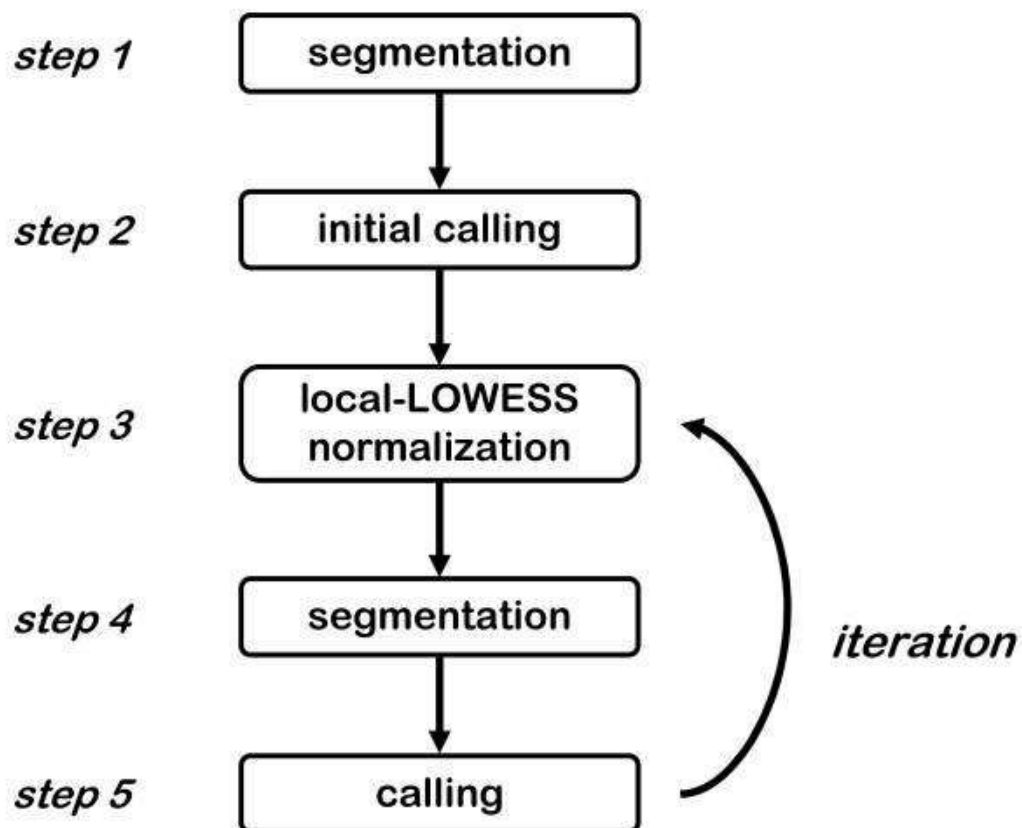


Figure 3 : Normalisation, segmentation et calling selon CGHnormaliter(320)



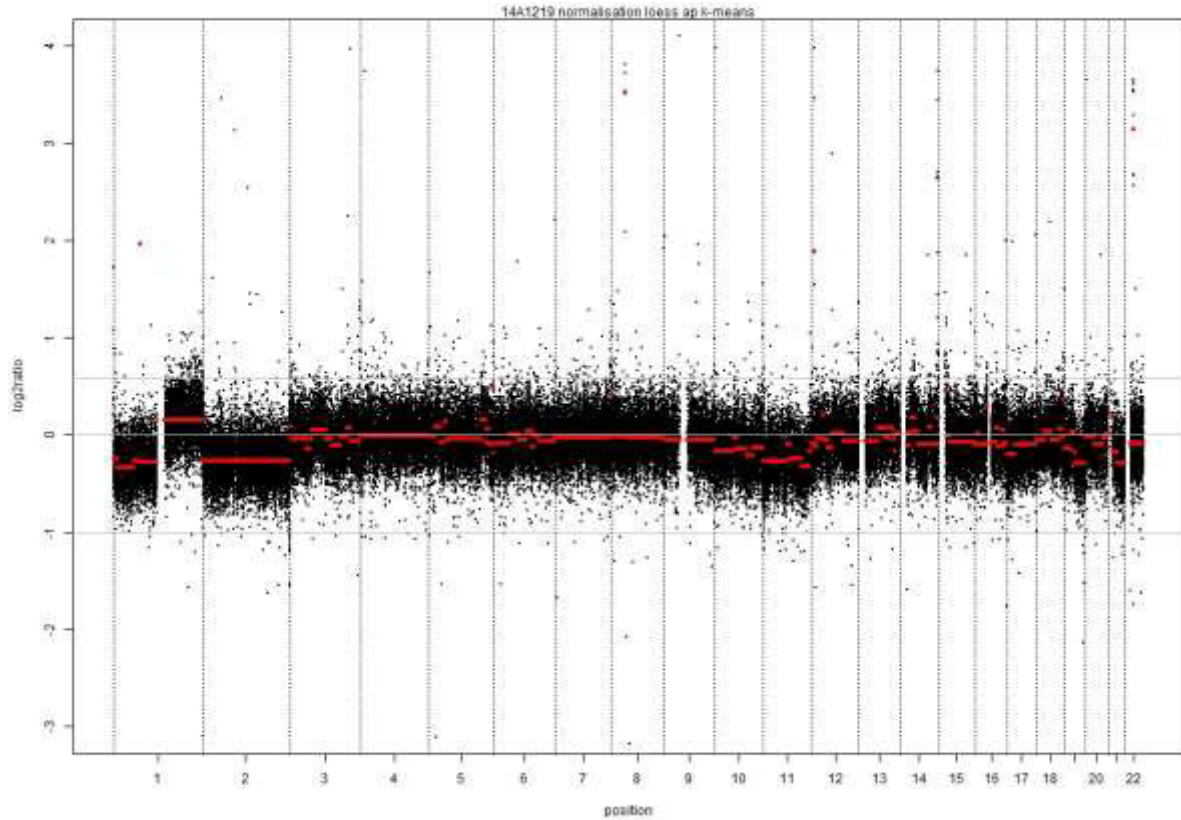
La segmentation correspond à l'identification de points de rupture afin que chaque sonde soit incluse dans un segment de \log_2 ratio constant (figure 4).

Plusieurs approches se distinguent, s'appuyant sur des maximisations de vraisemblance, sur des tests statistiques ou sur une approche bayésienne. Les approches basées sur une maximisation de la vraisemblance recherchent des points de cassure en maximisant la vraisemblance d'un modèle de régression linéaire pour chaque segment. La variance de chaque segment peut être considérée comme commune à tous les segments (modèle homoscedastique) ou propre à chaque segment (modèle hétéroscedastique) ; le modèle homoscedastique ayant tendance à créer plus de segments. Afin d'éviter de construire un modèle peu parcimonieux intégrant autant de points de cassure que de sondes, une pénalisation est nécessaire. Picard *et al* dans le package R CGHseg utilisent une vraisemblance pénalisée et optimisée par un algorithme dynamique(321). Plusieurs méthodes utilisent des tests statistiques pour localiser les points de cassure : Circular Binary Segmentation(322), ADM-2 utilisé par Cytogenomics® Agilent, Nexus copy number utilisé par BioDiscovery... Des corrections de la p-value sont nécessaires pour prendre en

compte la problématique des tests multiples. Des approches Bayésiennes modélisant la probabilité de localisation des points de cassure ont aussi été décrites (323,324)

Il semblerait que Circular Binary Segmentation (CBS) soit la méthode qui présente les meilleures performances (325,326). Son principal défaut, i.e. son incapacité à repérer les variations sur une seule sonde, n'est pas pénalisant en cancérologie. CBS est une méthode effectuant des tests de manière itérative, basée sur le principe des sommes partielles. Dans cette méthode, les chromosomes sont considérés comme circulaires. A chaque test, les moyennes des log₂ratios des sondes de part et d'autre des potentiels points de cassure évalués sont comparées selon un test t de Student. Si l'on considère la distribution de la variable non connue, la distribution de la statistique de test sous H₀ peut être estimée par méthode de permutation. Le test de deux nouveaux points de cassure sur données en cercle est ensuite effectué de manière itérative pour chaque nouveau segment identifié afin d'identifier tous les points de cassure. Le choix du seuil α à partir duquel l'hypothèse H₁ sera exclue est important. Dans la publication initiale, aucune correction de la p-value n'était effectuée malgré la multiplicité des tests réalisés, au profit d'une étape d'élagage des points de cassure. Cela est justifié par le fait que le nombre réel de points de cassure est inconnu et qu'une méthode de correction trop conservatrice de H₀ serait inadaptée. En effet, un résultat faussement négatif est considéré comme étant plus pénalisant qu'un résultat faussement positif, qui sera éventuellement corrigé lors de l'étape de calling ou lors de l'interprétation de la valeur du log₂ratio.

Figure 4 : Exemple de représentation des résultats de CGH array avec les log2ratio en fonction de la position génomique normalisés par régression loess (en noir) et segmentés par CBS (en rouge)



Le calling correspond à une catégorisation du log2ratio en variable soit binaire (présence d'une altération ou absence d'altération), soit ordinale de 3 classes (gain, perte, normal) ou plus (nombre de copies). Des méthodes statistiques basées sur la répartition de mélanges gaussiens existent mais la méthode la plus classique correspond au seuillage. Ainsi en théorie, une sonde

- présente en 2 copies présentera théoriquement un $\log_2\left(\frac{2}{2}\right) = 0$,
- déletée, présente en 1 copie, présentera théoriquement un $\log_2\left(\frac{1}{2}\right) = -1$,
- déletée de manière homozygote présentera théoriquement un $\log_2\left(\frac{0}{2}\right) \rightarrow -\infty$,
- gagnée, présente en 3 copies, présentera théoriquement un $\log_2\left(\frac{3}{2}\right) = 0.58$,
- gagnée présente en 4 copies présentera théoriquement un $\log_2\left(\frac{4}{2}\right) = 1$,
- etc....

Seulement, ceci ne tient pas compte de l'hétérogénéité cellulaire. En effet, lors de l'extraction d'ADN, les cellules ne sont pas individualisées. Les cellules tumorales sont alors analysées avec les cellules du microenvironnement (cellules immunes, endothéliales etc...). De même, les différentes populations tumorales, présentant des CNV différents, sont analysées ensemble. Il est donc nécessaire de définir un seuil de détection d'une anomalie. Par exemple, si un gain d'une copie concerne 20% des cellules, le log2ratio résultant correspondra à $\log_2\left(0.20 \times \frac{3}{2} + 0.80 \times \frac{2}{2}\right) = 0.14$; donc le seuil pour retenir un gain sera de 0.14.

2 OBJECTIFS

- 2.1 Le premier objectif était de mettre au point un plan d'analyse adapté afin d'utiliser la technique de CGH-array sur plateforme Agilent pour l'exploration de tumeurs hypophysaires.

- 2.2 Le second objectif était de rechercher des facteurs pronostiques de l'évolution des tumeurs hypophysaires en analyse par CGH-array.

3 RESULTATS

3.1 Importance du prétraitement des données de CGHarray pour l'interprétation

3.1.1 Contexte

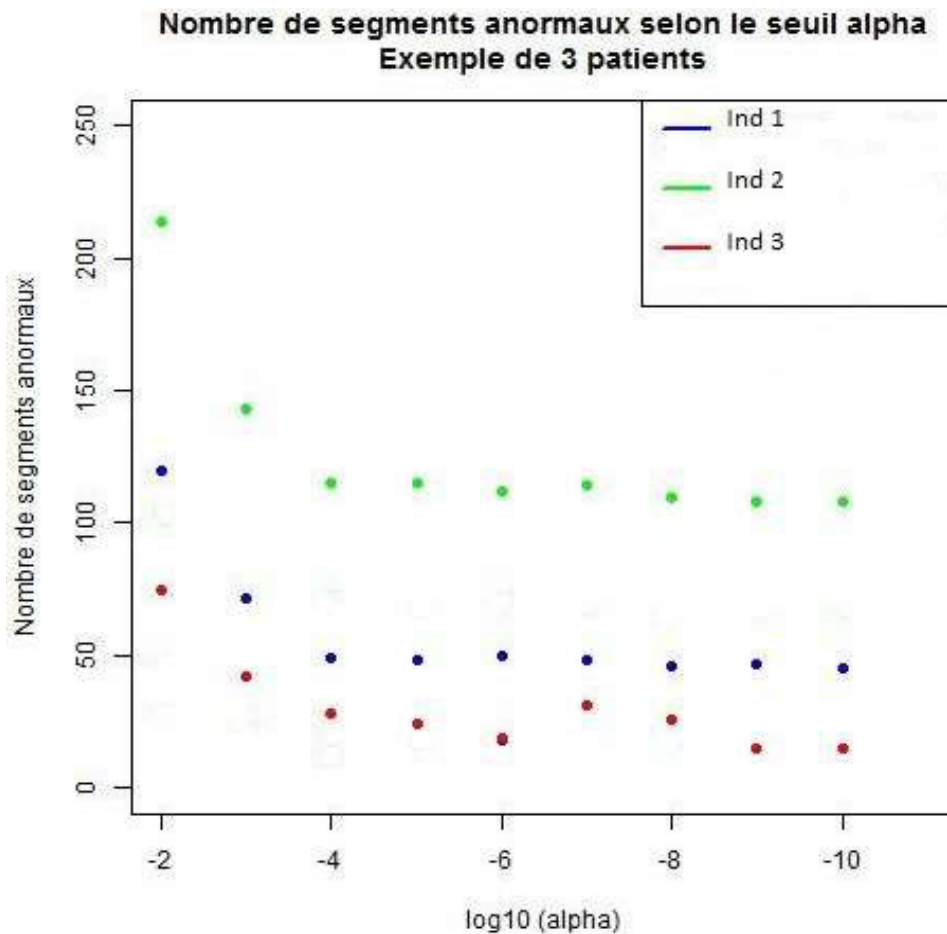
Nous avons débuté les analyses de CGHarray sur tumeurs hypophysaires congelées, que nous avons réalisées sur puce Agilent : SurePrint G3 Human genome CGH+SNP Microarray, 4x180K (Agilent Technologies, Santa Clara, CA, USA).

Le pré-traitement des données a été réalisé en utilisant le package CGHnormaliter (320) sur le logiciel R (<https://www.r-project.org>). Celui-ci réalise une itération des étapes de :

- normalisation par ajustement d'une régression loess sur le MA plot des sondes diploïdes, elles-mêmes sélectionnées suite à
- segmentation par méthode CBS (α fixé à 10^{-6})
- et calling par ajustement d'un mélange de lois normales.

Afin de préciser le choix du seuil α permettant de retenir les points de rupture des segments dans la méthode CBS, nous avons testé pour 3 patients différents seuil α et évalué le nombre de segments retenus. Nous avons observé que le nombre de segments variait de manière significative pour des valeurs élevées de α , alors qu'à partir de 10^{-4} , nous observions une stabilisation (figure 5).

Figure 5: Évaluation du nombre de segments obtenus avec la méthode CBS selon le seuil alpha choisi



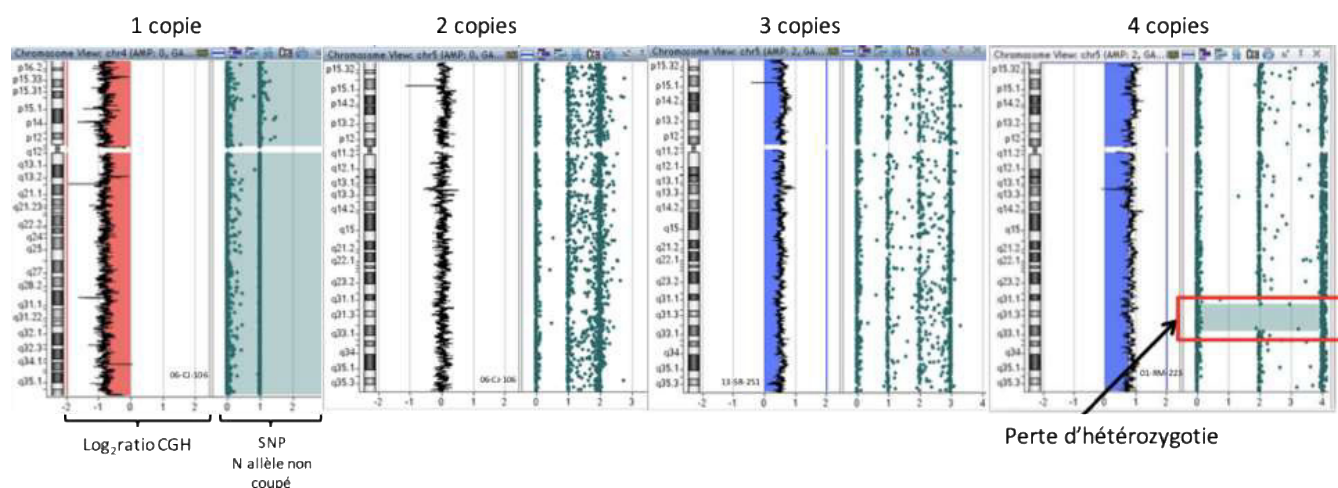
Parallèlement une analyse avec le logiciel CytoGenomics® été réalisée afin de comparer les résultats, en utilisant une centralisation sur le mode, et une segmentation suivant l'algorithme ADM-2 basé sur des tests statistiques de différences de moyennes.

Le critère de qualité de signal utilisé était le DLRS (Derivative Log Ratio Spread) donnant une estimation de la dispersion des valeurs de log2ratio en se basant sur les écarts de log2ratio de deux sondes adjacentes.

L'analyse des SNP a été réalisée en utilisant le logiciel CytoGenomics®. Sur la plateforme Agilent, les SNP sont analysées grâce à une enzyme de digestion qui permet de discerner un allèle clivé et un allèle non clivé. Seul l'allèle non clivé est comptabilisé, le nombre

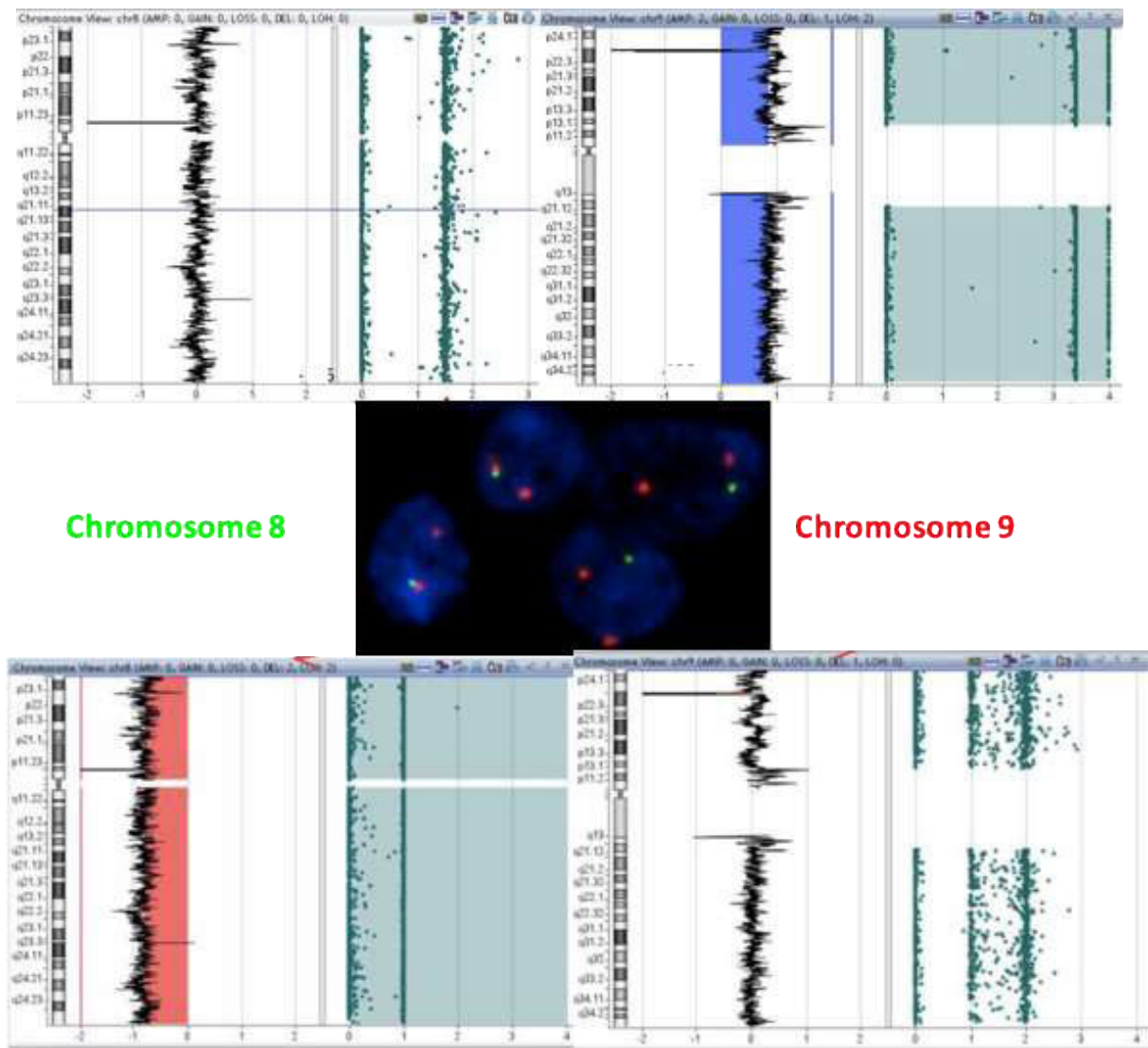
d'allèles clivés étant extrapolés en fonction du nombre de copies estimées par CGHarray (figure 6).

Figure 6 : Exemple de sortie du logiciel Cytogenomics CGH+SNP®



L'analyse des SNPs via le logiciel Cytogenomics® a montré des incohérences entre l'analyse du nombre de copies et l'analyse SNP, alors que la qualité évaluée par le DLRS était satisfaisante. Ainsi, sur la tumeur présentée, le chromosome 8 était présent en 2 copies selon l'analyse en CGHarray, et l'analyse SNP montrait 0 ou 1.5 allèles non coupés. Le chromosome 9 était présent en 4 copies et l'analyse SNP montrait 0, 3.5 ou 4 allèles non coupés (figure 7). Cette constatation nous a amenés à réaliser une analyse de vérification par FISH interphasique (Fluorescent In Situ Hybridization) et à mettre en évidence une anomalie de centralisation. Ainsi, le chromosome 8 était en fait présent en 1 copie et le chromosome 9 en 2 copies (figure 7). Le réaligement du profil CGH avec une centralisation selon la FISH a permis d'interpréter les analyses de SNPs. Il est à noter que l'analyse avec prétraitement via l'utilisation de CGHnormaliter n'avait pas permis de corriger la centralisation.

Figure 7 : Mise en évidence d'une erreur de centralisation avec le logiciel CytoGenomics®



Nous avons réalisé des analyses par FISH sur l'ensemble des tumeurs présentant au moins une altération de taille significative (> 5Mb) et disposant encore du matériel nécessaire. En effet, nous avons présumé qu'en cas de profil ne présentant pas d'altération, il était peu probable que cela corresponde à l'ensemble du génome altéré de manière constante mais plutôt à un génome diploïde sans altération.

Commo *et al* ont eux aussi mis en évidence des altérations de la centralisation et proposé une méthode de centralisation sur « le pic alternatif situé à gauche ». Cependant, cette méthode fait l'hypothèse que les gains sont toujours majoritaires et les critères de sélection du pic alternatif ne sont pas précisés(327).

Nous avons donc établi une méthode de centralisation intégrant les données de la FISH.

3.1.2 Article

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Bardel C.

Centralization errors in comparative genomic hybridization array analysis of pituitary
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RESEARCH ARTICLE

Centralization errors in comparative genomic hybridization array analysis of pituitary tumor samples

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Abstract

Reliable interpretation of comparative genomic hybridization array (aCGH) results requires centralization and normalization of the data. We evaluated the reliability of aCGH centralization by comparing aCGH results (with classical centralization-normalization steps) to fluorescence in situ hybridization (FISH) results. In addition, we propose a method to correct centralization bias. Sixty-six pituitary tumors were analyzed (Agilent aCGH + SNP 4 × 180K microarray). For each tumor, the FISH-based $\log_2(\text{ratios})$ of a subset of chromosomes were compared with the corresponding aCGH raw $\log_2(\text{ratios})$. With our new normalization-centralization process, this difference was added to all $\log_2(\text{ratios})$, before performing loess regression on non-altered probes only. Finally, the mean $\log_2(\text{ratio})$ and the percentage of normal probes were compared between CGHnormaliter and our new FISH-based method. For 11 tumors, FISH results and raw CGH $\log_2(\text{ratios})$ differed significantly. In addition, nine tumors showed discrepancies between results generated by CGHnormaliter and our new-method. Such discrepancies seemed to occur with tumours with many abnormalities (0%–40% normal probes), rather than in those tumours with fewer abnormalities (31%–100% normal probes). Five tumors had too few normal probes to allow normalization. In these tumors, which can exhibit many changes in DNA copy number, we found that centralization bias was frequent and uncorrected by current normalization methods. Therefore, an external control for centralization, such as FISH analysis, is required to insure reliable interpretation of aCGH data.

KEYWORDS

Agilent platforms, CGH array, fluorescent in situ hybridization, normalization, pituitary tumors

1 | INTRODUCTION

Comparative genomic hybridization array (aCGH) is a high-resolution cytogenetic technique that searches for variations in DNA copy number. First developed to detect germ-line variations (constitutional analysis), it is now used to assess variations in somatic DNA copy number likely to change the expression levels of genes involved in tumor development.

The interpretation of aCGH results requires important data pre-treatment steps to correct for bias, specifically normalization and centralization.¹ Normalization deals with intensity bias (ie, differences in fluorescence intensity between fluorochromes) and is often obtained

by applying loess regression to an MA plot (for each probe, $\log_2 \frac{\text{tumor DNA fluorescence}}{\text{reference DNA fluorescence}}$ or L2R, in function of $\log_2(\text{tumor DNA fluorescence}) + \log_2(\text{reference DNA fluorescence})/2$).² Because aCGH results are expressed as a ratio, centralization performs aCGH profile alignment based on zero (ie, two copies of a chromosome per cell) to allow interpretation of L2R.

In constitutional analysis, few DNA alterations are expected; centralization uses loess normalization, then L2R values are centralized on their mean. But, in tumors, gains and losses of DNA are not always balanced; thus loess normalization may introduce a centralization bias, mostly at extreme fluorescence intensities.³ Therefore, other methods have been developed including: (1) Agilent CytoGenomics software

which proposes several centralization methods based either on minimizing the number of aberrant probes or on centralizing on the L2R distribution mode;⁴ (2) loess regression on only "unaltered" probes selected by the larger k-means cluster (popLowess); and⁵ (iii) or by adjusting a mixture model (CGHnormaliter).³

These methods assume that most probes are non-altered, hence most centralization errors occur when most probes are actually altered. Commo et al.⁶ proposed centralizing on an "alternative" peak of L2R density to favor DNA gains, however this method cannot favor DNA deletions and is also not accurate at defining the alternative peak.⁶ Centralization using single nucleotide polymorphism (SNP) data is reliable on Affymetrix platforms,^{7,8} but is not possible for the widely used Agilent platforms⁹ that record only single allele fluorescence in SNP analysis. Hence, when using Agilent platforms¹⁰ for SNP analysis and aberration calling caution is recommended when conducting centralization and normalization steps.

In the present study, Agilent combined SNP/aCGH analysis was used to analyze DNA variations in pituitary tumor cells, and discrepancies were seen between aCGH and SNP results for some tumors. Moreover, SNPs could not be called in some tumor samples, despite an adequate signal quality. Hence, an external control was needed to confirm the number of DNA copies, and fluorescence in situ hybridization (FISH) seemed to offer the solution because it does not require DNA extraction and gives the absolute copy number at a given locus by hybridization of a fluorescent probe.

To our knowledge, centralization bias has seldom been discussed in the literature, and to date there is no optimal normalization method. Herein, we compare a classical centralization-normalization method (namely, CGHnormaliter) against FISH in order to assess the reliability of aCGH centralization in pituitary tumor cells which exhibit many DNA copy-number changes. We also propose a FISH-based centralization-normalization method able to correct centralization bias.

2 | MATERIALS AND METHODS

2.1 | Pituitary tumors

This work is part of PITUIGENE, a French multicenter retrospective analysis of 212 frozen pituitary tumor surgical samples. Here, presuming all chromosomes cannot be simultaneously subject to systematic homogeneous deletion or gain, only tumors with >5 Mb of copy number alterations at initial analysis (CGHnormaliter) and with enough material for FISH analysis were considered. As a result 66 tumors were analyzed, the clinical and pathological characteristics of which are presented in Table 1.

2.2 | Array-CGH

Tumor DNA was extracted from ~15 mg frozen tissue using Masterpure™ Complete DNA and RNA Purification Kit (Epicentre® Biotechnologies, Madison, WI, USA). Array-CGH was performed using SurePrint G3 Human genome CGH + SNP Microarray, 4 × 180K (Agilent Technologies, Santa Clara, CA, USA). After enzymatic digestion by

RsaI and *AluI* enzymes, 1.5 µg of tumor DNA and 0.6 µg of sex-matched human reference DNA (Agilent) were labelled by random priming with Cyanine 5 and Cyanine 3, respectively. Hybridization was performed at 65°C for 24 h and the arrays were scanned using an Agilent DNA Microarray Scanner. Fluorescence was quantified using Feature Extraction 11.5.11 software whose output is the L2R ($\log_2 \frac{\text{tumor DNA fluorescence}}{\text{reference DNA fluorescence}}$) of each probe. Data analysis was carried out using the CytoGenomics 3.0.3.3 software (Agilent).

2.3 | Fish

FISH was performed on interphasic nuclei from frozen tumor-tissue appositions. The tissue used was adjacent to the one used for DNA extraction. For each tumor, at least two centromeric probes (Cytocell, Cambridge, UK) were chosen to hybridize on an aneuploid and a diploid chromosome (as assessed from initial aCGH results). For tumors with only interstitial or telomeric variations specific regional probes were chosen. Two independent analyses of 50 cells were carried out. The FISH L2R of each chromosome was calculated as follows:

$$\text{FISH L2R} = \log_2 \left(\frac{\text{mean number of spots observed per cell}}{\text{standard number of copies}} \right).$$

The standard number of copies was two, except for chromosomes X and Y.

2.4 | CGHnormaliter centralization-normalization

An initial pre-treatment of the data was performed using R package CGHnormaliter:³ it consisted of repeating normalization, segmentation, and calling steps until values stabilized. Normalization was based on loess regression applied only to non-altered probes after segmentation (circular binary segmentation [CBS] $\alpha = 10^{-6}$)¹¹ and calling performed using a mixture model.¹² Segmented L2Rs were defined by assigning the segment mean to each probe. Probes were considered as normal, gained, or deleted after applying a mixture model to the segmented L2R distribution. The proportion of gained or deleted probes per tumor was calculated as: $\frac{\text{number of gained or deleted probes}}{\text{total number of probes analysed}}$. X and Y chromosomes were excluded from the analysis.

2.5 | FISH-based centralization-normalization

2.5.1 | Centralization

For each tumor and each chromosome submitted to FISH, we calculated the difference between FISH L2R and either the median of raw L2Rs for the whole corresponding chromosome (when centromeric probes were used) or the median of raw L2Rs for the corresponding chromosome arm or region (when specific probes were used). Whenever more than one chromosome per tumor was analyzed the following rules were applied: (1) if all differences were consistent (a discrepancy of <0.2 between differences), the mean of the differences per chromosome was considered the "centralization value;" (2) if there was a discrepancy of ≥0.2 between the differences, the chromosome with the closest number of copies to the physiological state in FISH analysis (two copies for autosomes) was selected and its corresponding

TABLE 1 Clinical, histological, and aCGH results for all tumors

Tumor	Sex	Age at diagnosis (years)	Tumor type	Tumor maximal diameter (mm)	Sinus invasion (Y/N)	Ki67 > 3% (Y/N)	raw			CGH normalizer			FISH-based centralization		
							Median L2R [Q1-Q3]	Proportion of 2 copies probes (%) ^a	Median L2R [Q1-Q3]	Proportion of 2 copies probes (%) ^b	Centralization value	Median L2R [Q1-Q3]	Proportion of 2 copies probes (%) ^c		
Tumor 54	F	50	Gonadotroph	38	N	N	-0.05 [-0.06-0.03]	93.5	0 [-0.01-0.02]	93.5	-0.01	0 [-0.01-0.01]	93.5		
Tumor 50	M	78	Gonadotroph	32	N	N	-0.07 [-0.14-0.07]	66.8	0 [-0.08-0.11]	90.9	-0.02	-0.02 [-0.1-0.1]	78.1		
Tumor 21	F	55	Prolactinoma	20	Y	Y	0.03 [-0.01-0.04]	84.3	-0.01 [-0.02-0.01]	88.7	-0.03	0 [-0.02-0.01]	88.8		
Tumor 64	F	59	Somatotroph	NA	Y	Y	0.05 [0.03-0.07]	95.6	0 [-0.01-0.01]	98.0	-0.03	0 [-0.01-0.02]	97.8		
Tumor 66	M	23	Somatotroph	15	Y	Y	-0.02 [-0.03-0]	85.1	0 [0-0.02]	85.3	-0.04	0 [-0.01-0.01]	85.3		
Tumor 23	F	50	Somatotroph	8	N	N	-0.01 [-0.06-0.04]	93.7	-0.01 [-0.05-0.05]	97.0	-0.05	-0.01 [-0.06-0.03]	94.2		
Tumor 31	M	48	Corticotroph	33	Y	N	0.02 [0-0.04]	94.9	0.01 [-0.01-0.02]	95.1	-0.05	0 [-0.02-0.01]	94.6		
Tumor 44	M	60	Prolactinoma	40	Y	Y	0.03 [0-0.04]	85.4	-0.01 [-0.04-0.01]	86.9	-0.05	-0.01 [-0.04-0.01]	86.9		
Tumor 65	M	53	Gonadotroph	30	Y	N	0.02 [-0.01-0.04]	95.1	0 [0-0.01]	95.9	-0.05	0 [-0.01-0.01]	95.9		
Tumor 26	M	46	Gonadotroph	>10	N	N	-0.03 [-0.09-0.02]	98.8	0 [-0.01-0.01]	99.7	-0.07	0 [-0.01-0.01]	99.7		
Tumor 40	F	2	Somatotroph	40	Y	Y	0.1 [0.03-0.11]	64.8	-0.01 [-0.04-0]	72.0	-0.08	-0.01 [-0.04-0]	71.9		
Tumor 45	M	54	Prolactinoma	15	Y	Y	0.06 [-0.01-0.08]	80.4	0.01 [-0.02-0.03]	87.0	-0.09	0 [-0.04-0.02]	87.0		
Tumor 52	F	47	Gonadotroph	>10	Y	N	0.02 [-0.02-0.06]	82.2	-0.01 [-0.04-0.02]	83.4	-0.12	0 [-0.04-0.03]	83.4		
Tumor 13	F	51	Somatotroph	19	N	N	0.03 [-0.06-0.09]	70.6	0 [-0.08-0.04]	77.1	-0.13	-0.05 [-0.14-0.02]	71.1		
Tumor 29	M	28	Corticotroph	14	N	Y	0.06 [-0.01-0.08]	82.6	0.01 [-0.03-0.03]	88.2	-0.14	0 [-0.03-0.01]	88.2		
Tumor 49	F	50	Somatotroph	21	Y	N	0.18 [0.11-0.21]	18.7	-0.02 [-0.07-0.01]	79.1	-0.18	-0.01 [-0.06-0.02]	78.2		
Tumor 51	M	62	Gonadotroph	28	Y	Y	0.06 [0.04-0.08]	93.1	0 [-0.02-0.03]	99.6	-0.22	0 [-0.02-0.02]	98.2		
Tumor 42	M	59	Somatotroph	15	Y	N	0.12 [0.02-0.15]	42.3	0.01 [-0.07-0.03]	76.6	-0.24	-0.01 [-0.09-0.01]	76.6		
Tumor 6	F	38	Corticotroph	17	Y	N	-0.17 [-0.19-0.06]	8.0	0 [-0.02-0.08]	76.4	-0.51	-0.93 [-0.95-0.86]	23.3		
Tumor 8	M	38	Corticotroph	>10	Y	Y	-0.36 [-0.39-0.46]	0.2	0.02 [0-0.92]	56.8	-0.52	-0.91 [-0.94-0.03]	40.1		
Tumor 16	F	21	Somatotroph	18	Y	Y	-0.02 [-0.03-0.01]	97.5	0 [-0.02-0.01]	97.5	0.00	0.01 [-0.01-0.02]	97.5		
Tumor 47	M	52	Corticotroph	10	N	N	0.02 [-0.03-0.04]	93.7	0.02 [-0.03-0.03]	94.9	0.00	0 [-0.04-0.02]	94.7		
Tumor 55	M	39	Somatotroph	30	Y	N	0 [-0.01-0.02]	94.6	0 [-0.02-0.02]	94.7	0.00	-0.01 [-0.02-0.01]	94.7		
Tumor 17	F	34	Prolactinoma	11	N	Y	0 [0-0.01]	98.5	-0.01 [-0.02-0]	99.1	0.01	0 [-0.01-0.01]	98.9		
Tumor 25	M	42	Prolactinoma	17	N	N	-0.11 [-0.15-0.26]	37.0	0.03 [0.01-0.47]	70.8	0.03	0.01 [-0.01-0.46]	70.7		
Tumor 41	M	60	Prolactinoma	18	N	Y	-0.06 [-0.08-0]	83.5	0 [-0.02-0.06]	85.1	0.03	0 [-0.03-0.07]	84.5		

(Continues)

TABLE 1 (Continued)

Tumor	Sex	Age at diagnosis (years)	Tumor type	Tumor maximal diameter (mm)	Sinus invasion (Y/N)	Ki67 > 3% (Y/N)	raw			CGH normaliter			FISH-based centralization		
							Median L2R [Q1-Q3]	Proportion of 2 copies probes (%) ^a	Proportion of 2 copies probes (%) ^b	Median L2R [Q1-Q3]	Proportion of 2 copies probes (%) ^a	Proportion of 2 copies probes (%) ^b	Centralization value	Median L2R [Q1-Q3]	Proportion of 2 copies probes (%) ^a
Tumor 30	M	57	Corticotroph	14	Y	N	-0.01 [-0.05-0.03]	95.0	0 [-0.04-0.04]	99.1	0.04	0 [-0.04-0.03]	97.4		
Tumor 62	F	32	Somatotroph	>10	Y	N	-0.12 [-0.14-0]	58.6	-0.01 [-0.02-0.11]	76.4	0.04	-0.01 [-0.02-0.12]	76.4		
Tumor 28	F	39	Gonadotroph	21	Y	N	-0.03 [-0.06-0]	99.8	0 [-0.03-0.02]	99.6	0.05	0 [-0.02-0.02]	99.5		
Tumor 39	F	45	Corticotroph	4	N	N	0.02 [-0.03-0.06]	80.8	-0.01 [-0.03-0.02]	87.4	0.05	-0.02 [-0.05-0]	87.3		
Tumor 18	F	33	Gonadotroph	>10	Y	Y	0 [-0.04-0.03]	91.7	0 [-0.02-0.02]	92.0	0.06	-0.01 [-0.03-0.01]	91.9		
Tumor 38	M	42	Somatotroph	17	Y	N	-0.18 [-0.19-0.33]	3.5	-0.01 [-0.02-0.36]	73.5	0.06	0.01 [-0.01-0.38]	73.0		
Tumor 56	F	60	Somatotroph	20	Y	N	-0.13 [-0.15-0.27]	47.4	0 [-0.02-0.51]	72.8	0.08	0 [-0.02-0.51]	72.1		
Tumor 37	M	54	Gonadotroph	17	N	N	-0.09 [-0.12-0.06]	80.2	0 [-0.02-0.02]	90.2	0.09	0 [-0.02-0.02]	90.0		
Tumor 24	F	35	Corticotroph	25	N	N	-0.16 [-0.19-0.35]	3.7	0.02 [-0.01-0.53]	53.7	0.11	0.03 [0-0.56]	53.5		
Tumor 36	M	72	Prolactinoma	10	Y	Y	-0.12 [-0.16-0.31]	35.1	0.02 [-0.01-0.46]	61.8	0.12	0.02 [-0.01-0.46]	61.8		
Tumor 63	F	50	Somatotroph	27	Y	N	-0.15 [-0.17-0.33]	29.8	0 [-0.01-0.56]	67.9	0.12	0 [-0.01-0.56]	67.9		
Tumor 14	M	63	Corticotroph	13	N	N	-0.15 [-0.17-0.12]	28.2	0 [-0.01-0.03]	80.3	0.13	0.01 [0-0.04]	80.2		
Tumor 22	M	68	Prolactinoma	28	Y	Y	-0.19 [-0.24-0.32]	4.4	0.06 [0.01-0.59]	51.4	0.13	0.05 [-0.01-0.58]	50.7		
Tumor 43	F	69	Corticotroph	25	Y	N	0.2 [-0.19-0.27]	8.1	0.44 [-0.01-0.49]	39.5	0.13	0.42 [-0.02-0.47]	39.4		
Tumor 48	F	69	Corticotroph	22	N	N	-0.19 [-0.23-0.3]	5.1	0.02 [0-0.55]	58.0	0.13	0.02 [-0.01-0.55]	58.0		
Tumor 59	M	43	Prolactinoma	>10	Y	N	-0.11 [-0.19-0.33]	17.1	0.04 [0-0.52]	54.8	0.13	0.05 [0-0.52]	54.6		
Tumor 15	M	45	Prolactinoma	20	Y	N	-0.12 [-0.17-0.34]	21.8	0.04 [-0.03-0.46]	63.6	0.15	0.05 [-0.02-0.47]	60.7		
Tumor 20	M	46	Corticotroph	20	Y	N	-0.11 [-0.14-0.33]	54.4	0.03 [0-0.55]	69.8	0.15	0.01 [-0.01-0.53]	69.8		
Tumor 46	M	39	Prolactinoma	35	N	Y	-0.15 [-0.23-0.31]	9.7	0.05 [-0.02-0.52]	57.3	0.15	0.05 [-0.02-0.52]	56.5		
Tumor 58	M	47	Corticotroph	13	N	N	-0.18 [-0.27-0.26]	0.9	0.13 [0.02-0.59]	50.7	0.16	0.1 [-0.01-0.56]	50.7		
Tumor 60	M	33	Prolactinoma	>10	Y	N	-0.19 [-0.23-0.27]	6.2	0.03 [0-0.51]	57.2	0.17	0.02 [-0.01-0.5]	57.2		
Tumor 53	M	26	Prolactinoma	52	Y	N	-0.19 [-0.27-0.26]	5.9	0.06 [-0.02-0.54]	50.6	0.19	0.06 [-0.02-0.53]	49.6		
Tumor 5	M	51	Somatotroph	16	N	N	-0.02 [-0.24-0.29]	42.5	0.03 [-0.19-0.37]	47.7	0.23	0.21 [-0.01-0.52]	11.8		
Tumor 32	F	48	Prolactinoma	40	Y	N	0.11 [-0.32-0.19]	14.0	0.49 [-0.01-0.54]	44.1	0.24	0.5 [0-0.54]	44.1		
Tumor 57	M	28	Somatotroph	35	Y	Y	0.1 [-0.34-0.19]	9.9	0.47 [0.01-0.55]	46.8	0.24	0.47 [0.01-0.55]	46.8		
Tumor 33	M	66	Prolactinoma	22	Y	Y	0.05 [-0.27-0.29]	8.2	0.31 [-0.01-0.54]	43.6	0.25	0.35 [0.03-0.57]	43.3		

(Continues)

TABLE 1 (Continued)

Tumor	Sex	Age at diagnosis (years)	Tumor type	Tumor maximal diameter (mm)	Sinus invasion (Y/N)	K67 > 3% (Y/N)	raw		CGH normalizer		FISH-based centralization		
							Median L2R [Q1-Q3]	Proportion of 2 copies probes (%) ^a	Median L2R [Q1-Q3]	Proportion of 2 copies probes (%) ^a	Centralization value	Median L2R [Q1-Q3]	Proportion of 2 copies probes (%) ^a
Tumor 61	F	54	Somatotroph	30	Y	N	0.09 [-0.29-0.2]	10.1	0.39 [0-0.49]	47.1	0.25	0.38 [-0.01-0.48]	46.9
Tumor 19	F	29	Prolactinoma	19	Y	Y	-0.03 [-0.25-0.25]	4.2	0.35 [-0.02-0.53]	49.9	0.26	0.36 [-0.01-0.53]	49.7
Tumor 35	F	59	Corticotroph	18	Y	N	-0.01 [-0.09-0.01]	75.5	0.04 [-0.05-0.06]	73.3	0.26	0.07 [-0.02-0.09]	76.1
Tumor 27	F	25	Prolactinoma	10	N	N	0.1 [-0.32-0.2]	7.0	0.44 [0-0.53]	49.7	0.27	0.44 [0-0.53]	49.6
Tumor 34	M	39	Somatotroph	15	Y	N	-0.25 [-0.38-0.11]	29.5	0.12 [-0.01-0.56]	50.7	0.28	0.12 [-0.01-0.56]	50.7
Tumor 1	M	45	Corticotroph	30	Y	N	-0.36 [-0.39-0.49]	2.7	0.02 [-0.01-0.88]	56.5	0.31	0.03 [0-0.89]	56.7
Tumor 3	M	39	Somatotroph	19.4	N	N	-0.02 [-0.09-0.24]	47.1	0.01 [-0.04-0.33]	47.3	0.32	0.48 [0.44-0.8]	24.3
Tumor 2	M	24	Prolactinoma	35	N	Y	-0.13 [-0.15-0.35]	44.1	0 [-0.02-0.48]	61.5	0.32	0.01 [-0.01-0.49]	61.3
Tumor 4	M	45	Prolactinoma	33	Y	N	0 [-0.43-0.22]	41.5	0.51 [0.03-0.75]	31.1	0.45	0.51 [0.02-0.75]	31.1
Tumor 7	M	44	Prolactinoma	47	Y	N	0.01 [-0.4-0.28]	31.1	0 [-0.46-0.34]	32.8	0.51	0.52 [0.07-0.86]	26.4
Tumor 9	F	21	Prolactinoma	17	N	N	0.04 [-0.22-0.29]	22.2	0.25 [0-0.49]	30.7	0.58	0.62 [0.36-0.87]	9.0
Tumor 12	M	53	Prolactinoma	24	Y	Y	-0.01 [-0.25-0.16]	39.5	-0.01 [-0.26-0.18]	46.8	0.74	0.72 [0.49-0.89]	6.3
Tumor 10	F	58	Prolactinoma	>10	Y	Y	0.05 [-0.29-0.27]	43.9	0 [-0.37-0.26]	46.7	0.84	0.89 [0.55-1.11]	2.9
Tumor 11	F	20	Somatotroph	22	N	N	-0.03 [-0.07-0.24]	55.2	0 [-0.04-0.26]	55.2	0.87	0.84 [0.8-1.11]	0.0

^aProportion of probes with an L2R between -0.15 and 0.14 after the CBS segmentation process. Total of 99659 probes.

^bProportion of normal probes after the CGH-normalizer calling process. Total of 99659 probes.

TABLE 2 Raw, CGHnormaliter, and FISH-based centralization-normalization results for tumors with high centralization values

Tumor	Chromosome	FISH L2R	Median raw L2R	Pre-segmentation median CGHnormaliter L2R	Pre-segmentation median FISH-based normalization L2R	Centralization value
Tumor 1 ^a	8	-0.081	-0.371	0.012	0.016	0.31
Tumor 2 ^a	18	-0.700	-0.962	-0.809	-0.800	0.32
Tumor 4 ^a	13	-0.01	-0.40	0.03	0.03	0.45
Tumor 3	16	-0.157	-0.480	-0.477	0.000	0.32
Tumor 5	15	-0.31	-0.54	-0.51	-0.34	0.23
Tumor 7	10	0.00	-0.509	-0.562	-0.034	0.51
Tumor 9	13	-0.014	-0.595	-0.353	-0.014	0.58
Tumor 10	10	0.556	-0.324	-0.398	0.514	0.84
Tumor 11	6	0.485	-0.384	-0.356	0.485	0.87
Tumor 12	11	0.02	-0.72	-0.76	-0.00	0.74
Tumor 8 ^b	9	-0.105	0.435	0.919	-0.030	-0.52
Tumor 6 ^b	7	0.202	0.709	0.954	0.014	-0.51

^aRaw centralization error corrected by CGHnormaliter.

^bRaw centralization errors accentuated by CGHnormaliter. FISH: Fluorescent in situ hybridization. L2R: Log₂ ratio of tumor DNA fluorescence/reference DNA fluorescence. CGH: comparative genomic hybridization.

difference was considered the "centralization value." Indeed, this non-altered chromosome is probably less likely to exhibit L2R differences between cells, which can be caused by the presence of several cell clones or by a mixture of both normal and tumor cells in the sample.

Finally, centralized L2R values were computed for each probe:

$$\text{Centralized L2R} = \log_2 \frac{\text{raw patient probe fluorescence}}{\text{reference probe fluorescence}} + \text{centralization value}$$

2.5.2 | Normalization of FISH centralized data

For each tumor, after L2R centralization based on FISH data, loess regression was estimated on non-altered probes only, so to avoid centralization on the mean and over-correction of alterations due to a side-effect of loess regression on all probes. This probe selection step relied on an in-house method inspired by popLowess,⁵ and not on CGHnormaliter which assumes that 80% of the probes are in the "non-altered" class (in their starting values). This selection consisted of dividing the L2Rs of each tumor into three groups (deleted, normal, and gained) using k-means clustering (for details, see Supplementary data). The loess regression was then estimated on the centralized intensities of the probes present in the closest cluster to zero (normal group), and this was finally used to normalize all centralized L2Rs. Whenever the number of probes close to zero was not large enough to create a cluster and allow estimation of loess regression, normalization was omitted, and only the centralized L2Rs were used (X and Y chromosomes excluded).

2.5.3 | Segmentation and calling of FISH centralized-normalized data

Segmentation using CBS was then performed ($\alpha = 10^{-6}$) on centralized and normalized L2Rs. Segmented L2Rs were defined by

assigning to each probe its segment mean. When the segmented L2R was higher than 0.14 ($\log_2(0.80 \times \frac{1}{2} + 0.20 \times \frac{3}{2})$), the probe was considered as gained. When the segmented L2R was lower than -0.15 ($\log_2(0.80 \times \frac{1}{2} + 0.20 \times \frac{3}{2})$), the probe was considered as deleted. Therefore, we allowed for a 20% mosaic consideration. Here too, the proportion of gained or deleted probes was calculated as $\frac{\text{number of gained or deleted probes}}{\text{total number of probes analysed}}$.

All analyses were performed using R software version 3.0.1.

3 | RESULTS

3.1 | FISH-based centralization

The number of chromosomes analyzed per patient ranged from 1 to 7 (median [1st-3rd quartile]: 2).^{2,3}

Twenty-four tumors showed disparities between chromosomes in the difference between the FISH L2R and the corresponding median of the raw L2R. In these tumors, only the chromosome whose number of copies was the closest to two with FISH analysis was used for centralization.

The median of "centralization value" of raw L2Rs was 0.09 [-0.03; +0.24] (range -0.52 to +0.87). Twenty-six tumors showed a centralization value >0.15 and 11 (17%) displayed a marked centralization abnormality (centralization value >0.30) (Table 2). For each tumor the repartition of L2R depending on normalization method is presented in Table 1.

3.1.1 | Comparison between CGHnormaliter and FISH-based centralization-normalization

The proportion of normal probes was generally higher with CGHnormaliter than with FISH-based normalization-centralization; the mean

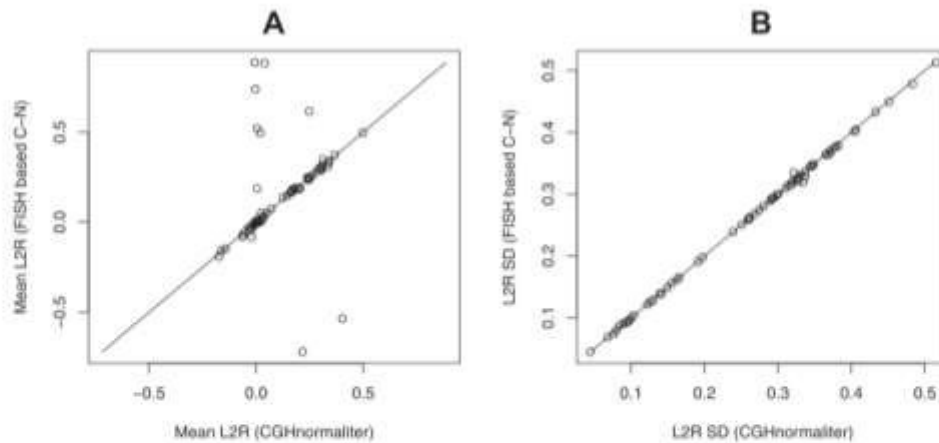


FIGURE 1 A, Comparison per tumor of the mean L2Rs obtained with FISH-based centralization-normalization versus CGHnormaliter. B, Comparison per tumor of the standard deviations (SD) of the L2Rs obtained with FISH-based centralization-normalization (C-N) versus CGHnormaliter

(\pm standard deviation [SD]) proportion of normal probes per patient was 70% (\pm 21) and 65% (\pm 27), respectively.

In each tumor, the mean values of all segmented L2Rs ranged from -0.17 to $+0.50$ (median 0.04) with CGHnormaliter and from -0.72 to $+0.89$ (median 0.16) with FISH-based centralization-normalization. These means tended to be positive, thus gains were more numerous than losses.

For most tumors, the segmented L2R means from the two methods were similar (Figure 1A). Nine tumors (14%) presented marked differences (>0.15 , range: -0.89 to $+0.94$) between the two means (Table 2, Tumors 3 and 5–12). Hence, CGHnormaliter tended to shrink the L2R to zero, whereas FISH-based centralization-normalization allowed for more variability across patients.

Figure 2 shows the aCGH profiles of Tumors 8 and 10. For Tumor 8, FISH showed only one copy of chromosome 8 in 90% of tumor cells and two copies of chromosome 9 in 79% of tumor cells, whereas CGHnormaliter indicated that chromosome 8 was unaltered and chromosome 9 was gained. For Tumor 10, FISH showed three copies of chromosome 10 in 81% of cells analyzed, whereas CGHnormaliter indicated that this chromosome had been deleted.

To check whether FISH-based centralization-normalization changed only centralization and did not alter L2R variability in given tumors, we compared the SDs of the segmented L2Rs given by the two methods. Figure 1B shows that there is no difference between CGHnormaliter and FISH-based centralization-normalization; only two tumors showed slight differences. These two tumors did not undergo normalization after FISH-based centralization, because of an insufficient number of normal probes. Hence, our in-house normalization did not alter the intra-patient variability of L2Rs. Three other tumors were not normalized, because of an insufficient number of normal probes.

Centralization discrepancies seemed to occur when a majority of the probes were altered. Indeed, those tumors that showed discrepancies between the two normalization methods had between 0% and 40% normal probes (mean: 16%, SD: 13, min: 0%, max: 40%, computed with FISH-based centralization-normalization), and those that showed no discrepancies between the normalization methods had between

31% and 100% normal probes (mean: 73%, SD: 19, min: 31%, max: 100%).

Table 2 shows that eight of the nine tumors whose centralization value differed between the two normalization methods already displayed a marked centralization abnormality in raw L2R versus FISH (centralization value >0.3), the ninth (Tumor 5) had a centralization value <0.3 ($=0.23$).

In two cases (Tumors 6 and 8, Table 2), CGHnormaliter increased the centralization bias. In other cases, the bias was partially (Tumor 9) or even totally (Tumors 1, 2, and 4) corrected. The tumors corrected by CGHnormaliter had a higher proportion of normal probes (mean: 50%, SD: 16, min: 31%, max: 61%) than tumors whose centralization errors could not be corrected by CGHnormaliter (mean: 17%, SD: 14, min: 0, max: 40%).

4 | DISCUSSION

Our work highlights the prevalence of centralization bias in aCGH analysis of tumor cells with a high proportion of copy number alterations. In order to correct this bias, we propose a centralization-normalization method based on FISH data. In pituitary tumors (cells with highly altered DNA), strong discrepancies were seen between raw aCGH and FISH results in nearly 17% of the tumors, thus highlighting the key role of centralization in aCGH analysis.

Centralization errors were first suspected when SNP and CGH analyses were found discordant with Agilent CytoGenomics software. Here, we confirm the existence of such errors, and show that they often occur in tumors with a high proportion of altered probes. We believe this bias occurs because aCGH is not a per-cell analysis. It requires DNA extraction and hybridization of the same weights of patient and reference DNA. When a cell possesses too many copy alterations, its DNA weight is significantly changed. Hybridizing equal weights acts then like a comparison between two different cell quantities, which may confound the interpretation of the number of copies present in patient tumor cells.

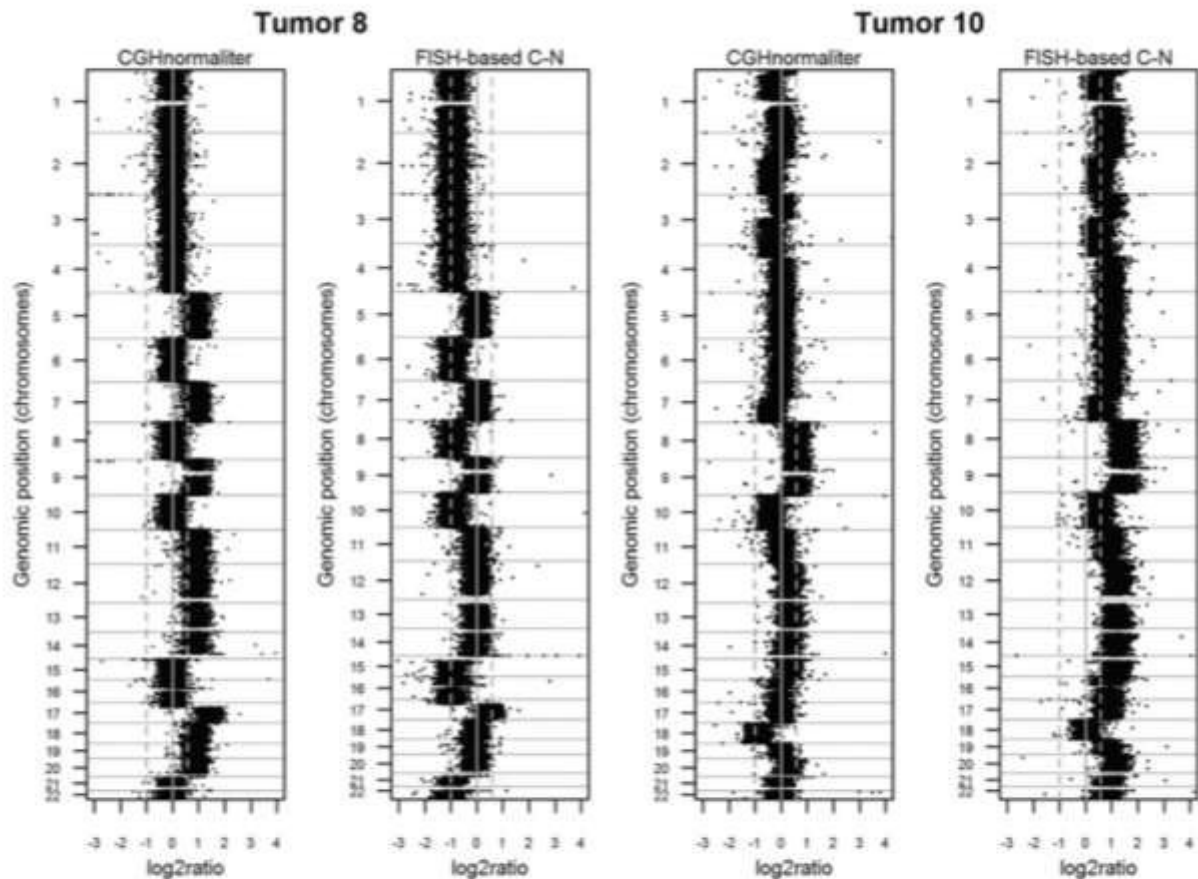


FIGURE 2 The aCGH profiles obtained using CGHnormaliter and FISH-based centralization-normalization (C-N) for Tumors 8 and 10

When comparing raw L2R with FISH results, 11 tumors showed noticeable discrepancies. Comparing FISH-based centralization-normalization with CGHnormaliter, eight of these 11 tumors showed centralization abnormalities, of which two showed great disparities. The calling step is probably involved. This step consists of applying a mixture model to centralize the L2R using non-altered probes supposing, to start with, that 80% of the probes are in the normal group (the mean L2R of this group equals zero). This calling step cannot correct the centralization when a majority of probes are gained or deleted; it tends to rank the altered probes in the normal group.

Commo et al.⁶ have already highlighted the lack of methods available that can be adapted to Agilent data, whereas many publications discuss centralization methods for Affymetrix data. These authors proposed a method based on L2R density analysis and centralization on a major "alternative left peak", but they did not consider tumor samples containing many deletions (eg. DNA from pituitary tumor cells). Moreover, there is currently no single or reliable criterion that justifies the use of this "alternative left peak" for centralization. Commo et al.⁶ proposed to use this peak when its height is greater than a given proportion of the major peak (50%). In our tests, centralization differed highly according to the threshold set to define the alternative peak.

Array-CGH provides a relative measure of DNA quantity according to the genomic position, whereas FISH is an absolute measure. Thus, in

the absence of a gold standard for CGH interpretation, we considered FISH data to be more reliable. Tumor heterogeneity is the major limitation to the use of FISH. The material we used for aCGH and FISH came from the same samples, but not strictly the same cells, suggesting tumor mosaicism (genomic alterations affecting only some tumor cells) or a mixture of tumor and non-tumor cells in the sample. Therefore, we cannot exclude the possibility of different DNA alterations between FISH- and aCGH-analyzed cells. Moreover, the centralization value may depend on the chromosome analyzed. When the difference between centralization values of the chromosomes analyzed in a tumor sample was over 0.2, only the chromosome with the number of copies closest to two was analyzed, because it was probably less concerned by mosaicism. We also tested a threshold value of 0.1, but it did not significantly change the results.

After centralization, normalization should correct for intensity bias. However, the widely used loess regression on all centralized L2Rs may alter the previous centralization step.^{3,5,6} To normalize the aCGH profiles while maintaining a non-biased centralization, we selected a set of normal probes by *k*-means clustering as in the popLoess method,⁵ but instead of considering the major set as that of normal probes, we chose the set with the closest L2R to zero and applied the widely used loess regression to this set of normal probes.

We also tested the Agilent CytoGenomics software. Its default centralization algorithm could not correct centralization abnormalities,

and in fact it gave more inadequate centralizations than CGHnormaliter (11 tumors vs. 9). The centralization method of this workbench consists of adding to all L2Rs a constant value that minimizes the number of aberrant probes. This leads to centralization errors when the majority of probes are subject to gains and losses. A manual centralization may help, whereby centralization can be adjusted according to FISH results and the calling process can re-start. The new "zero" peak of the L2R distribution can be chosen based on the graphical representation of L2R density, and then a value added to all L2Rs. However, this also requires FISH and will fail in the absence of probes in two copies. Indeed, this happened with one of our samples: one tumor had more than two copies of all the chromosomes (see Supplementary data).

5 | CONCLUSION

In our set of tumors, which had highly altered DNA, centralization errors in aCGH tumor analysis occurred quite frequently. In such cases, an absolute number of copies should be obtained by FISH analysis or karyotyping. Here, we propose a centralization method based on reliable FISH data, and in addition adapt a normalization method that preserves this centralization whilst correcting the intensity bias.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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SUPPLEMENTARY DATA

Selection of non-altered probes

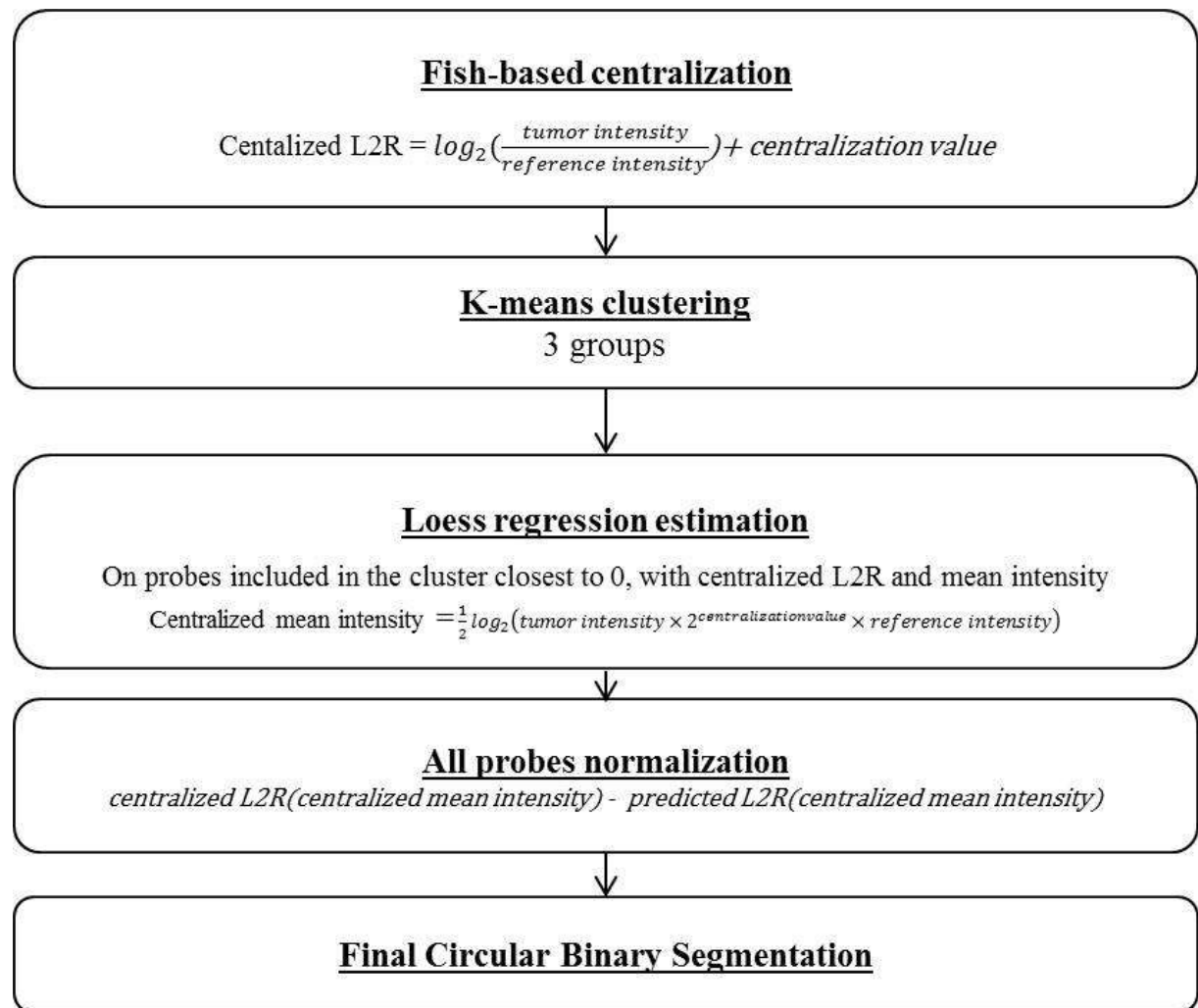
In a first step, we defined the initial means (centers) of the three groups after iterative clustering (R function: k-means) to limit the variability due to the random choice of the initial-group centers. We retained the three most frequent centers obtained after iterative k-means clustering of segment L2Rs of each tumor (for each probe, mean of corresponding segment from circular binary segmentation).

In a second step, we defined the optimal number of groups (1 to 3). The centers were merged and their mean calculated when they were less distant than 0.3. This allowed considering only 1 or 2 centers in case of entirely normal profile, absence of deleted probes, etc. The final k-means clustering using these selected centers (one to three) was then performed on segment L2R. Finally, loess regression was estimated on centralized M and A values of the probes whose center is the closest to 0 in the cluster before normalizing all centralized L2Rs:

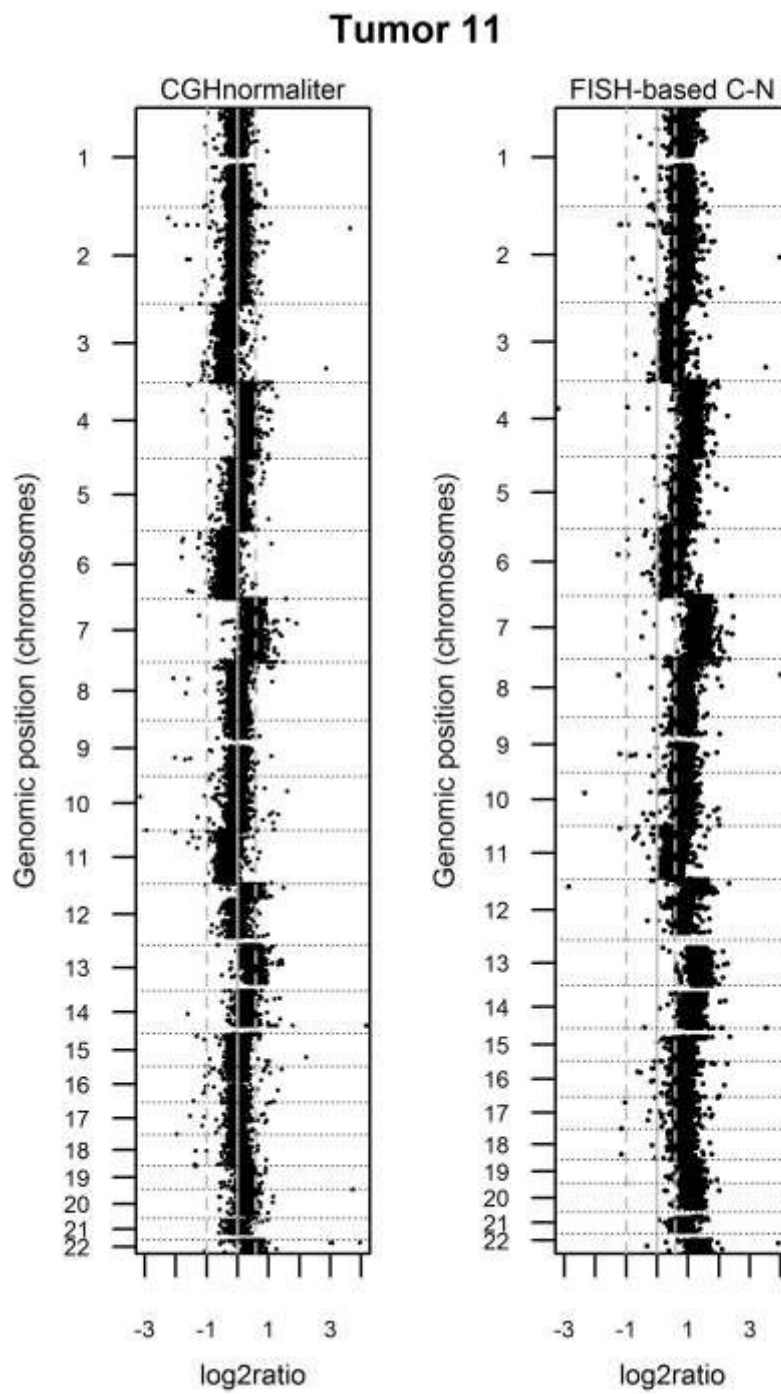
$$\text{Centralized M} = \log_2 \left(\frac{\text{raw fluorescence} \times 2^{\text{centralization value}}}{\text{reference fluorescence}} \right)$$

$$\text{Centralized A} = \frac{1}{2} \log_2 \left(\text{raw fluorescence} \times 2^{\text{centralization value}} \times \text{reference fluorescence} \right)$$

Supplementary figure 1 - FISH-based centralization- normalization steps



Supplementary figure 2 - Profiles obtained with CGHnormaliter and FISH-based centralization-normalization (C-N) for Tumor 11.



3.1.3 Discussion

En utilisant l'analyse par FISH, nous avons identifié 9/66 tumeurs dont la centralisation était franchement erronée en utilisant CGHnormaliter ou Cytogenomics®. Parmi ces 9 tumeurs, 7 présentaient une majorité de gains, 2 présentaient une majorité de délétions. Ces anomalies de centralisation surviennent sur des génomes très altérés. En effet, la CGH array est une technique de mesure relative et nous supposons que ces anomalies sont liées à la modification de la masse de l'ADN par cellule en cas de remaniements multiples. Par exemple, un génome entièrement triploïde verra la masse de l'ADN multipliée par 1.5 dans chaque cellule. Or, dans la technique de CGH array, la quantité d'ADN initiale est sélectionnée sur la masse donc la quantité de cellules analysées du génome référent ne sera pas identique à celle du génome tumoral. Ainsi, le \log_2 ratio réel devrait correspondre à $\log_2 \left(\frac{\text{fluorescence ADN tumeur observée} \times 1.5}{\text{fluorescence ADN référence observée}} \right) = \log_2 \text{ratio observé} + \log_2(1.5)$. Le \log_2 ratio observé correspond à un profil entièrement décalé.

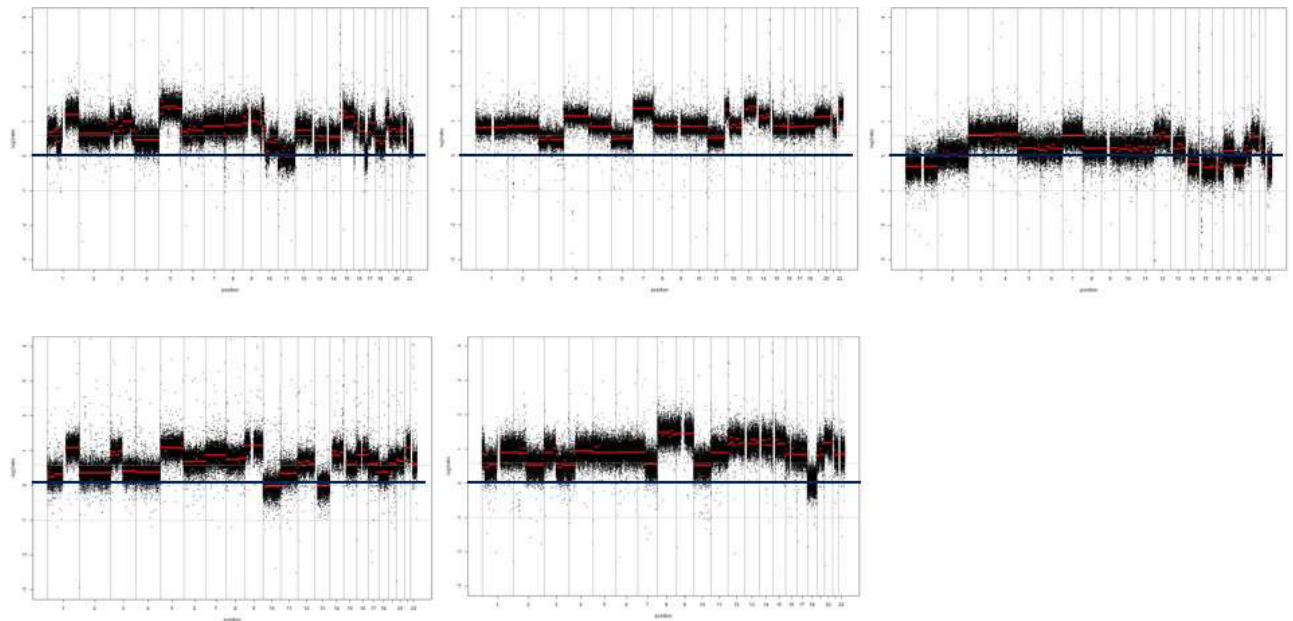
Les méthodes de normalisation classiques ne sont pas en mesure de corriger ces biais de centralisation et nous n'avons pas trouvé d'autre solution que de réaliser un contrôle externe par FISH. Il est à noter que les puces de la plateforme Affymetrix ne posent pas ce type de problème. En effet, dans les puces Affymetrix, l'analyse SNP est au centre de la technique et pour chaque SNP, les 2 allèles sont hybridés à des oligonucléotides et détectés. Le nombre de copies est estimé à partir de l'analyse des SNP et non par une comparaison à un ADN référence. Dans les puces Agilent, seul l'allèle non coupé des SNPs est hybridé et détecté, le nombre du second allèle étant estimé à partir du nombre de copies.

La limite principale de la technique de centralisation mise au point concerne l'hétérogénéité tumorale. En effet, l'analyse FISH nécessite l'utilisation de matériel tumoral qui n'est pas exactement celui utilisé pour l'analyse de CGHarray. Ainsi, une différence entre les populations tumorales ne peut pas être exclue. Pour limiter au maximum ce biais, nous avons sélectionné pour l'analyse FISH un fragment adjacent à celui utilisé pour l'extraction d'ADN utilisé pour l'analyse par CGH-array. De plus, les analyses par FISH ont été réalisées de manière indépendante par 2 opérateurs qui ont

rapporté pour chaque chromosome analysé 50 cellules chacun, et nous avons utilisé le nombre moyen de copies observées. Pour certaines tumeurs, nous avons observé des différences de normalisation selon le chromosome utilisé faisant suspecter de multiples populations tumorales. Dans ce cas, nous avons utilisé pour la centralisation le chromosome présent en 2 copies, supposant qu'il était sujet à une variabilité intercellulaire moins importante.

Par ailleurs, la normalisation n'était pas possible pour les tumeurs ne présentant pas suffisamment de sondes diploïdes pour l'estimation de la régression loess sur celles-ci. Ainsi, 5 tumeurs étaient concernées et n'ont pas été normalisées (figure 8).

Figure 8 : Profil CGH des tumeurs sans normalisation (en raison d'un nombre insuffisant de sondes diploïdes)



3.2 Analyse pronostique

3.2.1 Contexte

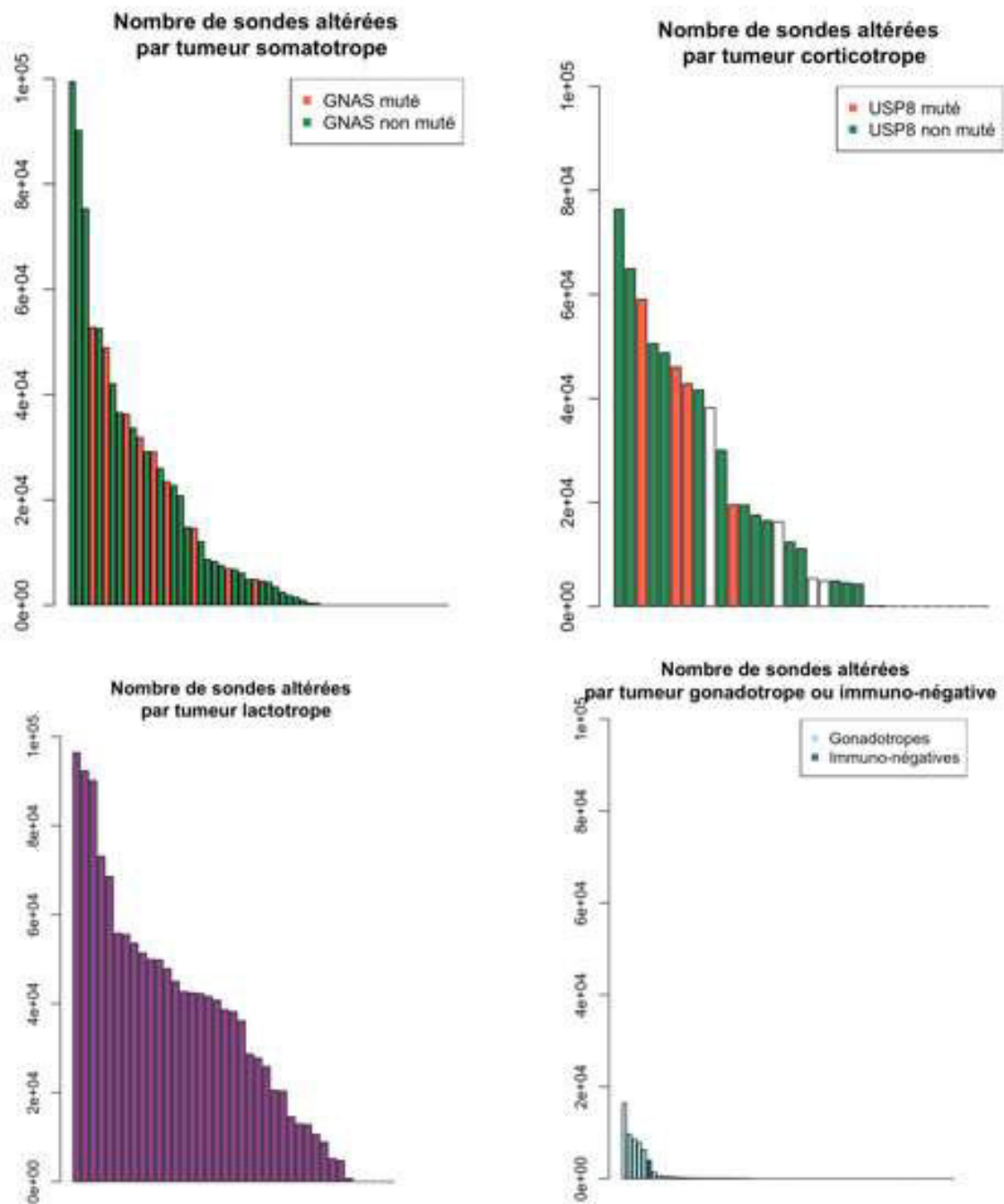
Comme cité précédemment, la littérature a montré que le taux de mutations ponctuelles était globalement très faible dans les tumeurs hypophysaires, alors que les altérations chromosomiques et notamment les variations du nombre de copies étaient fréquentes. La plupart des études distinguent 2 types de tumeurs, certaines avec une grande quantité d'altérations et d'autres avec très peu ou pas d'altérations. Le seuil permettant de définir une tumeur altérée ou non varie selon les auteurs. Hage *et al.* définissaient les tumeurs peu altérées présentant < 12% du génome altéré, et celles altérées présentant > 24% du génome altéré(71). Ben Shlomo *et al* ont utilisé un score (SCNA score utilisant la somme des gains et délétions) en choisissant comme seuils 0,2 et 2 pour définir 3 degrés d'altérations (72). Song *et al.* ont défini les tumeurs altérées présentant > 80% du génome altéré, vs non altérées < 10%(69). Les seuls facteurs associés à la quantité d'altérations retrouvés dans la littérature sont le caractère fonctionnel et l'absence de mutation de *GNAS* dans les tumeurs somatotropes.

Seules 2 études ont analysé le lien entre altérations chromosomiques et pronostic en utilisant un suivi standardisé homogène et une définition de la récurrence : Buch *et al.* en analyse LOH sur 96 tumeurs (311) et Wierinckx *et al* sur 13 tumeurs lactotropes en CGHarray (198).

L'objectif de notre travail était de rechercher un lien entre variations du nombre de copies, phénotype et pronostic des tumeurs hypophysaires sur une cohorte de taille significative ayant un suivi homogène. Après exclusion des données manquantes et tumeurs dont la qualité ne permettait pas l'analyse, 195 tumeurs bénéficiant d'un suivi d'au moins 5 ans ont été analysées. Dans cette cohorte, la quantité d'altérations s'associait de manière évidente au type tumoral. La quantité d'altérations par tumeur était continue, ne nous permettant pas de définir clairement un seuil pour classer les tumeurs comme dans les études précédemment citées (figure 9) ; que ce soit sur l'ensemble des tumeurs ou par type tumoral.

L'analyse pronostique est détaillée dans l'article ci-après.

Figure 9 : Barplots présentant la quantité de sondes altérées par tumeur par type tumoral



3.2.2 Article

Chromosomal instability in the prediction of pituitary tumor prognosis.

Hélène Lasolle, Mad-Hélénie Elsensohn, Anne Wierinckx, Eudeline Alix, Clément Bonnefille, Alexandre Vasiljevic, Christine Cortet, Bénédicte Decoudier, Nathalie Sturm, Stephan Gaillard, Amandine Ferrière, Pascal Roy, Emmanuel Jouanneau, Philippe Bertolino, Claire Bardel, Damien Sanlaville, Gérald Raverot.

En révision

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[Click here to view linked References](#)

1 **Chromosomal instability in the prediction of pituitary neuroendocrine tumors prognosis.**

2

3

Running title: chromosomal instability of pituitary neuroendocrine tumors

4

5

Hélène Lasolle, Mad-Hélène Elsensohn, Anne Wierinckx, Eudeline Alix, Clément Bonnefille, Alexandre

6

Vasiljevic, Christine Cortet, Bénédicte Decoudier, Nathalie Sturm, Stephan Gaillard, Amandine Ferrière, Pascal

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43 **Abstract**

1
2 44 The purpose of this study was to analyze the impact of copy number variations (CNV) on sporadic pituitary
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4 45 neuroendocrine tumors (PitNETs) prognosis, to identify specific prognosis markers according to the known
5
6 46 clinico-pathological classification.

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8
9 47 CGH array analysis was performed on 195 fresh-frozen PitNETs (56 gonadotroph, 11 immunonegative, 56
10
11 48 somatotroph, 39 lactotroph and 33 corticotroph), with 5 years post-surgery follow-up (124 recurrences),
12
13 49 classified according to the five-tiered grading classification (invasion, Ki-67, mitotic index and p53 positivity).
14
15 50 Effect of alterations on recurrence was studied using logistic regression models. Transcriptomic analysis of 32
16
17 51 lactotroph tumors was performed.

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20 52 The quantity of CNV was dependent on tumor type: higher in lactotroph (median(min-max)=38%(0-97) of
21
22 53 probes) compared to corticotroph (11%(0-77)), somatotroph (5%(0-99)), gonadotroph (0%(0-10)) and
23
24 54 immunonegative tumors (0%(0-17)). It was not predictive of recurrence in the whole cohort. In lactotroph tumors,
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26 55 genome instability, especially quantity of gains, significantly predicted recurrence independently of invasion and
27
28 56 proliferation (p-value=0.02, OR=1.2). However, no specific CNV was found as a prognostic marker.

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30 57 Transcriptomic analysis of the genes included in the CNV and associated with prognosis didn't show
31
32 58 significantly overrepresented pathway. In somatotroph and corticotroph tumors, USP8 and GNAS mutations
33
34 59 were not associated with genome disruption or recurrence respectively.

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37 60 To conclude, CGH array analysis showed genome instability was dependent on PitNET type. Lactotroph tumors
38
39 61 were highly altered and the quantity of altered genome was associated with poorer prognosis though the
40
41 62 mechanism is unclear, whereas gonadotroph and immunonegative tumors showed the same 'quiet' profile,
42
43 63 leaving the mechanism underlying tumorigenesis open to question.

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46 64 **Keyword**

47
48 65 pituitary neuroendocrine tumors, prognosis, genomic instability, copy number variations, pituitary adenoma
49
50 66

67 Introduction

68 Pituitary neuroendocrine tumors (PitNETs) represent 10-15% of intra-cranial tumors among which most are
69 benign and controlled by current therapeutic strategies. While surgery is the first-line treatment, it can also be
70 associated with medical therapies. Despite these strategies, approximately 25-40% of PitNETs present a
71 regrowth after surgery [15]. PitNETs that are recurrent and resistant to conventional treatment are considered as
72 aggressive, however there is no standardized criteria to define them [21]. Various approaches have been
73 proposed for the prediction of tumor behavior, including the study of pathological markers. While the recent
74 WHO 2017 classification of PitNETs did not propose individual markers, it did identify a group of tumors with a
75 high risk of recurrence, including sparsely granulated somatotroph tumors, lactotroph tumors in men, Crooke's
76 cell tumors, silent corticotroph tumors (SCT), and the newly introduced pluri-hormonal Pit-1-positive tumor
77 [10]. Although interesting, the clinical impact of these groups of tumors is still limited since they represent a
78 small number of cases and are not representative of the outcomes of the most common types of PitNETs.
79 Combining radiological and pathological characteristics, we have proposed a clinico-pathological prognosis
80 classification, based on five grades, that associate proliferative (mitosis, Ki67, p53 expression) and invasiveness
81 (cavernous or sphenoid sinus) criteria [32]. According to this classification grade 2b (invasive and proliferative
82 tumors) presented higher risk of recurrence or progression on medical treatment compared to non-invasive non-
83 proliferative tumors (grade 1a). The prognostic value of this classification has been validated by independent
84 prospective study [22] and retrospective studies[1, 14] in all PitNETs types.
85 In parallel to studies on pathological markers, numerous studies have been conducted to identify genomic
86 alterations leading to pituitary tumorigenesis and/or associated with PitNETs behavior [27]. Familial forms of
87 PitNETs due to germinal mutations are more prone to resistance to medical treatment, however, the incidence of
88 aggressive PitNETs or carcinomas did not appear to be higher compared to sporadic tumors [21]. Studies
89 focusing on somatic mutations identified a low mutation rate [4, 16, 26]. *GNAS* gain-of-function mutations have
90 been identified in 30 % of somatotropinomas and *USP8* or *USP48* mutations in about 40 % of corticotroph
91 PitNETs [6, 23, 25]. However, none of these mutations has been clearly associated with tumor behavior.
92 Since chromosome imbalance is frequent in tumors and associated with prognosis especially in brain tumors [2],
93 several studies have analyzed the impact of such mechanisms on PitNETs. CGH analysis, performed using
94 metaphase control chromosomes in a limited number of PitNETs, suggested that many alterations occur in
95 PitNETs [19, 31]. Interestingly, those alterations may be preferentially found in functioning [28, 31] and in
96 invasive PitNETs [28]. Whole exome sequencing analysis has pointed to the existence of 2 groups of PitNETs,

97 defined as “disrupted” or “quiet”, depending on the quantity of copy number variations (CNV)[4]. These 2
1 98 groups were specifically associated with functional characteristics, and with GNAS mutation status in
2
3 99 somatotroph tumors [3, 4, 9, 16, 26]. However, in most of these studies the non-functioning PitNETs were
4
5 100 classified based on their clinical presentation and not on their histopathology. Such an approach led to a mix of
6
7 101 gonadotroph, silent-corticotroph, -somatotroph, -lactotroph and non-immunoreactive tumors, as reported by
8
9 102 Neou *et al* [16].
10
11 103 Few studies have explored the direct association between CNV and recurrence. While a LOH analysis in non-
12
13 104 functioning PitNETs found an increased frequency of 2 allelic losses on chromosome 1q in recurrent tumors [5],
14
15 105 a CGH array performed on 13 lactotroph tumors showed recurrent loss of chromosome 11p in aggressive tumors
16
17 106 compared to indolent tumors [36]. More recently, Neou *et al* reported the lack of association between
18
19 107 aggressiveness and chromosomal alterations in 86 PitNETs of all types using whole exome sequencing [16].
20
21 108 Unfortunately, this latter study was based on a cohort of patients with variable clinical data follow-up (1 to 120
22
23 109 months) and the definition of aggressiveness was not standardized. Finally, an association of chromosomal
24
25 110 instability and markers of aggressiveness has also been reported in the subgroup of pediatric corticotroph
26
27 111 PitNETs [30].
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29 112
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31 113 Since the genetic mechanisms underlying PitNETs growth and behavior are not fully understood, we conducted
32
33 114 a large-scale CGHarray study to analyze the impact of CNV on sporadic PitNETs prognosis. Our aim was to
34
35 115 identify specific markers associated with prognosis, in a large cohort of 195 PitNETs, taking into account the
36
37 116 known clinico-pathological five-tiered classification and at least 5 years post-surgery follow-up.
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119 **Methods**

120 **Study design**

121 The study is part of PITUIGENE, a French multicentric retrospective study (ClinicalTrials.gov Identifier:
122 NCT01903967) based on 212 frozen surgical samples of PitNETs. Data are registered according the French data
123 protection agency CNIL. Written informed consent was given by all patients, and the procedure was in
124 accordance with the ethical standards and approved by a local ethics committee (committee for the protection of
125 persons CPP SUD-EST IV LYON). Recruited patients presenting a lactotroph (PRL), somatotroph (GH),
126 corticotroph (ACTH) and gonadotroph (FSH/LH) immunoreactive PitNET, were selected from 10 different
127 centers in France. All patients were operated, via trans-sphenoidal route, between 1988 and 2010. Patients were
128 selected based on a clinical follow-up of at least 5-years combined with the availability of matching frozen tumor
129 samples. Patients who underwent adjuvant post-operative radiotherapy or presented germinal *MEN1* or *AIP*
130 mutation were excluded. Forty-six patients were part of the HYPOPRONOS (PHRC 27-43) French multicenter
131 retrospective study [22]. Tumors were classified as functional or silent PitNETs according to hormonal levels,
132 *i.e.* plasma PRL for lactotroph tumors, IGF1 and GH levels for somatotroph tumors and urinary free cortisol and
133 response to suppression tests for corticotroph tumors. For each patient, functional subtype and data on
134 proliferation were recorded from histological evaluations as previously described [32]. Tumor with no hormone
135 expression using immunohistochemistry analysis were classified as immunonegative.

137 **Definition of recurrence**

138 Disease-free patients or patients controlled by medical treatment and/or with a stable remnant on MRI up to 5
139 years, were considered as non-recurring. Patients who presented recurrence/tumor progression on MRI and/or a
140 significant increase of plasma hormone levels requiring therapeutic changes in the five years post-surgery were
141 considered as recurring.

143 **CGH data**

144 Tumor DNA was extracted from ~15 mg frozen tissue using Masterpure™ Complete DNA and RNA
145 Purification Kit (Epicentre® Biotechnologies, Madison, WI, USA). aCGH was performed using SurePrint G3
146 Human genome CGH+SNP Microarray, 4x180K (Agilent Technologies, Santa Clara, CA, USA). After
147 enzymatic digestion by *RsaI* and *AluI* enzymes, 1.5 µg of tumor DNA and 0.6 µg of sex-matched human
148 reference DNA (Agilent) were labelled by random priming with Cyanine 5 and Cyanine 3, respectively.

149 Hybridization was performed at 65°C for 24 h and the arrays scanned on Agilent DNA Microarray Scanner.
150 Fluorescence was quantified with Feature Extraction 11.5.11 software whose output is the L2R (\log_2 (tumor
151 DNA fluorescence)/(reference DNA fluorescence)) of each probe.
152
153 *USP8* and *GNAS* sequencing
154 *GNAS* activating somatic mutations and *USP8* gain-of-function somatic mutations were determined using
155 conventional Sanger DNA-sequencing in somatotroph and corticotroph tumors respectively. Genomic DNA was
156 extracted from frozen tissue using Masterpure™ Complete DNA and RNA Purification Kit (Epicentre®
157 Biotechnologies, Madison, WI, USA). DNA sequences were amplified by PCR with the CORE 10 (Mpbio
158 NH4(SO4)2 Kit (MP Biomedicales) using forward 5'-CTATGTGCCGAGCGATCAGG-3' and reverse 5'-
159 CCGTGTGAATGCTTGGGAGA -3' primers for *GNAS*, and forward 5'-CAACCTGAGATGCTGGCTAC-3'
160 and reverse 5'- CCAACTCCCTGACACTAACA-3' primers for *USP8*. Sanger sequencing of PCR products was
161 performed on a 3130xl Genetic Analyzer (Applied Biosystems®) following the use of BigDye™ Terminator
162 v3.1 Cycle Sequencing Kit (Applied Biosystems®). Results were interpreted using Seqscape V3 software.
163
164 Transcriptomic analysis
165 Transcriptomic analysis of 32 lactotroph PitNETs was done using CodeLink Uniset Human Whole Genome
166 bioarrays containing 55,000 human oligonucleotide gene probes (GE Healthcare Europe GmbH, Freiburg,
167 Germany), of which 16 were also analyzed by CGH array analysis. Technical details were previously described
168 by Wierinckx *et al* [35].
169
170 Statistical analysis
171 *CGH data preparation.*
172 For each patient, raw CGH data were normalized and subsequently centralized as reported previously [13]. The
173 centralization step based on FISH analysis was applied on patient profiles with at least one alteration longer than
174 5Mb. Circular binary segmentation was applied on the centralized and normalized Log2Ratios (L2R) (with
175 significance level for the test to accept change-points= 10^{-6}) [18]. X and Y chromosomes were excluded from the
176 analysis. All identified CNV were manually reviewed by an experienced cytogenetician (EA). Loss of
177 heterozygosity (LOH) calling was performed using Cytogenomics 3.0.3.3 software (Agilent) for tumors with
178 derivative log ratio spread (DLRS) less than 0.3.

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180 *Descriptive analysis.*

181 Genome instability was determined using the number of altered (deleted+gained) probes compared to the total
182 number of probes. The association between clinical data and quantity of altered probes was tested using
183 Wilcoxon rank tests. Non-supervised hierarchical clustering was done using Jaccard distance and Ward criterion,
184 by tumor type and in the whole cohort. The associations between clinical data and clusters, and between clinical
185 data and *USP8* or *GNAS* mutations, were tested using Fisher and Kruskal-Wallis tests.

186

187 *Analysis of prognosis.*

188 The association between tumor recurrence and the number of altered probes (deleted + gained, then deleted +
189 gained + copy neutral LOH) was studied using univariate logistic regression models in the whole cohort, and
190 then for each tumor type. Subsequently, multivariate analysis adjusted for the main known factors of recurrence
191 (tumor type, histological grade, age at surgery and sex) were performed. In the per-type analysis, histological
192 grades 1a and 1b were grouped due to the small number of 1b samples. For corticotroph and somatotroph
193 tumors, an analysis adjusted to the presence of *USP8* and *GNAS* mutations respectively was also done.

194 Likelihood ratio tests (LRT) were calculated to compare the models.

195 Specific alterations associated with recurrence were searched for through univariate and multivariate (including
196 tumor type, histological grade, age at surgery and sex) logistic regressions for each probe (deleted, normal state
197 or gained) in the whole cohort and in each tumor type separately. LRT were calculated to compare the models
198 with and without the probe status. P-values were adjusted for dependent multiple testing using the Benjamini-
199 Yekutieli approach.

200 In order to perform transcriptomic analysis comparisons in lactotroph tumors, genes included in CNV were listed
201 using the Hg19 reference genome. For each gene (altered or non-altered), we performed univariate and
202 multivariate (including clinical data) logistic regression models. LRT were calculated to compare models with
203 and without the gene status. P-values were adjusted for multiple testing using the Benjamini-Hochberg approach.

204

205 *Transcriptomic analysis.*

206 mRNA transcripts showing at least 2-fold variation were considered as differentially expressed after statistical
207 analysis using Student's t-test with a p-value ≤ 0.05 . Gene Set enrichment analysis (GSEA) was performed to
208 search for implicated biological pathways, following the recommended protocol from the Broad Institute Gene

209 Set Enrichment Analysis website (<https://www.gsea-msigdb.org/gsea>). GSEA software v7.0.0 and the Molecular
1
2 210 Signatures Database v7.0. were used for running GSEA. A ranked-list metric was generated by calculating the
3
4 211 signal-to-noise ratio. The number of permutations was set to 1000. Nominal p-values < 0.05 and adjusted q-
5
6 212 values (FDR) < 0.25 were considered as significant.
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8 213
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10 214 All analyses were performed with R software version 3.5.2. P-values and adjusted p-values less than 0.05 were
11
12 215 considered as significant.
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16 217 **Data Availability**
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18 218 CGHarray data will be available at the EMBL-EBI Array Express under accession number E-MTAB-9237.
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20 219 Transcriptomic data are available at Gene Expression Omnibus (GEO) under accession number GSE120350.
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222 Results

223 Cohort description

224 Of the 212 PitNETs initially included, 17 tumors were excluded due to unsatisfactory sequencing
225 quality (DLRS>0.47) (n= 7); the presence of alterations larger than 5MB and the lack of material to confirm
226 centralization by FISH (n=9), or missing information concerning their pathological grade (n=1). A total of 195
227 PitNETs were analyzed, including 56 gonadotroph, 11 immunonegative, 56 somatotroph, 39 lactotroph and 33
228 corticotroph (8 being silent) tumors. Clinico-pathological characteristics of those tumors are presented in Table
229 1, their mean (sd) post-operative follow-up was 8.3(3.5) years and tumor recurrence/progression occurred in 124
230 patients (64%) within 1.4(1.6) years after initial surgery.

231 Somatic pathogenic mutations of *USP8* were detected in 5/27 (19%) corticotroph tumors (heterozygous
232 p.Ser718Pro (n=2), p.Ser718Cys, p.Ser719del and p.Pro720Arg). In addition, two of the wild type *USP8*
233 corticotroph tumors harbored a respective gain and deletion of 15q21.2 region. Somatic pathogenic mutations of
234 *GNAS* were identified in 13/53 (25%) somatotroph tumors (11 heterozygous missense p.Arg201Cys mutations
235 and 2 p.Gln227Leu). In addition, 13 *GNAS* wild-type somatotroph tumors showed a gain including the 20q13.32
236 region. These gains were confirmed using FISH analysis in 5/5 studied tumors (supplemental Figure 1). Tumors
237 with *GNAS* mutation, tumors with gain of the *GNAS* region and tumors with no alteration (*GNAS* wt and no gain)
238 were comparable in terms of sex, age at surgery, grade, secretion and tumor size.

239

240 Genomic instability description

241 Genomic instability was dependent on the tumor type (Figure 1&2). Median (min-max) percentage of
242 altered probes per tumor (total=99,659 CGH probes) was 0% (0-9.7) in gonadotroph and 0% (0-16.5) in
243 immunonegative, compared to 4.8% (0-99.8) in somatotroph, 11.1% (0-76.6) in corticotroph and 38.3% (0-96.7)
244 in lactotroph tumors. Gains were globally more frequent than deletions: 0% (0-9.7) vs 0% (0-4.3) in
245 gonadotroph, 0% (0-6.1) vs 0% (0-10.4) in immunonegative, 0.4% (0-99.8) vs 0% (0-32.1) in somatotroph, 2.3%
246 (0-65.2) vs 0% (0-76.6) in corticotroph and 36.2% (0-96.7) vs 0% (0- 23.3) in lactotroph tumors (Figure 2). LOH
247 were less frequent and detected in 13/56 gonadotroph, 2/11 immunonegative, 17/56 somatotroph, 9/33
248 corticotroph and 15/39 lactotroph tumors (median 0% for all types). Large alterations of entire chromosomes or
249 chromosome arms were frequent. Entire chromosomes 9, 5, 7, 12, 19, 20 were gained in 41 (21%), 38 (19%), 38,
250 37 (19%), 37 and 37 patients respectively, while short arms of chromosomes 7, 19, and 9 were gained in 43

251 (22%), 42 (22%) and 42 (22%) patients respectively. Common deletions were rarely found and whole
252 chromosomes 18, 11 and 13 were deleted in 10 (4%), 9 (3%) and 8 (3%) patients respectively.
253 In gonadotroph tumors, whole chromosome 7 was gained only in 3/56 patients and no large recurrent alterations
254 were detected in immunonegative tumors. In somatotroph tumors, the large alteration most frequently found
255 concerned chromosome 9, with 14/56 patients showing a whole chromosome gain and 1/56 patients presenting a
256 gain of the entire short arm. In corticotroph tumors, the most frequent large alteration concerned chromosome 12
257 (gain of the entire chromosome in 11/33 (33%) patients). Regarding lactotroph tumors, gain of whole
258 chromosomes 9, 12, 7 and 19 were found in 21/39, 19/39, 19/39 and 18/39 patients respectively, whereas a gain
259 of their short arms were found in another 0/39, 1/39, 3/39 and 3/39 tumors. Deletion of chromosome 18 was
260 observed in 4/39 lactotroph tumors.

261

262 The quantity of altered probe per tumor was extremely variable and no evident threshold could be
263 identified to classify tumors (Figure 1). Clinical and pathological characteristics of tumors showing alterations,
264 defined as tumors with at least one CNV, compared to tumors lacking CNVs are presented for each tumor type
265 in Table 2.

266 We found that the quantity of altered probes in somatotroph tumors was not associated with *GNAS* mutation and
267 alterations were found in both *GNAS*mut and *GNAS*wt tumors. The median (min-max) of altered probes was 6%
268 (0-15) in *GNAS*mut compared to 5% (0-100) in *GNAS*wt (p-value=0.57). However, the quantity of altered probes
269 associated with secretion. All 4 silent tumors had no altered probe compared to 6% (0-100) of altered probes in
270 functioning somatotroph tumors (p-value=0.02).

271 In lactotroph tumors, the quantity of altered probes did not associate with secretion (median (min-max) of altered
272 probes were 21% (1-42) in silent tumors vs 38% (0-97) in functioning tumors, p-value=0.5).

273 For corticotroph tumors, most of the identified alterations concerned macroadenomas, while microadenomas
274 appeared less altered (median (min-max) of altered probes were 20% (0-77) vs 0.1% (0-17)), but this was not
275 statistically significant (p-value=0.15). The quantity of altered probes was not different between *USP8*mut and
276 *USP8*wt (11%(0-59) vs 17%(0-77); p-value=0.75), silent and functioning (10%(0-46) vs 11%(0-77); p
277 value=0.69) or invasive and non-invasive corticotroph tumors (17%(0-77) vs 5%(0-49); p-value=0.46).

278

279 **Non-supervised analysis**

280 Clustering analysis performed on the whole cohort identified 3 major clusters based on the number of
281 altered probes (Figure 3). 74 tumors were included in the 'quiet' cluster (no alteration), and 42 tumors were in
282 the most altered cluster (med (min-max) of altered probes = 49% (26-99.8%)). The last 79 tumors were in the
283 intermediate cluster (5% of altered probes (0-37%)). As presented in Figure 3, while clusters were significantly
284 associated with tumor type (p-value<0.001), they were not associated with other pathological (grade, invasion,
285 proliferation) or clinical criteria (size, age, sex), confirming that cell lineage was the strongest factor influencing
286 genomic instability. Indeed, no gonadotroph or immuno-negative PitNETs were found in the most altered cluster
287 whereas only 5 lactotroph tumors were in the 'quiet' cluster.

288

289 Prognostic analysis

290 While the quantities of altered, gained, deleted or copy neutral LOH probes were not associated with
291 prognosis in univariate analysis or in multivariate analysis in the whole cohort, the pathological classification (p-
292 value LRT <0.001) and age at surgery (p-value LRT < 0.001) were associated with prognosis. Hence, grades 2a
293 and 2b were associated with higher risk of recurrence compared to grade 1a in multivariate analysis adjusted for
294 age, sex, and tumor type (OR = 4.3 IC95% [2.1; 9.3] and OR = 8.7 [2.9-30.5] respectively), as well as a younger
295 age at surgery (OR for 10 years older = 0.6 IC95% [0.5;0.8]). The analysis did not reveal an association of
296 specific CNV with tumor recurrence when tested on the whole cohort.

297 In lactotroph tumors, the quantities of altered (gained and deleted) probes, and more specifically gained
298 probes, were associated with recurrence in univariate analysis (p-value LRT = 0.004 and 0.02 respectively) and
299 multivariate analysis (p-value LRT = 0.003 and 0.02 respectively). In multivariate analysis, the risk of
300 recurrence was multiplied by 1.3 for doubling of altered probes (OR=1.3 IC95% [1.1;1.6]) and 1.2 for doubling
301 of gained probes (OR=1.2 IC95% [1.0;1.3]). Similar results were obtained when considering altered probes as a
302 combination of gained, deleted and copy neutral LOH. We also found that the quantities of deleted probes and
303 copy neutral LOH were not individually associated with tumor recurrence. No specific CNV was found to be
304 significantly associated with recurrence after regression on each probe and correction for multiple testing. The
305 number of genes included in the CGHarray alterations in lactotroph tumors was 18,577. The numbers of
306 recurrent and non-recurrent tumors related to the alteration for each gene are listed in Supplemental Table 1.
307 Logistic regression on genes included in the alterations found 2,189 genes significantly associated with
308 prognosis after p-value adjustment using univariate analysis, and 1,329 using multivariate analysis. The genes

309 and their p-values are listed in Supplemental Table 2. These genes were included in CNV which concerned
1 310 chromosomes 1 to 16.

2 311 In corticotroph tumors, the quantity of deleted probes tended to be associated with fewer recurrences,
3
4 312 though was not statistically significant (OR= 0.9 IC95% [0.8;1.0], p-value LRT =0.08 in univariate analysis,
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6 313 OR=0.9 IC95% [0.8;1.0], p-value LRT=0.07 in multivariate analysis), while *USP8* mutations were not
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8 314 associated with prognosis (p-value LRT=0.82).

9
10 315 In somatotroph tumors the quantity of altered probes, as well as *GNAS* mutations, were not associated
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12 316 with tumor recurrence. However, while considering tumors with *GNAS* mutation or gain of the *GNAS* region,
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14 317 tumors with none of these alterations were significantly more likely to show recurrence (p-value of univariate
15
16 318 LRT = 0.02; OR = 4.2 IC95% [1.2;17.6]). Note, that this association was not found in multivariate analysis (p-
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18 319 value LRT=0.11).

19
20 320 In gonadotroph and immunonegative tumors, quantity of deleted probes was limited and not associated
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22 321 with tumor recurrence.

23
24 322 We did not find specific CNV associated with recurrence after regression on each probe and correction
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26 323 for multiple testing in somatotroph, corticotroph and gonadotroph tumors (Figure 4).

27 324

32 325 **CGH array and transcriptomic analysis in lactotroph tumors**

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34 326 To extend our work, we performed a transcriptomic analysis of 32 lactotroph tumors (25 recurrent, 7 non-
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36 327 recurrent, 16 of whom had CGHarray analysis). The top 100 genes associated with recurrent phenotype were
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38 328 ranked according to signal-to-noise ratio (Supplemental Figure 2). No gene sets appeared significantly associated
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40 329 with recurrent phenotype using GSEA analysis based on Hallmark gene sets. Among the 2,189 and 1,329 genes,
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42 330 which were found altered in lactotroph tumors by CGHarray and associated with prognosis by univariate and
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44 331 multivariate analysis, 40 and 29 were significantly differentially expressed between recurrent and non-recurrent
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46 332 tumors in our transcriptomic analysis respectively (Table 3). These genes localized mostly on chromosomes 1
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48 333 and 11.

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335 **Discussion**

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2 336 Here, we report the first study of a large multicentric cohort of PitNETs patients with standardized
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4 337 clinical follow-up, clear definition of recurrence and available pathology data, in which we analyzed the impact
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6 338 of chromosome instability on tumor prognosis. As shown previously[4, 16], our CGHarray results confirmed the
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8 339 large number of CNV that can be detected in PitNETs. We report that the amount of genome alteration is
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10 340 associated with tumor types but not with the prognosis in the whole cohort. However, our data also support that
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12 341 the number of genomic alterations found in lactotroph tumors are associated with a poor prognosis,
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14 342 independently of the tumor's invasive and proliferative status.

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16 343 We observed a wide range of proportions of altered genome, varying from 0 to almost 100% of the whole
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18 344 genome. While gains were more frequently observed compared to deletions, copy neutral LOH were rare
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20 345 compared to CNV. This result is rather surprising for this type of frequently indolent tumor, in view of large
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22 346 numbers of genomic alterations being generally a key feature of more aggressive tumors with metastatic spread
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24 347 [11]. Unlike other studies [4, 9, 16, 26], we did not find a clear threshold to categorize PitNETs as “altered” or
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26 348 “quiet” in our entire cohort, nor in each tumor type, as the quantity of altered genome per tumor was continuous.

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30 350 Analysis of prognosis for the whole cohort did not show any association between the quantity of
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32 351 alterations or specific CNV, and 5 years' recurrence status. As previously reported, histological classification,
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34 352 which associates invasiveness and proliferation criteria (Ki67 index, p53 expression and mitotic index), was
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36 353 associated with prognosis, as well as age at surgery [22, 32].

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38 354 Interestingly, using univariate analysis and multivariate analysis adjusted for age, sex, and histological
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40 355 classification, we found that the quantity of alterations was an independent risk factor for recurrence for
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42 356 lactotroph tumors. However, the exact consequences of these alterations that underlie recurrence remain unclear.
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44 357 Alterations of chromosomes 1p, 11 and 17 were exclusively found in recurrent tumors however, we did not find
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46 358 CNV statistically associated with recurrence after adjustment of p-values. We cannot exclude the possibility that
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48 359 the combination of a series of specific CNVs may lead to an increased risk of recurrence, while a lack of power
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50 360 regarding our study should not be excluded. The number of genes included in the alterations being high, we
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52 361 failed to identify specific and relevant target genes using CGHarray analysis. It is important to emphasize that
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54 362 the consequences of CNV on gene expression are difficult to predict, especially in the case of gains, which can
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56 363 localize anywhere on the genome and impact the functionality of long range enhancers and silencers. We
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58 364 evaluated the impact of CNV on gene expression thanks to transcriptomic analysis of 32 lactotroph tumors.
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365 Through this approach, we found 29 genes, among the 1,329 identified with CGHarray using multivariate
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2 366 analysis, that were significantly differentially expressed between recurrent tumors vs non-recurrent tumors.
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4 367 However, further analysis of these 29 candidates, did not identify an over-represented pathway.
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6 368 Various genes have been suspected to be associated with PitNETs aggressiveness. While *TP53* mutations have
7
8 369 been described in pituitary carcinomas [29], no deletions including *TP53* were found in lactotroph tumors in our
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10 370 cohort. Mutations of the protooncogene *HRAS* have been reported in metastasis [20] and alterations including
11
12 371 *HRAS* (chromosome 11) were found in 8 recurrent lactotroph tumors with various mechanism (4 gains, 3
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14 372 deletions, 1 copy neutral LOH), while expression was not different in recurrent tumors. Lastly, reduced
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16 373 expression of *D2R* is suspected to be associated with dopamine agonist resistance in prolactinomas [37]. Here,
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18 374 we found the *DRD2* gene deleted and in copy neutral LOH in 5 and 2 recurrent tumors respectively. However,
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20 375 *DRD2* expression was not different in our transcriptomic analysis. The consequences of these CNV are thus
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22 376 difficult to evaluate.
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24 377 Our results underline that the quantity of alterations is associated with PitNET type. Whereas lactotroph
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26 378 tumors were the most altered tumors, gonadotroph and non-immunoreactive tumors only present a small number
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28 379 of short CNVs.
29
30 380 *Bi et al.* [4], *Salomon et al.* [24] and *Neou et al.* [16] also described an association between functional
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32 381 characteristics and quantity of alterations whereas *Song et al* did not [26]. In these studies, non-functional tumors
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34 382 included not only gonadotroph and immunonegative tumors, but also silent corticotroph and, occasionally,
35
36 383 thyrotroph, somatotroph and lactotroph tumors. The mechanisms of tumorigenesis of the gonadotroph and
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38 384 immunonegative tumors remain unclear as they present a few short CNV and no mutations in sequencing
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40 385 studies [17].
41
42 386 One may also question why some corticotroph and somatotroph tumors were found to present no alterations.
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44 387 Similar results have been previously reported by others [4, 7, 9, 16, 24], while the association with secretory
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46 388 phenotype [4, 16], and the role of *PTTG1* [27] or hypomethylation [16] have also been suggested.
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48 389 In our cohort, 8/33 corticotroph tumors were clinically silent and the aCGH profile of these tumors was not
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50 390 distinguishable from those of the clinically functional corticotroph tumors. 9/33 corticotroph tumors showed no
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52 391 alteration, whereas 9/33 were in the most altered cluster. We found no association between alteration
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54 392 numbers/clusters and tumor invasion, tumor recurrence or *USP8* mutations. We did not find the quantity of
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56 393 alterations in *USP8*wt tumors to be associated with tumor invasion, unlike the findings of *Tatsi et al.* [30].
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58 394 However, the small number of invasive *USP8*wt corticotroph tumors present in our cohort (n=9) and in the *Tatsi*
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395 *et al* cohort (n=2), means that our results should be taken with caution [30]. In accordance with Tatsi *et al*,
1 396 larger tumors seemed to be more altered though this did not reach statistical significance in our study.
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4 397 Contrary to Hage *et al.* and Valimaki *et al*, we did not find a significant association between *GNAS* mutations
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6 398 (found in 13/53 tumors) and the quantity of genomic alterations in somatotroph tumors [9, 33]. We identified 13
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8 399 *GNAS*wt tumors presenting gains of the 20q region, these tumors further presenting a high quantity of
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10 400 alterations. On the contrary, none of the *GNAS* mutated tumors harbored gains of the 20q region. Duplication of
11
12 401 the *GNAS* gene has been proposed as an alternative mechanism in somatotroph tumorigenesis [9]. Neou *et al.*
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14 402 described that *GNAS* mutated tumors were associated with fewer chromosomal alterations and DNA
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16 403 hypomethylation, whereas hypomethylation was associated with chromosomal alterations in other
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18 404 *POU1F1/PIT1* lineage tumors [16]. Data regarding the association between *GNAS* mutation and prognosis in the
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20 405 literature are inconsistent [8, 12, 34]. In our study, *GNAS* mutation was not associated with recurrences.
21
22 406 However, somatotroph tumors with *GNAS* mutation or gain of the *GNAS* region presented significantly less
23
24 407 recurrence than tumors lacking *GNAS* alteration by univariate analysis.
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27
28 409 While our conclusion is appealing, some caution is required due to study limitations, such as the
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30 410 detection sensitivity of the CNV which may be affected through the pre-treatment of our CGH data. Hence the
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32 411 Log₂(ratio) threshold to define a gain or a deletion is particularly important. A more sensitive threshold allows
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34 412 the detection of small cell populations, but also risks exposure to artifacts and false discoveries. Moreover,
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36 413 lactotroph tumors included in our study are not representative of the usual clinical presentation. Most indolent
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38 414 lactotroph lesions are medically managed and most that are operated are associated with an aggressive behavior.
39
40 415 This could partly explain the high quantity of alterations observed in those tumors. In addition, macro-
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42 416 corticotroph tumors are likely overrepresented whereas typical microadenomas may be underrepresented due to
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44 417 the limited material available for analysis
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47
48 419 In conclusion, our study confirms the association between genomic alterations and PitNET type,
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50 420 suggesting that mechanisms associated with pituitary tumorigenesis and behavior are specific for each tumor
51
52 421 type. In lactotroph tumors, genomic instability can partly explain tumorigenesis and mechanism of progression,
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54 422 whereas the mechanism of tumorigenesis and recurrence in gonadotroph tumors remains unclear and requires
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56 423 further exploration of other mechanisms, such as the role of the micro-environment, epigenetic mechanisms and
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58 424 cellular heterogeneity.
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8 430

9
10 431 **Author contributions**

11
12 432 Conception of the work: GR, PR, DS

13
14 433 Acquisition of data: AV, CC, BD, NS, SG, AF, EJ

15
16 434 Pathological analysis: AV

17
18 435 Molecular analysis: AW, EA, CBo, DS

19
20 436 Data treatment and statistical analysis: HL, MHE, AW, PB, CBa, PR

21
22 437 Interpretation of data: HL, MHE, AW, PB, CBa, DS, GR

23
24 438 Draft redaction: HL, MHE, AW, EA, AV, PB, CBa, GR

25
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36 444

37
38 445 **Conflict of interest statement**

39
40 446 The authors declare no potential conflicts of interest.

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43
44 448 **Ethics Approval**

45
46 449 The procedure was in accordance with the ethical standards and approved by a local ethics committee

47
48 450 (committee for the protection of persons CPP SUD-EST IV LYON). Data are registered according the French

49
50 451 data protection agency CNIL.

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52 452

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54 453 **Consent to participate**

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56 454 Written informed consent was given by all patients.

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456 Data Availability

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2 **457** CGHarray data will be available at the EMBL-EBI Array Express under accession number E-MTAB-9237.
3
4 **458** Transcriptomic data are available at Gene Expression Omnibus (GEO) under accession number GSE120350.
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587 **Figure legends:**

- 1
- 2 588 Figure 1: Barplot of the quantities of altered probes per tumor
- 3
- 4 589 Figure 2 : Boxplot of the quantities of altered, deleted and gained probes per tumor type
- 5
- 6 590 Figure 3: Heatmap of the non-hierarchical clustering in the whole cohort
- 7
- 8 591 Figure 4: Heatmap representation of the CNV in each tumor type
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Tables

Table 1: Description of patients and tumors

		Recurrence	No recurrence	Analyzed cohort	Excluded
N		124	71	195	17
Age mean ± sd (yrs)		44.8 (14.4)	51.1 (13.4)	47.1 (14.3%)	43.3 (16.3)
Sex					
	F	50 (40.3%)	34 (47.9%)	84 (43.1%)	8 (47.1%)
	M	74 (59.7%)	37 (52.1%)	111 (56.9%)	9 (52.9%)
Tumor type					
	Gonadotroph	33 (26.6%)	23 (32.4%)	56 (28.7%)	2 (11.7%)
	Immunonegative	5 (4.0%)	6 (8.5%)	11 (5.6%)	1 (5.9%)
	Somatotroph	40 (32.2%)	16 (22.5%)	56 (28.7%)	13 (76.5%)
	GNASwt	31	9	40	/
	GNAS mutation	7	6	13	/
	GNAS Not available	2	1	3	/
	Lactotroph	28 (22.6%)	11 (15.5%)	39 (20.0%)	0 (0%)
	Corticotroph	18 (14.6%)	15 (21.1%)	33 (17.0%)	1 (5.9%)
	USP8wt	12	10	22	/
	USP8 mutation	3	2	5	/
	USP8 Not available	3	3	6	/
Grade					
	1a	26 (21.0%)	36 (50.7%)	62 (31.9%)	2 (13.3%)
	1b	4 (3.2%)	5 (7.0%)	9 (4.6%)	1 (6.7%)
	2a	64 (51.6%)	25 (35.3%)	89 (45.6%)	7 (46.7%)
	2b	30 (24.2%)	5 (7.0%)	35 (17.9%)	5 (33.3%)
Size					
	Microadenomas	4 (3.2%)	9 (12.7%)	13 (6.7%)	2 (11.8%)
	Macroadenomas	111 (89.6%)	62 (87.3%)	173 (88.7%)	15 (88.2%)
	Giant adenomas	8 (6.4%)	0	8 (4.1%)	0
	Not available	1 (0.8%)	0	1 (0.5%)	0

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Table 2: Comparison of altered vs non-altered tumors

		Alterations	No alterations
Gonadotroph		24	32
Age		54.19 (14.1)	54 (14.1)
Sex			
	F	10 (41.7%)	11 (34.4%)
	M	14 (58.3%)	21 (65.6%)
Recurrence			
	Yes	15 (62.5%)	18 (56.3%)
	No	9 (37.5%)	14 (43.7%)
Grade			
	1a	7 (29.2%)	11 (34.4%)
	1b	1 (4.2%)	1 (3.1%)
	2a	12 (50.0%)	16 (50%)
	2b	4 (16.6%)	4 (12.5%)
Size			
	Microadenomas	0 (0%)	0 (0%)
	Macroadenomas	24 (100%)	31 (96.9%)
	Giant Adenomas	0 (0%)	1 (3.1%)
Secretion			
	Silent	24 (100%)	32 (100%)
	Functioning	0 (0%)	0 (0%)
Immunonegative		2	9
Age		52.49 (7.5)	58.36 (11.6)
Sex			
	F	1 (50%)	3 (33.3%)
	M	1 (50%)	6 (66.7%)
Recurrence			
	Yes	1 (50%)	4 (44.4%)
	No	1 (50%)	5 (55.6%)
Grade			
	1a	0 (0%)	2 (22.2%)
	1b	0 (0%)	1 (11.1%)
	2a	2 (100%)	6 (66.7%)
	2b	0 (0%)	0 (0%)
Size			
	Microadenomas	0 (0%)	0 (0%)
	Macroadenomas	2 (100%)	8 (88.9%)
	Giant Adenomas	0 (0%)	1 (11.1%)
Secretion			
	Silent	2 (100%)	9 (100%)
	Functioning	0 (0%)	0 (0%)
Somatotroph		37	19
Age		44.36 (12.9)	41.84 (10.8)
Sex			
	F	18 (48.7%)	12 (63.2%)
	M	19 (51.3%)	7 (36.8%)
Recurrence			
	Yes	23 (62.2%)	17 (89.5%)
	No	14 (37.8%)	2 (10.5%)
Grade			
	1a	12 (32.4%)	4 (21.1%)
	1b	2 (5.4%)	0 (0%)
	2a	16 (43.3%)	10 (52.6%)
	2b	7 (18.9%)	5 (26.3%)
Size			
	Microadenomas	3 (8.1%)	1 (5.6%)
	Macroadenomas	33 (89.2%)	17 (94.4%)
	Giant Adenomas	1 (2.7%)	0 (0%)

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GNAS				
		WT	25 (67.6%)	15 (78.9%)
		Mutation	10 (27.0%)	3 (15.8%)
		Not available	2 (5.4%)	1 (5.3%)
Secretion		Silent	0 (0%)	4 (21.1%)
		Functioning	37 (100%)	15 (78.9%)
Lactotroph			34	5
Age			42.06 (14.7)	40.1 (6.9)
Sex				
		F	11 (32.4%)	2 (40.0%)
		M	23 (67.6%)	3 (60.0%)
Recurrence				
		Yes	27 (79.4%)	1 (20.0%)
		No	7 (20.6%)	4 (80.0%)
Grade				
		1a	9 (26.5%)	1 (20.0%)
		1b	2 (5.9%)	1 (20.0%)
		2a	12 (35.3%)	1 (20.0%)
		2b	11 (32.3%)	2 (40.0%)
Size				
		Microadenomas	1 (2.9%)	1 (20.0%)
		Macroadenomas	28 (82.4%)	4 (80.0%)
		Giant Adenomas	5 (14.7%)	0 (0.0%)
Secretion		Silent	2 (5.9%)	0 (0%)
		Functioning	32 (94.1%)	5 (100%)
Corticotroph			24	9
Age			45.99 (12.0)	39.37 (18.7)
Sex				
		F	11 (45.8%)	5 (55.6%)
		M	13 (54.2%)	4 (44.4%)
Recurrence				
		Yes	13 (54.2%)	5 (55.6%)
		No	11 (45.8%)	4 (44.4%)
Grade				
		1a	12 (50.0%)	4 (44.5%)
		1b	1 (4.2%)	0 (0.0%)
		2a	11 (45.8%)	3 (33.3%)
		2b	0 (0%)	2 (22.2%)
Size				
		Microadenomas	5 (20.8%)	2 (22.2%)
		Macroadenomas	19 (79.2%)	7 (77.8%)
		Giant Adenomas	0 (0.0%)	0 (0.0%)
Secretion				
		Silent	5 (20.8%)	3 (33.3%)
		Functioning	19 (79.2%)	6 (66.7%)
USP8				
		WT	17 (70.8%)	5 (55.6%)
		Mutation	4 (16.7%)	1 (11.1%)
		Not available	3 (12.5%)	3 (33.3%)

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Table 3: List of genes associated with recurrence in lactotroph tumors selected using CGH array (univariate and multivariate analysis) and transcriptomic analysis.

Gene	Chromosome	aCGH univariate logistic regressions		aCGH multivariate logistic regressions**		Transcriptomic analysis	
		LRT raw p-values	LRT adjusted p-values*	LRT raw p-values	LRT adjusted p-values*	Fold-Change	T-test p-values
<i>ACTL8</i>	1	0.003	0.041	0.003	0.046	10.955	0.004
<i>ALDH3B1</i>	11	0.005	0.041	0.012	0.099	2.194	0.033
<i>AMBRA1</i>	11	0.002	0.041	0.002	0.046	3.642	0.033
<i>ATPAF1</i>	1	0.005	0.041	0.003	0.046	-2.104	0.016
<i>CEL</i>	9	0.001	0.041	0.003	0.046	2.392	0.004
<i>CLCNKA</i>	1	0.003	0.041	0.003	0.046	9.043	0.012
<i>DLG2</i>	11	0.005	0.041	0.012	0.099	-2.146	0.027
<i>DOCK7</i>	1	0.005	0.041	0.003	0.046	2.100	0.000
<i>DPAGT1</i>	11	0.005	0.041	0.012	0.099	6.217	0.001
<i>ELAVL4</i>	1	0.005	0.041	0.003	0.046	2.786	0.039
<i>FPGT-TNNI3K</i>	1	0.003	0.041	0.003	0.046	2.064	0.036
<i>FRMD8</i>	11	0.005	0.041	0.012	0.099	25.132	0.007
<i>GSTM4</i>	1	0.003	0.041	0.003	0.046	19.111	0.019
<i>HECTD3</i>	1	0.005	0.041	0.003	0.046	2.465	0.002
<i>HMGCL</i>	1	0.001	0.041	0.003	0.046	-2.135	0.017
<i>HSD17B12</i>	11	0.002	0.041	0.002	0.046	-2.021	0.016
<i>HSPB7</i>	1	0.003	0.041	0.003	0.046	10.999	0.012
<i>HTATIP2</i>	11	0.002	0.041	0.002	0.046	-2.014	0.008
<i>KCNJ5</i>	11	0.005	0.041	0.012	0.099	-2.514	0.002
<i>LPTM5</i>	1	0.003	0.041	0.003	0.046	-2.272	0.034
<i>LPL</i>	8	0.000	0.041	0.001	0.046	2.578	0.003
<i>MAST2</i>	1	0.005	0.041	0.003	0.046	2.094	0.023
<i>MDK</i>	11	0.002	0.041	0.002	0.046	-2.180	0.041
<i>NRIP3</i>	11	0.002	0.041	0.002	0.046	2.076	0.020
<i>PAMR1</i>	11	0.002	0.041	0.002	0.046	-2.563	0.048
<i>PGM2L1</i>	11	0.005	0.041	0.012	0.099	2.222	0.013
<i>PHOX2A</i>	11	0.005	0.041	0.012	0.099	52.058	0.007
<i>PIFO</i>	1	0.003	0.041	0.003	0.046	2.751	0.001
<i>PLA2G2F</i>	1	0.003	0.041	0.003	0.046	3.403	0.047
<i>POMGNT1</i>	1	0.005	0.041	0.003	0.046	15.479	0.047
<i>PRKAA2</i>	1	0.005	0.041	0.003	0.046	2.568	0.002
<i>RHCE</i>	1	0.003	0.041	0.003	0.046	-2.184	0.017
<i>RPUSD4</i>	11	0.005	0.041	0.012	0.099	2.011	0.001
<i>RUNX3</i>	1	0.003	0.041	0.003	0.046	-2.438	0.003
<i>SCGB1D2</i>	11	0.005	0.041	0.012	0.099	2.155	0.005
<i>SOX6</i>	11	0.001	0.041	0.001	0.046	37.554	0.000
<i>SRSF8</i>	11	0.005	0.041	0.012	0.099	-2.223	0.010
<i>ST3GAL3</i>	1	0.005	0.041	0.003	0.046	11.519	0.000
<i>TARDBP</i>	1	0.003	0.041	0.003	0.046	2.117	0.001
<i>TMEM216</i>	11	0.005	0.041	0.012	0.099	-2.006	0.009

LRT: likelihood ratio test

*Benjamini-Hochberg

** adjusted for age at diagnosis, sex, histologic grade

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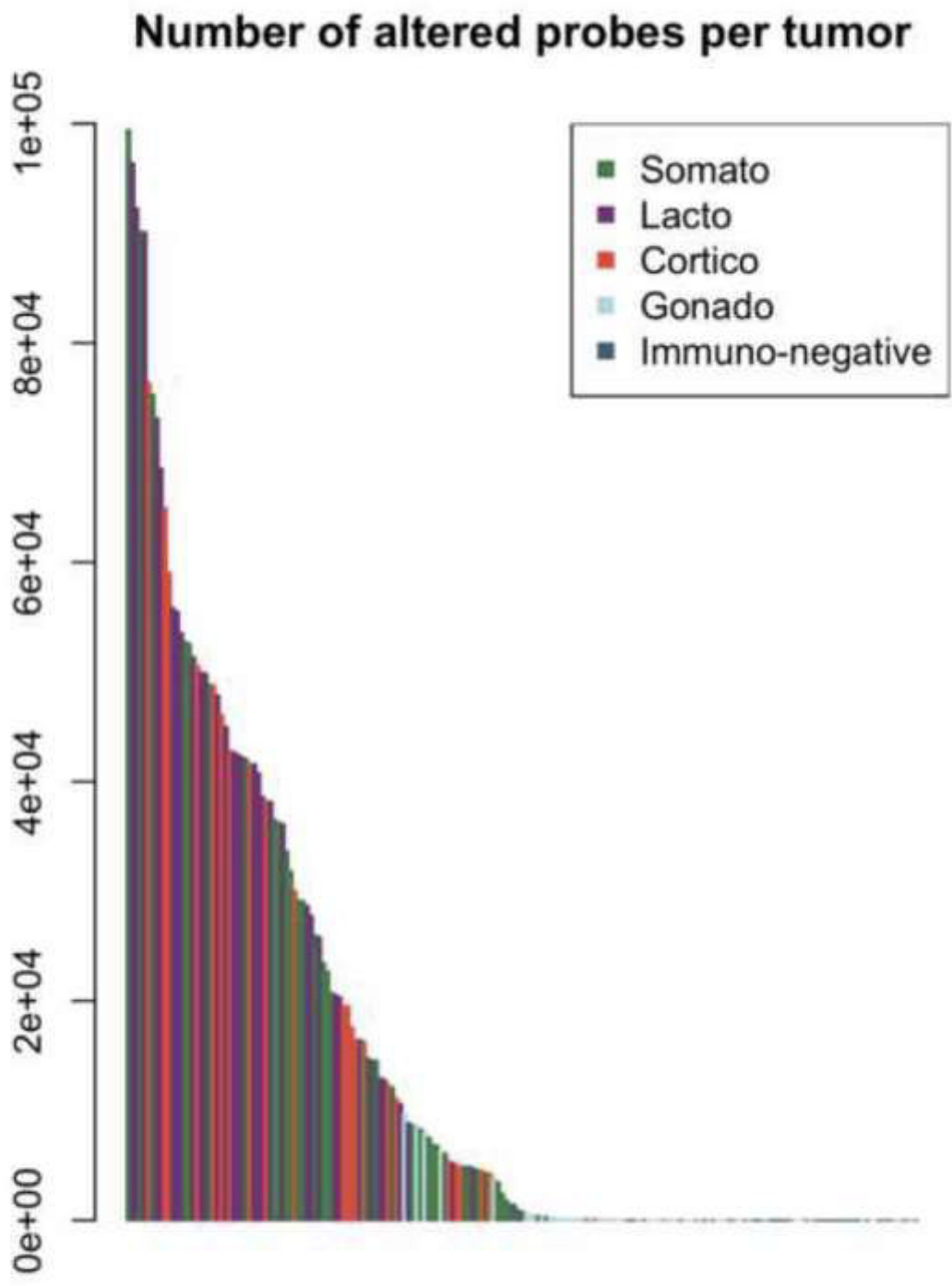
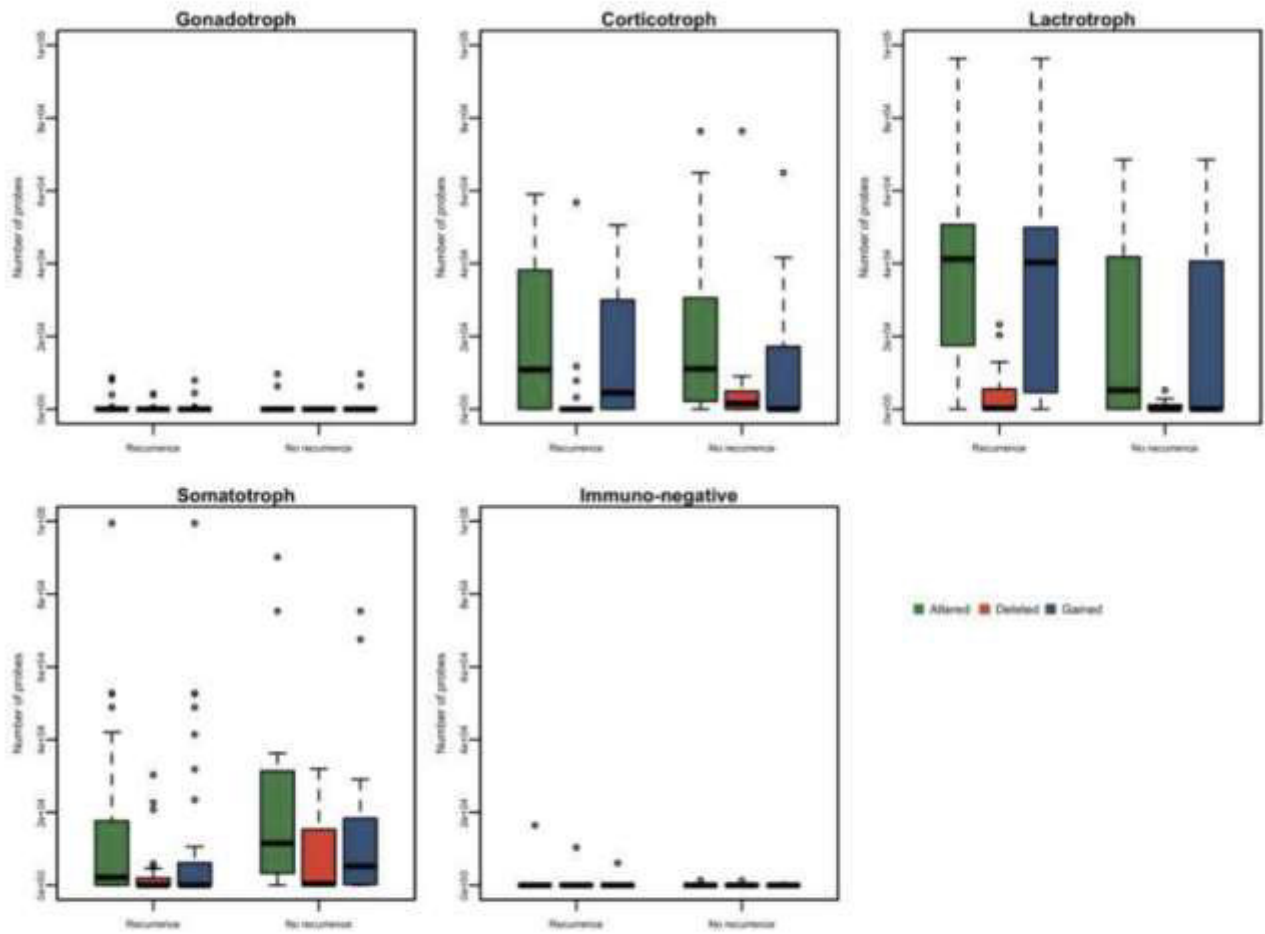


Figure 2

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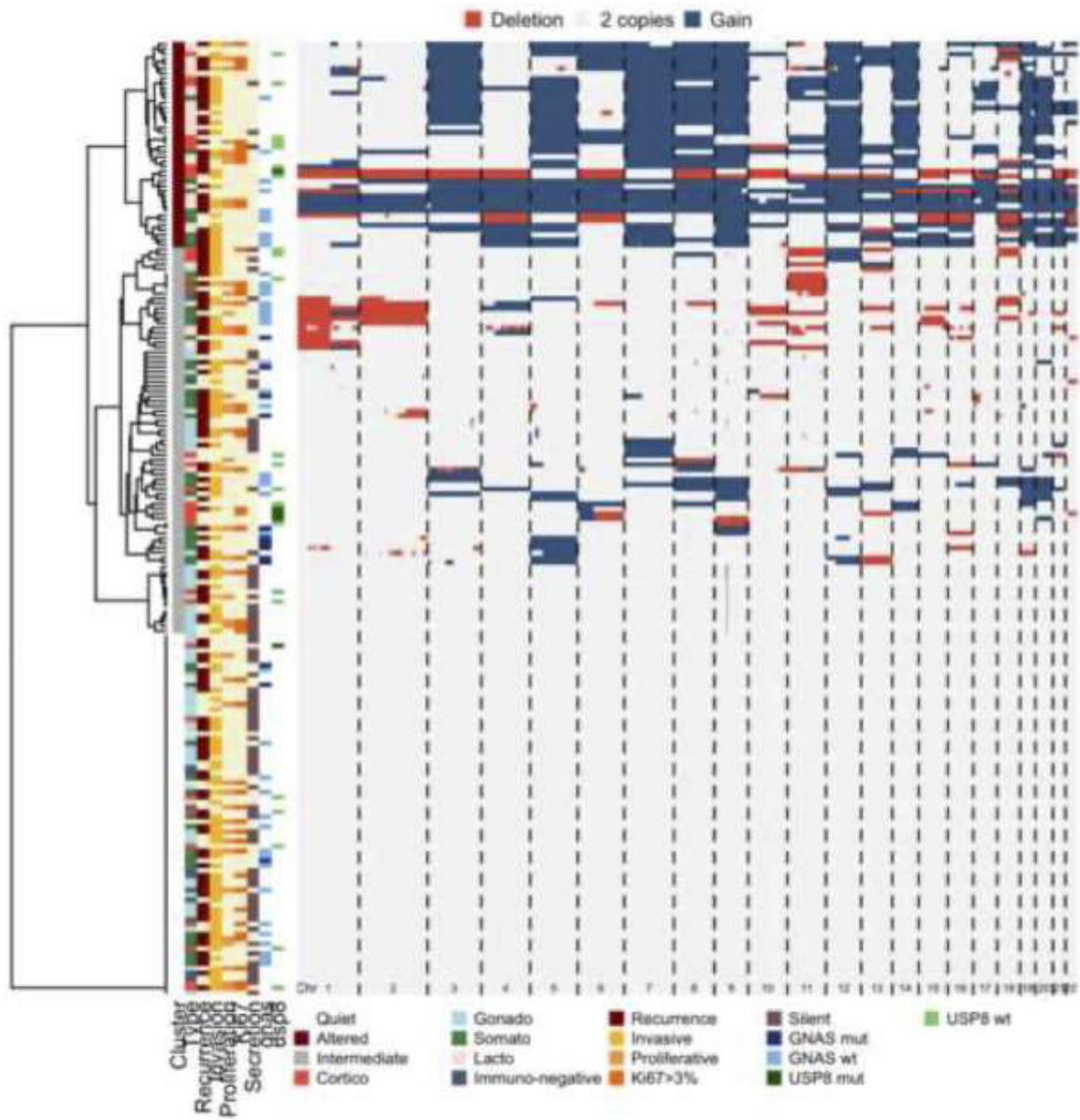
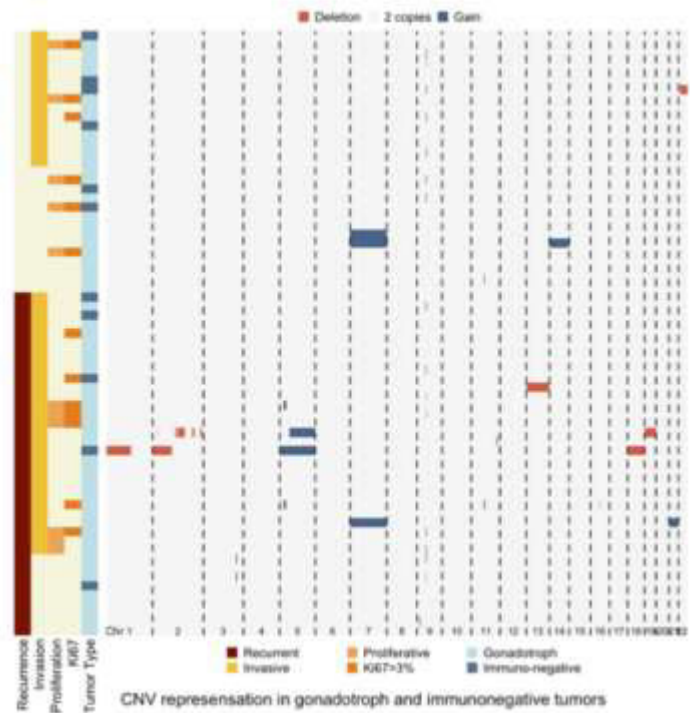
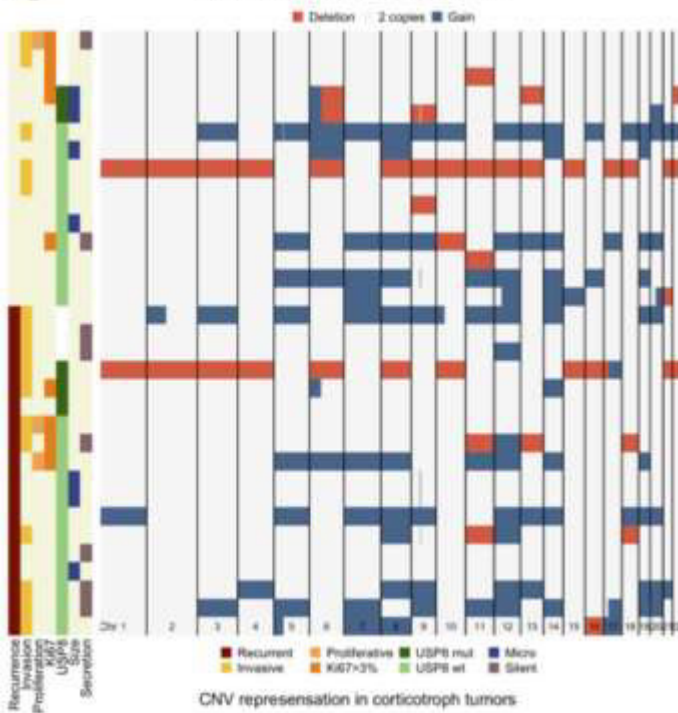
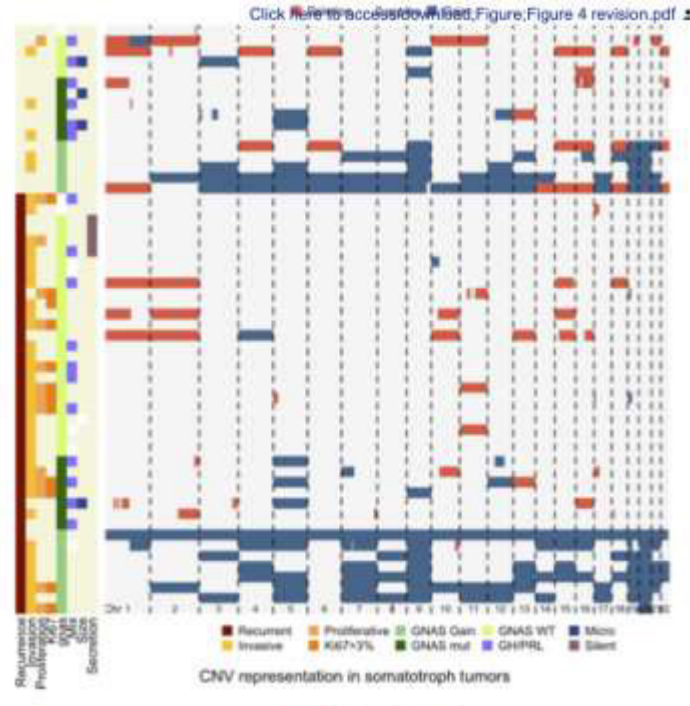
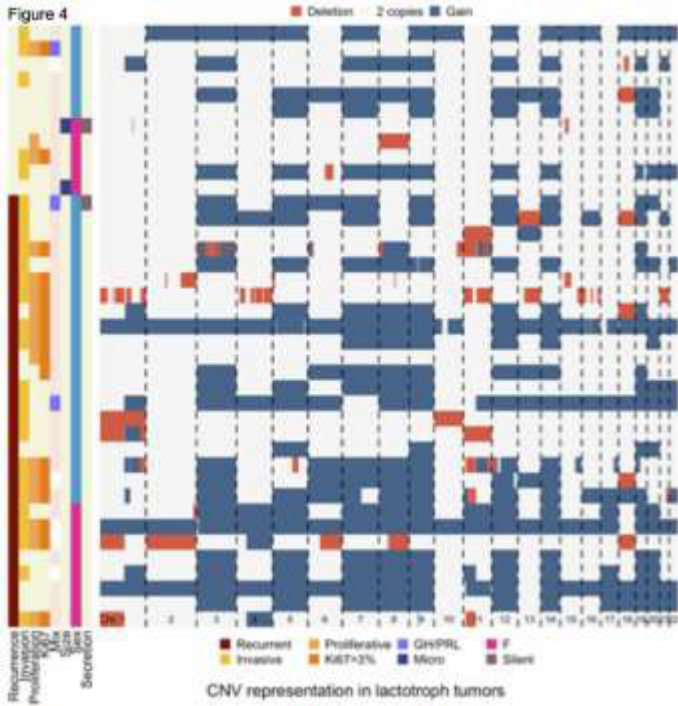


Figure 4

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Chromosomal instability in the prediction of pituitary tumor prognosis.

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Supplementary material

Table S1: Number of recurrent and non-recurrent lactotroph tumors concerned by deletion, gain and copy-neutral LOH for each altered gene.

Table S2: LRT p-values of univariate and multivariate logistic regressions on the genes significantly associated with recurrence in lactotroph tumors.

Figure S1 : FISH analysis in 8 tumors harboring gains of the GNAS locus.

A. Heatmap representation of the CNV in the somatotroph tumors with GNAS locus duplication which were analyzed using FISH.

B. Illustration of the results obtained with FISH analysis

FISH was performed on interphasic nuclei from frozen tumor-tissue appositions.

Probes used for FISH studies included 11pter (D11S2071) subtelomere probe (Cytocell, Cambridge, UK) in green and BAC RP1-309F20 encompassing *GNAS* locus in red.

Independent analyses of more than 20 cells were carried out for each tumor.

For tumor 1 to 5, at least 3 red dots are observed in each nucleus confirming the *GNAS* locus duplication identified by CGHarray. FISH analysis confirmed chromosome 11 gains in tumor 2 and tumor 4, as shown using CGHarray. For tumor 6 to 8, FISH analysis confirmed the absence of chromosome 20 and chromosome 11 gains. Tumor 6 and 8 harbored *GNAS* mutation while tumor 7 did not.

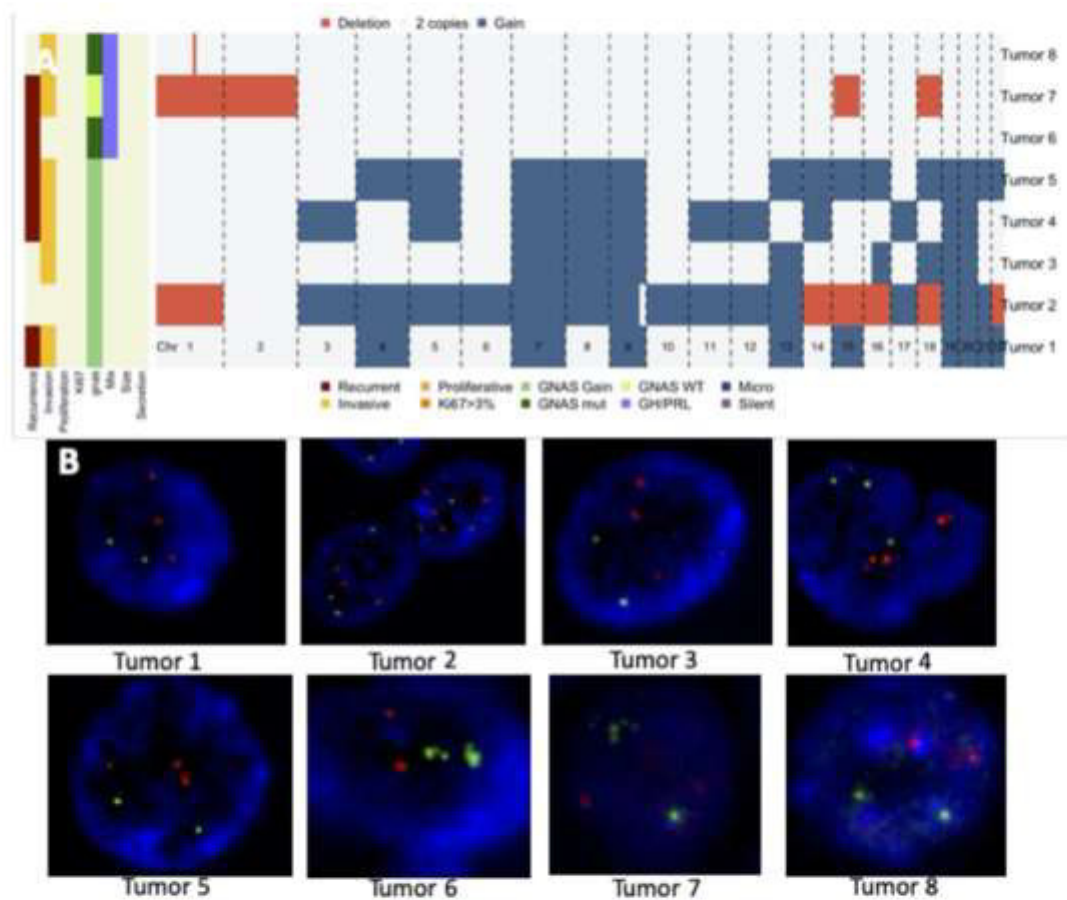
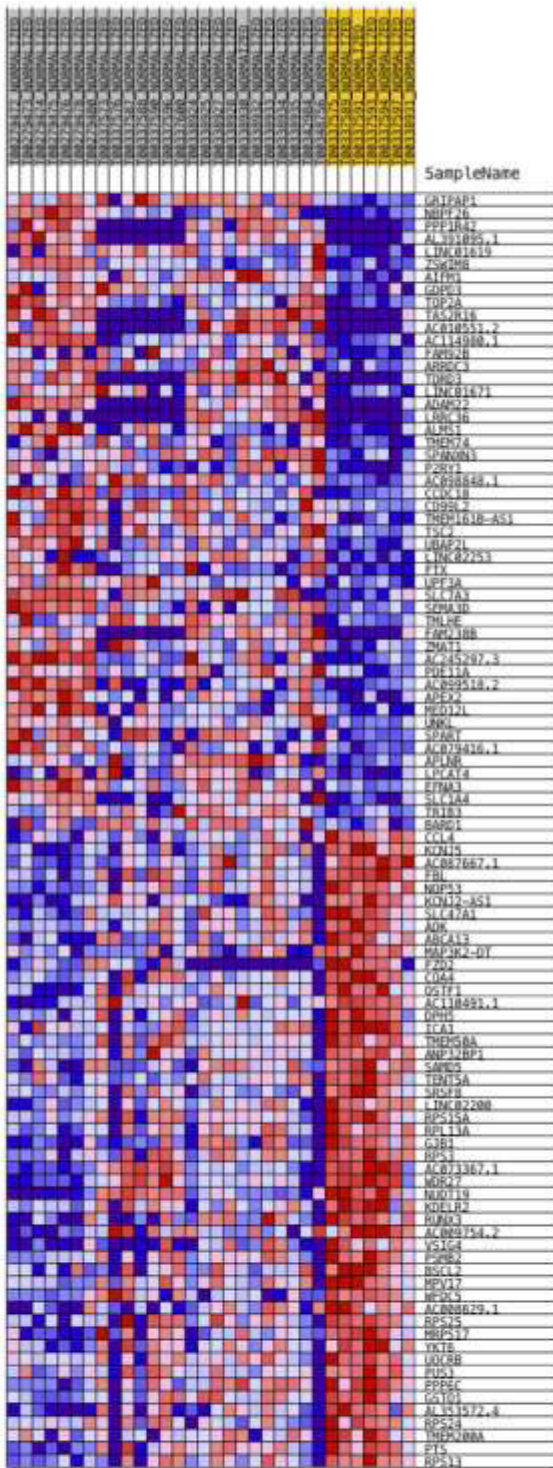


Figure S2: Heatmap representation of the transcriptomic analysis of 32 lactotroph tumors using GSEA with hallmark gene set (100 top genes associated with recurrent tumors in grey and non-recurrent in yellow. Upregulated genes are presented in blue, downregulated genes are presented in red)

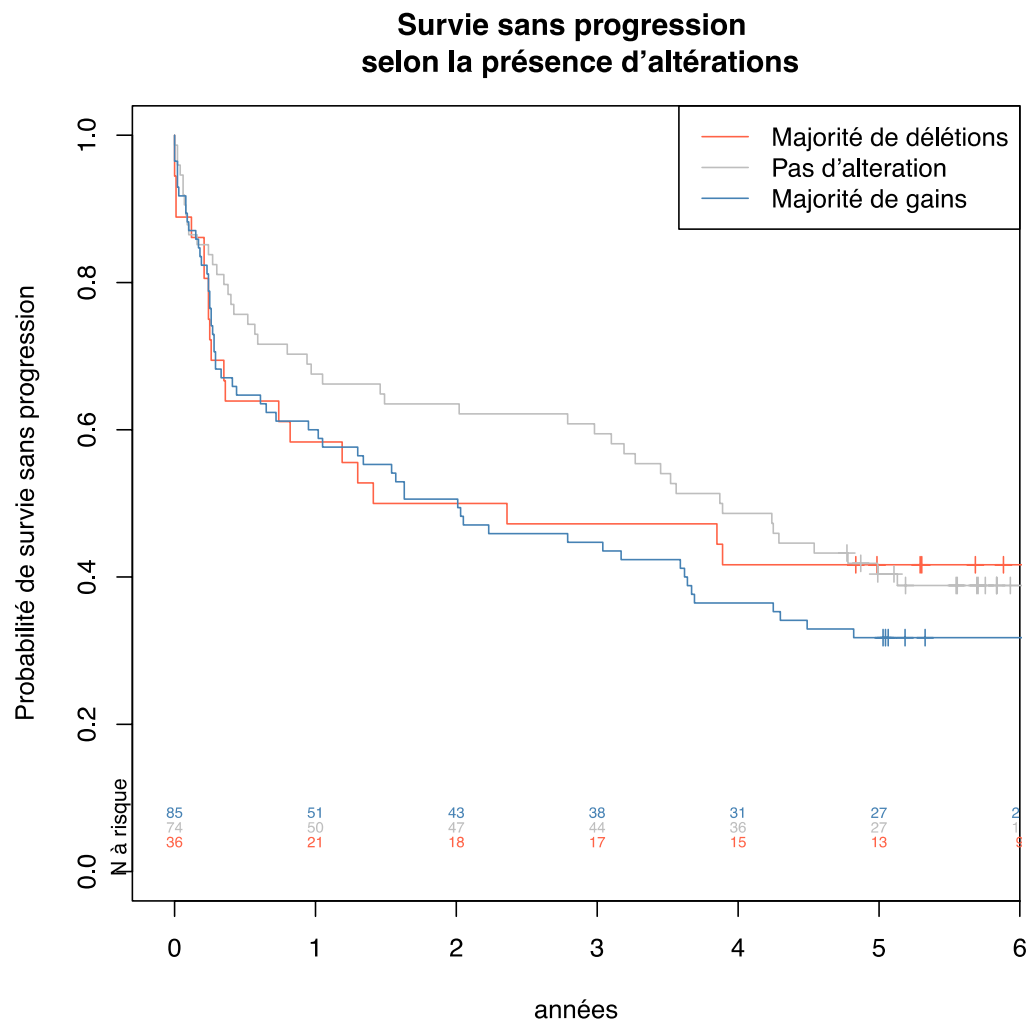


3.2.3 Discussion

L'instabilité chromosomique semble liée fortement au type tumoral et non au pronostic sur la cohorte entière.

Nous avons complété les analyses faites par régression logistique par des analyses de survie. L'analyse par modèle de Cox avec test de Wald, Score et du rapport de vraisemblance n'a pas retrouvé d'association entre l'estimation des taux de survie sans progression et la quantité de sondes altérées, délétées ou gagnées. L'analyse par méthode de Kaplan-Meier avec test du log-rank n'a pas retrouvé d'association entre le taux de survie sans progression et la quantité d'altérations catégorisée en 3 classes : absence d'altérations, présence d'altérations avec un maximum de gains ou présence d'altérations avec un maximum de délétions (figure 10). Trois cas de carcinomes hypophysaires ont été identifiés dans la cohorte, présentant 43, 59 et 93% de leur génome altéré, dont 2 avec majorité de gains et 1 avec majorité de délétions.

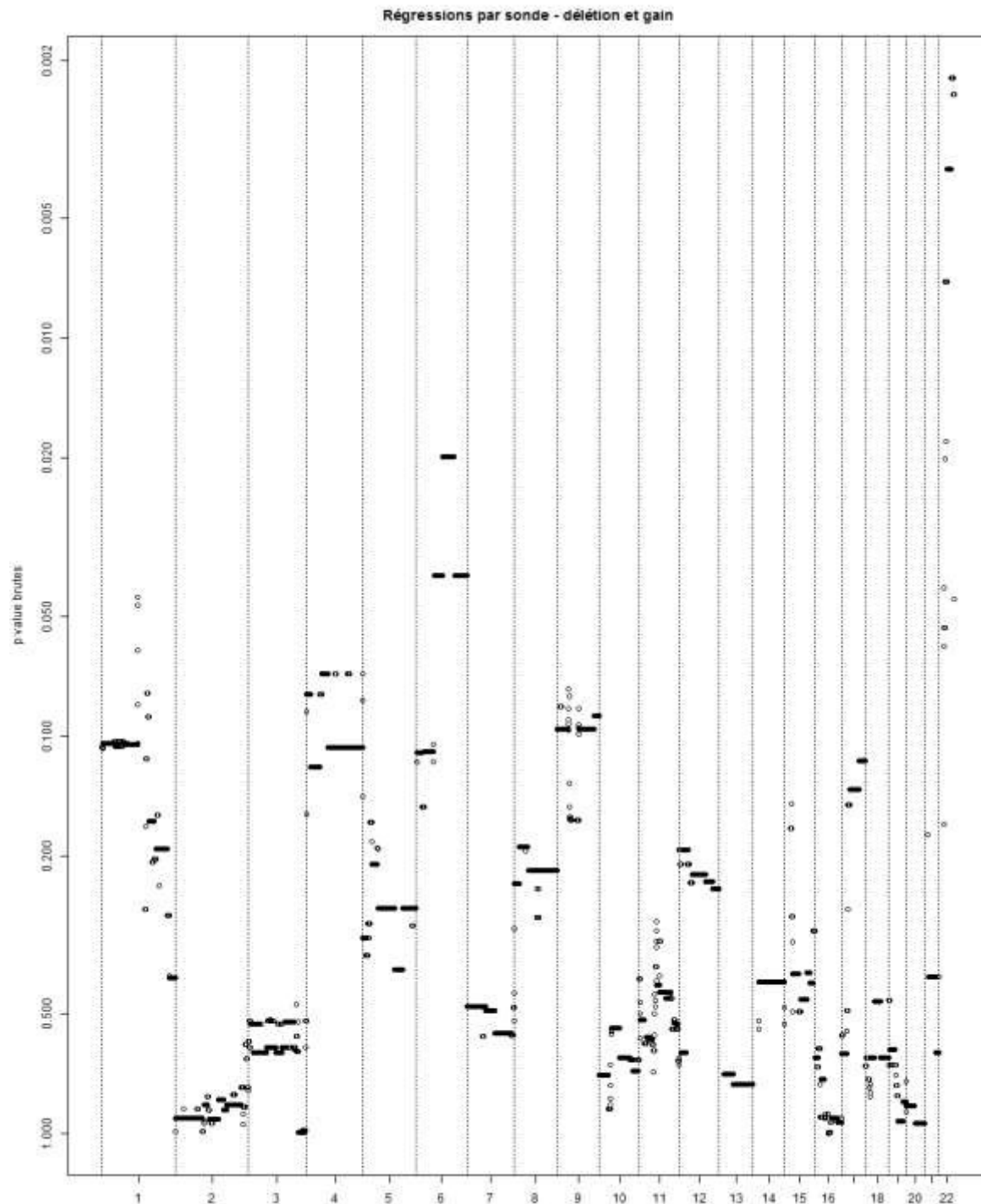
Figure 10 : Estimation du taux de survie sans progression par méthode de Kaplan-Meier dans l'ensemble de la cohorte en fonction de la présence d'altérations.



La recherche de CNV spécifiquement associés au pronostic grâce à des modèles de régression logistique par sonde n'a pas permis d'identifier de sonde associée à la récurrence de manière significative. La figure 11 présente les p-values non corrigées de chacun des tests de rapport de vraisemblance en fonction de la position génomique. Les rapports de vraisemblance comparent, pour chaque sonde, le modèle de régression logistique modélisant la récurrence en fonction de l'âge, du sexe, du grade et du type, et celui incluant en plus le log2ratio de la sonde classée comme gagnée, déletée ou sans altération. Seules quelques sondes du chromosome 22 semblent présenter des p-values plus faibles qui ne se maintiennent pas après correction des p-values en raison des tests

multiples. Les sondes plutôt que les segments ont été analysés, les bornes des segments étant propre à chaque patient.

Figure 11 : Représentation de valeurs de p-values des tests de rapport de vraisemblance des régressions logistiques multivariées par sonde en fonction de la position génomique sur l'ensemble de la cohorte.



L'analyse au sein de chaque type grâce à des modèles de régression logistique a retrouvé une association significative entre récurrence et quantité d'altérations, notamment de gains, dans les tumeurs lactotropes. L'analyse de survie par méthode de Kaplan Meier a

retrouvé une association significative entre taux de survie sans progression et présence d'altérations (p -value test de log-rank = 0.04). Les tumeurs étaient classées en 2 catégories selon la présence ou l'absence d'altérations. La séparation des tumeurs présentant des altérations en 2 catégories, avec majorité de gains ou majorité de délétions n'apportait par contre pas d'information supplémentaire (figure 12). La schématisation des différents CNV entre les tumeurs récidivantes et non récidivantes a montré que les altérations des chromosomes 1p, 11 et 17 survenaient exclusivement parmi les tumeurs récidivantes (figure 13).

Seulement, il est à noter que les tumeurs lactotropes sont traitées médicalement par agonistes dopaminergiques en première intention dans la très grande majorité des cas (328). La chirurgie est en général réservée aux tumeurs résistantes entraînant un biais de sélection dans les cohortes chirurgicales au profit de tumeurs plus agressives. L'estimation des taux de survie sans progression et de la proportion de récidives n'est donc pas à interpréter de manière absolue mais uniquement en analyse comparative.

Figure 12: Estimation de la probabilité de survie sans récidence selon la méthode de Kaplan Meier en fonction de la présence d'altérations dans les tumeurs lactotropes

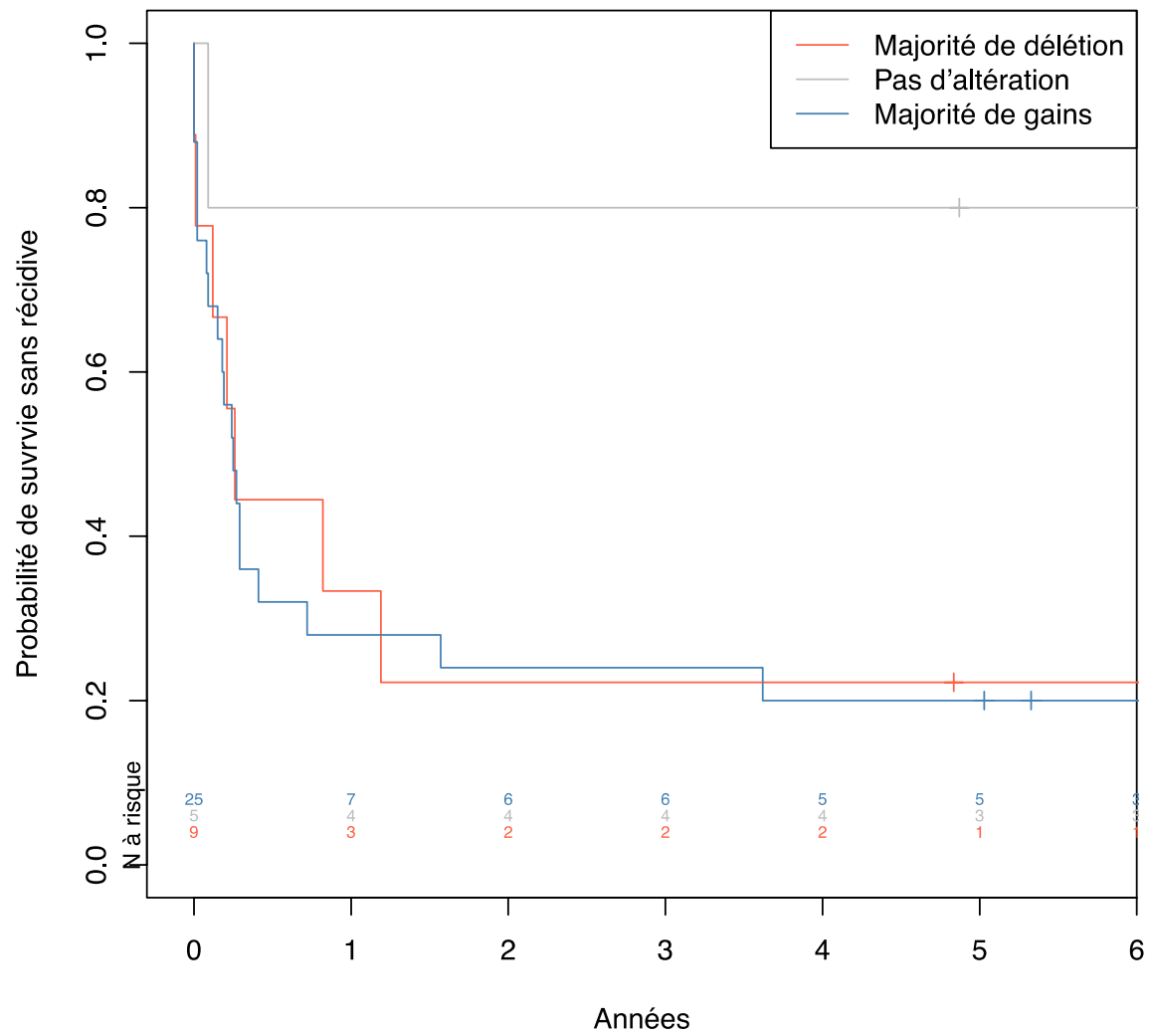
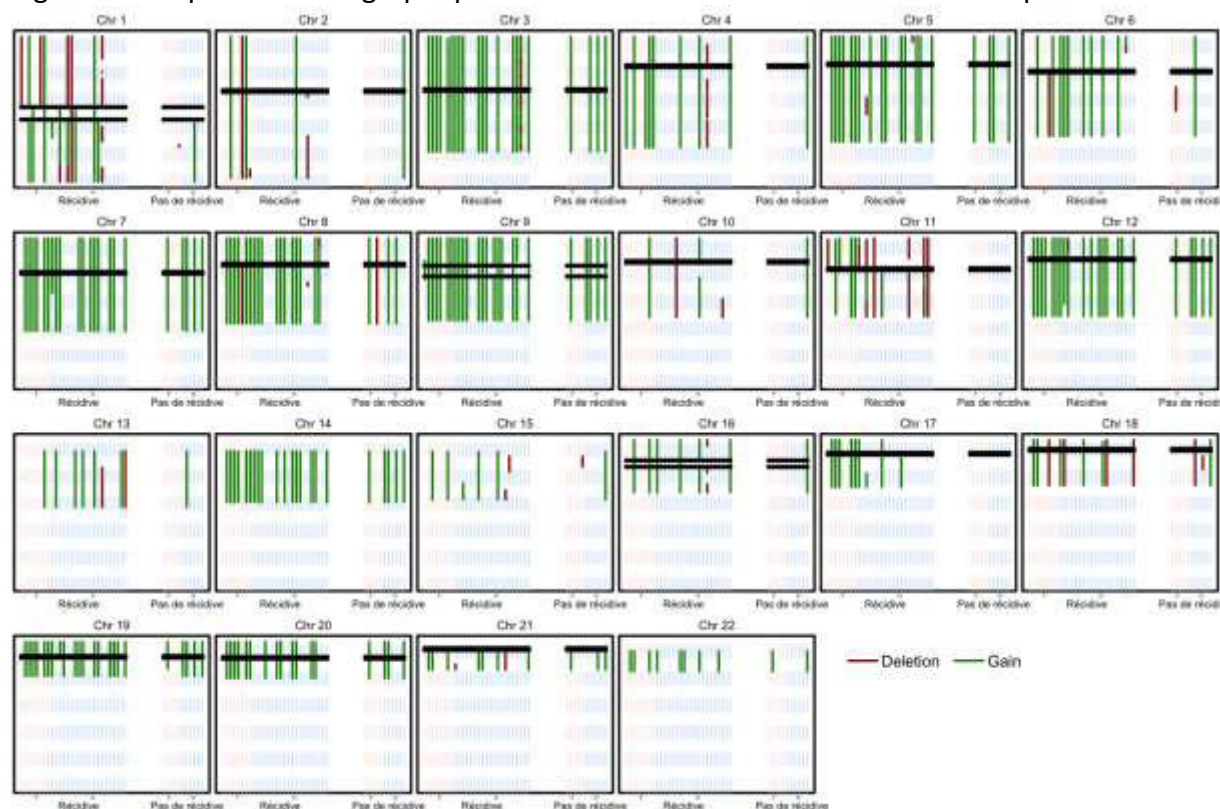


Figure 13 : Représentation graphique des différents CNV des tumeurs lactotropes



L'origine de l'instabilité chromosomique dans les tumeurs hypophysaires, très majoritairement de bon pronostic, pose question.

Le rôle de la surexpression de *PTTG1* est évoqué par certains auteurs. La protéine PTTG1 est exprimée au niveau du noyau et du cytoplasme. Elle stimule l'apoptose via p53 et participe à l'attache des chromatine sœurs lors de la division cellulaire (261). Sa surexpression pourrait participer à des phénomènes d'aneuploïdie. Ainsi, Valimaki *et al.* ont retrouvé une surexpression de PTTG1 en IHC dans les tumeurs somatotropes avec aneuploïdie (130), vs sans aneuploïdie.

Neou *et al.* ont évoqué le rôle de l'hypo-méthylation et de l'hyper-expression d'éléments transposables dans leur étude pangénomique (15). Valimaki *et al.* dans l'analyse de 21 tumeurs somatotropes retrouvaient un lien entre méthylation et mutation de *GNAS* mais pas de lien entre méthylation et altérations chromosomiques.

Ben Shlomo *et al.* supposent un rôle, dans l'instabilité chromosomique des tumeurs somatotropes, de l'activation de la voie AMPc via une surexpression de p53/p21^{Cip1/Wif1}, ainsi qu'une diminution de l'expression des gènes de la voie de l'anémie de Fanconi impliqués dans les mécanismes de réparation de l'ADN (72,170). Valimaki *et al.* dans leur

étude n'ont pas retrouvé de lien entre aneuploïdie et expression de pRB1 ou totRB1 en IHC(130).

Dans d'autres types tumoraux, l'aneuploïdie pourrait être associée à l'impureté cellulaire. Alors que l'aneuploïdie est classiquement associée à un faible infiltrat immunitaire, Taylor *et al.* ont retrouvé sur les données du TCGA, une corrélation positive entre aneuploïdie et impureté cellulaire faisant suspecter un impact de l'infiltrat non immunitaire (329). En effet, dans les méthodes classiques de séquençage, de puce SNP et CGHarray, l'étape d'extraction d'ADN ne permet pas de différencier les populations cellulaires tumorales et non tumorales, et l'analyse concerne l'ensemble des cellules du fragment tumoral. Ceci est une limite majeure de l'analyse par CGHarray. Les différences marquées de profil en CGHarray parmi les types tumoraux analysés pourraient être le reflet de différences de pureté cellulaire et d'infiltrat immunitaire. De plus, dans d'autres types tumoraux, le nombre d'altérations chromosomiques est habituellement inversement corrélé au nombre de mutations ponctuelles, en l'absence d'instabilité microsatellite ou de mutation germinale de P53 (329). Les tumeurs avec peu d'altérations chromosomiques et une charge mutationnelle forte, présentent souvent un infiltrat immunitaire important et une meilleure réponse aux immunothérapies; à l'inverse des tumeurs avec aneuploïdie (316,330). A l'heure où le traitement par immunothérapie commence à être proposé dans les tumeurs hypophysaires agressives et carcinomes (331,332), il pourrait être intéressant d'étudier le lien entre instabilité chromosomique et infiltrat immunitaire.

La technique de CGHarray ne permet pas non plus l'exploration de l'hétérogénéité intra-tumorale. Ainsi, lors de l'analyse, l'ADN des différents clones tumoraux est poolé et le log₂ratio finale est la résultante des log₂ratio de chacun des clones.

Par ailleurs, le lien entre variations du nombre de copies et modification d'expression des gènes n'est pas évident, faisant intervenir d'autres mécanismes de régulation de l'expression génique. Une amplification génique n'entraîne pas toujours une surexpression de la protéine, et inversement pour une délétion. Lin *et al* par exemple ont analysé le lien entre amplification de *PIK3CA* et expression en IHC (87). L'expression en IHC était significativement associée au nombre de copies mais seule 26/99 tumeurs avec une amplification > 4 copies de *PIK3CA*, exprimaient PIK3CA en IHC. Dans notre travail, seules 16 tumeurs lactotropes ont bénéficié de l'analyse commune transcriptomique et

par CGHarray. Sur ces tumeurs, nous n'avons pas identifié de lien évident entre les variations du nombre de copies à un locus donné et fold-change en analysant les gènes précédemment sélectionnés.

La nécessité d'une analyse par type tumoral paraît évidente au vu de l'impact du type tumoral sur le profil de CGHarray. Seulement, nous avons été limités par le nombre de cas inclus par sous-type. Ainsi, la majorité des tumeurs analysées correspondent à des tumeurs gonadotropes montrant très peu d'évènements. Le nombre de tumeurs lactotropes et corticotropes est notamment limité, ne permettant pas d'analyse fiable à l'intérieur de chaque type de caractéristiques telles que le caractère silencieux, pluri-hormonal, le sexe etc...

4 CONCLUSIONS ET PERSPECTIVES

Les tumeurs hypophysaires sont des tumeurs habituellement de bon pronostic. Seulement, 30% environ présentent des récurrences et 40% envahissent les structures adjacentes. Parmi celles-ci, un faible pourcentage peut présenter un comportement agressif. Les mécanismes qui sous-tendent cette agressivité sont à l'heure actuelle insuffisamment compris. Leur exploration est rendue difficile par plusieurs points. D'une part, l'évolution lente et la faible proportion de tumeurs agressives nécessitent le suivi prolongé d'un grand nombre de tumeurs. D'autre part, le manque de standardisation dans la définition de l'agressivité, de même que l'hétérogénéité des tumeurs hypophysaires rendent difficile des analyses méthodologiquement robustes.

Ce travail a montré que les tumeurs hypophysaires, malgré leur évolution habituellement favorable, présentent une instabilité chromosomique importante.

Cette instabilité importante a mis en défaut la technique de CGHarray. Ainsi, nous avons montré que la technique d'hybridation comparative à un ADN témoin pouvait faire l'objet d'un biais de centralisation en cas d'altérations importantes. Il nous a été nécessaire de mettre au point une technique de centralisation prenant en compte une mesure absolue du nombre de copie grâce à une analyse par FISH. La mise en évidence de ce biais nous montre l'importance du prétraitement des données pour l'interprétation en analyse omique.

L'instabilité est extrêmement variable selon les tumeurs, certaines tumeurs ne présentant aucune altération, alors que d'autres présentent un génome entièrement aneuploïde voire des phénomènes de chromothripsis. Le facteur qui s'associait le plus à cette instabilité chromosomique était le type tumoral faisant suspecter des mécanismes de tumorigénèse propres à chaque type tumoral. Dans des études futures, une analyse par type tumoral sera à privilégier.

La quantité d'altérations ne s'associait pas au pronostic, ni à l'invasion, dans la cohorte entière. Parmi les prolactinomes, l'instabilité chromosomique était associée à plus de récurrences en analyse multivariée et à une survie sans récurrence plus courte. Les conséquences des CNV sont difficile à évaluer et 1,329 gènes inclus dans les altérations étaient associés avec la récurrence.

Cette instabilité chromosomique peut potentiellement être le reflet du microenvironnement tumoral et de l'infiltrat immunitaire plus que de la population cellulaire tumorale. La poursuite des explorations par une analyse couplée de l'instabilité tumorale et du microenvironnement tumoral par séquençage sur cellule unique par exemple pourrait être intéressante.

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Original article

Pasireotide: A potential therapeutic alternative for resistant prolactinoma

Pasiréotide, une alternative thérapeutique pour les prolactinomes résistants ?

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Abstract

Contexte.—About 10% of prolactinomas are resistant to dopamine-agonists (DAAs). The only alternatives for tumor and prolactin control are surgery or radiotherapy. While studies on first generation somatostatin analogs have shown an efficacy against prolactinomas, no study has been conducted on the new multireceptor-targeted somatostatin receptor ligand pasireotide, which presents high affinity for 5, 3, 2 and 1 receptor subtypes. **Cas clinique.**—A 41-year-old woman presented with a macroprolactinoma showing resistance to all available DAAs. She was first diagnosed at 17 years old after which she had undergone two incomplete debulking surgeries. Under pasireotide long-acting release (LAR) treatment, plasma prolactin levels normalized and symptoms disappeared within one month after initiation. The clinical benefits of the monotherapy (specifically, prolactin levels within normal range and stable tumor volume) were maintained for seven years. Glucose tolerance was satisfactory. Pathological analysis of the tumor revealed high SSTR5 and low SSTR2 expression (25 and 5% of cells respectively). **Conclusion.**—This is a promising first report of a patient with a DA-resistant macroprolactinoma who achieved long-term control, in terms of prolactin normalization and tumor volume, under pasireotide treatment alone. Pasireotide could thus be an alternative in prolactinomas resistant to DA. SSTR expression analysis on pathology could guide patient selection.

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Keywords: Pituitary tumor; Prolactinoma; Aggressive tumor; Somatostatin receptor; Pasireotide

Résumé

Contexte.—Dix pour cent des prolactinomes sont résistants aux agonistes dopaminergiques (DAAs), offrant comme seules possibilités thérapeutiques, pour contrôler la tumeur et la prolactinémie, la chirurgie ou la radiothérapie. Alors que les analogues de la somatostatine de première génération n'ont pas montré d'efficacité, aucune publication ne rapporte l'effet du pasiréotide, analogue de la somatostatine de nouvelle génération présentant un profil d'affinités plus large, notamment pour les récepteurs des sous-types 5, 3, 2 et 1. **Description.**—Une patiente âgée de 41 ans a été adressée pour un macroprolactinome évoluant depuis l'âge de 17 ans, résistant aux agonistes dopaminergiques, ayant nécessité deux chirurgies d'excision incomplètes. Après introduction de pasiréotide longue durée d'action (LAR), la prolactinémie s'est normalisée en 1 mois, permettant une disparition des symptômes. L'effet thérapeutique était constant au dernier suivi, 7 ans après l'introduction, avec une stabilisation du volume tumoral et une prolactinémie normale. La tolérance glucidique était correcte. L'analyse histologique de la tumeur a retrouvé une forte expression de SSTR5 associée à une faible expression de SSTR2 (25 et 5 % des cellules respectivement). **Conclusion.**—Nous rapportons le premier cas de macroprolactinome résistant aux agonistes dopaminergiques, pour lequel un traitement par pasiréotide seul a permis un contrôle de la prolactinémie et du volume tumoral sur le long terme. Ainsi, le pasiréotide pourrait être une alternative thérapeutique dans les prolactinomes résistants aux agonistes dopaminergiques. L'analyse histologique de l'expression de SSTR pourrait aider à sélectionner les patients.

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Mots-clés : Tumeur hypophysaire ; Prolactinome ; Tumeur agressive ; Récepteurs à la somatostatine ; Pasiréotide

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1. Introduction

Prolactinomas are the most frequently encountered pituitary tumor, representing around 50% of all pituitary tumors. Dopamine-agonist (DA) therapy, especially cabergoline, is the first-line treatment and is able to normalize prolactin levels and tumor volume in most cases. However, prolactin levels cannot be normalized by conventional doses in around 10% of prolactinomas [1]. These are considered as DA-“resistant” and present more often as invasive macroadenomas [2]. A tumor size reduction less than 50% maximal diameter is more rarely used as a criterion of resistance. In cases of resistance, or more rarely of intolerance to dopamine-agonists, alternative treatments are surgery and radiotherapy. However, they may not be indicated, depending on the size, localization and firmness of the tumor. Moreover, surgical excision is not always curative, especially in case of invasive tumors, while radiotherapy exposes to side effects such as hypopituitarism.

The new multireceptor-targeted somatostatin transmembrane receptor (SSTR) ligand pasireotide is marketed for patients with acromegaly or Cushing's disease. It can bind to G-protein coupled SSTRs of normal or neoplastic pituitary cells and inhibits hormone secretion and cell growth. Unlike first generation somatostatin analogs (lanreotide or octreotide) which bind preferentially SSTR2, pasireotide binds to multiple SSTRs with a high affinity for SSTR5 followed by SSTR2, SSTR3, and SSTR1 [3].

We report here the first patient with a DA-resistant macroprolactinoma to present long-term prolactin normalization on pasireotide treatment alone.

2. Case-report

In 2010, a 41 year-old woman was referred to us for treatment of a DA-resistant macroprolactinoma.

She was diagnosed in 1986 with a macroprolactinoma measuring 15 mm and responsible for galactorrhea and amenorrhea. Initial plasma prolactin levels were 116 ng/mL. The pituitary and visual function assessment were normal. Bromocriptine therapy was progressively increased up to 15 mg/day. In the absence of control over plasma prolactin levels, surgical removal of the tumor was decided five years later in 1991. The pathological examination revealed a grade 2b pituitary neuroendocrine tumor (Fig. 1A) with 100% of cells expressing prolactin (Fig. 1B), high mitotic count ($n=4$), and positive P53 expression. Ki67 index was not interpretable because of technical issues.

After an initial period of postoperative remission, her initial symptoms recurred one year after surgery. At this point, plasma prolactin levels had risen to 94 ng/mL and a CT-scan revealed a macroadenoma measuring 20 mm in the right sellar region. Bromocriptine (25 mg per day) and quinagolide (225 µg per day) separately failed to normalize plasma prolactin levels. A second partial surgery was required in 2003 to treat a third cranial nerve palsy caused by tumor regrowth that had occurred once DA had been stopped. Cabergoline treatment permitted a temporary and incomplete decrease in plasma prolactin levels which allowed a spontaneous pregnancy in 2007. The patient gave birth to a

normal girl but menstruation was not restored despite a continued and intensified treatment with cabergoline up to 4.5 mg per week. Plasma prolactin levels rose to 127 ng/mL while MRI showed global stability of the large right sellar residue measuring 33 mm. Moreover, this higher dose of cabergoline was badly tolerated inducing asthenia and dizziness. Radiotherapy was then proposed but refused by the patient.

Re-examination of the tumor fragment from the first surgery showed expression of SSTR5 in 25% of cells (Fig. 1C) whereas only 5% expressed SSTR2 (Fig. 1D). The analysis of genes *AIP* and *MEN1* revealed no mutation.

As part of a clinical trial (ClinicalTrials.gov Identifier: NCT00958841, approved by ethical committee of Hospices Civils de Lyon), pasireotide LAR was begun in October 2010 at 60 mg every 28 days. This normalized plasma prolactin levels within one month (Fig. 2) and allowed the stopping of cabergoline three months later. At this point, amenorrhea-galactorrhea recovered and MRI revealed a global stability of tumor size with necrotic transformation suggestive for an anti-tumor effect (Fig. 2). The treatment was well tolerated without any digestive disorders. There was no diabetes mellitus, but glucose intolerance occurred as shown by HbA1c rising from 5.7% to 6.2% and fasting plasma glucose level from 0.90 to 1.20 g/L then to 1.27 g/L six months after pasireotide initiation. However, this required no pharmacological treatment and the implementation of dietary measures was sufficient.

Treatment was progressively decreased to 20 mg every five weeks. At the last follow-up seven years after pasireotide initiation, the plasma prolactin levels were maintained within the normal range and MRI showed a stable residual tumor. Tolerance was correct, fasting plasma glucose was 0.97 g/L and HbA1c was 5.7%.

3. Discussion

We report here the first case of a DA-resistant macroprolactinoma demonstrating a clinical and hormonal response to pasireotide treatment allowing its long-term control.

Pasireotide is a new somatostatin receptor ligand currently prescribed in patients with acromegaly, Cushing's disease and neuroendocrine tumors. Compared to lanreotide and octreotide, it has a broader binding profile and significantly higher *in vitro* affinity for SSTR 1, 3 and 5 receptors (respectively 30, 5 and 40 times), and a lower one for SSTR 2 receptors (2.5 times) [3].

The expression of somatostatin receptor subtypes has already been described in human prolactinomas [4–6] and in light of our results presented here, may justify the potential use of somatostatin analogs in the treatment of prolactinomas. Both immunohistochemistry studies, via use of a scoring system (associating % of immunopositive cells and membrane staining pattern), and mRNA studies have shown the expression of three subtypes of SSTR in human prolactinomas: SSTR1, 2 and 5. The level of expression is highly variable depending on the tumor, but all studies found higher SSTR5 expression followed by lower and variable levels of expression of SSTR2A and SSTR1 [4–6]. The expression pattern has never been associated with DA resistance, tumor size or aggressiveness [5].

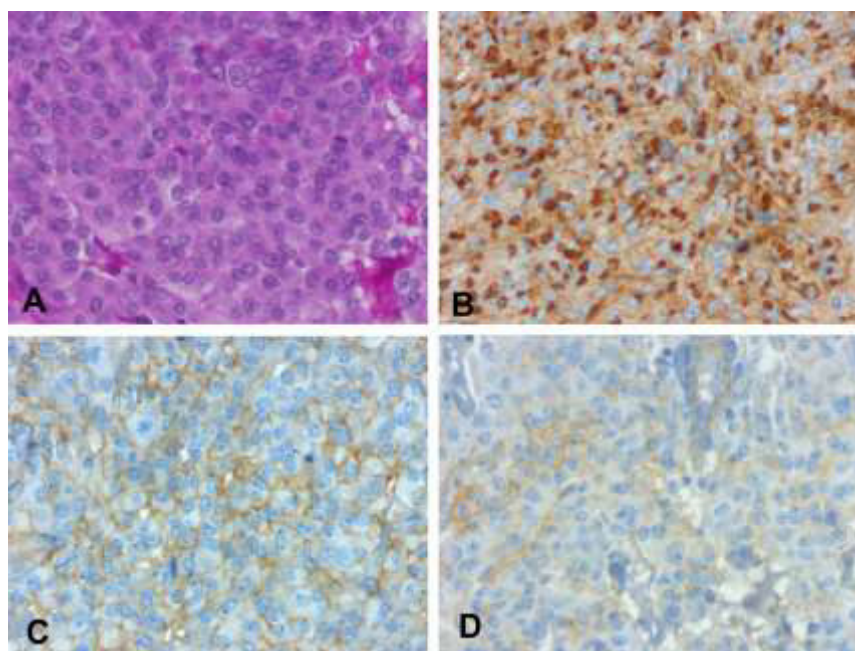


Fig. 1. Histopathological features of the first surgical specimen (original magnification $\times 400$). A. On hematoxylin phloxine saffron staining, the tumor shows a diffuse growth pattern. Anisokaryosis is moderate and numerous nuclei show a prominent nucleolus. B. The majority of neoplastic cells show typical paranuclear prolactin immunoreactivity. C. A moderately intense membranous immunoreactivity of SSTR5 is observed in 25% of neoplastic cells. D. Only 5% of neoplastic cells show a membranous immunopositivity for SSTR2.

A role for SSTRs in prolactin secretion is suspected. However, results from *in vitro* studies on use of somatostatin analogs in prolactinomas are inconsistent. In some studies, octreotide significantly reduced prolactin secretion of primary human prolactinoma and mixed growth hormone and prolactin (GH-PRL)-secreting cultured cells [7,8]. Other studies showed an uncertain and modest reduction of prolactin secretion and cell viability, especially using octreotide [6,9,10]. These inconsistencies could be due to differences in SSTR2 expression, as indicated by Acunzo et al. who observed prolactinomas becoming sensitive to octreotide only after *SSTR2* gene transfer [10,11]. Evidence suggests that pasireotide may be more effective than other somatostatin analogs at reducing prolactin secretion. In 1999, Jaquet et al. demonstrated that another somatostatin agonist (BIM-23268) with high affinity for SSTR5 could inhibit prolactin release from human prolactinomas in primary culture. This 26 to 90% reduction was higher compared to the one induced by octreotide [5]. More recently, pasireotide and octreotide effects on human prolactinoma and GH/PRL-secreting tumor were compared *in vitro*. Pasireotide could reduce PRL secretion between 38 and 74% in GH/PRL and between 23 and 80% in PRL tumors, compared to the percent reduction achieved with octreotide of between 16 and 66% in GH/PRL and 0 and 30% in PRL tumors [7]. An *in vivo* study in mouse GH/PRL adenomas then showed not only a greater reduction in prolactin secretion with pasireotide compared to with octreotide but also a superior anti-tumor effect [12]. However, the effects of pasireotide did not differ significantly from octreotide in other recent studies [6,8,9]. Moreover, in these latter studies, the effect

of somatostatin analogs on prolactin secretion was weaker than that of DAs, even in prolactinomas considered as resistant [5–7].

As suggested by *in vitro* studies, the SSTR profile of prolactinomas may explain the mainly disappointing results of first generation somatostatin analogs in treating prolactinomas. Only one successful case of octreotide treatment has been reported [13] in a young man who had previously undergone two surgical excisions. Initiation of octreotide at 20 mg every 28 days in association with cabergoline led to normalized plasma prolactin levels at the 12 month follow-up and stability of the pituitary tumor at 2 years, as shown by MRI. All other studies using cyclic synthetic somatostatins or octreotide reported therapeutic failures [14].

Clinical effects of the second-generation somatostatin analog pasireotide on prolactinomas have not yet been reported in the literature. Our report demonstrates the efficacy of pasireotide to normalize prolactin secretion, achieve long-term control over tumor volume and restore the menstrual cycle in a woman with a resistant prolactinoma ineffectively treated over the previous 24 years with high doses of all available dopamine-agonists (bromocriptine, quinagolide, cabergoline). In such cases of DA-resistant prolactinoma, this treatment could be proposed before considering radiotherapy or temozolomide treatment options.

No significant adverse effects of pasireotide were observed in this patient. Digestive disorders and hyperglycemia are usually the most frequent adverse effects of this drug. As pasireotide is associated with a reduction in the secretion of the hormones insulin and incretin, hyperglycemia and diabetes are observed in 30% of acromegalic patients and up to 70% of patients with

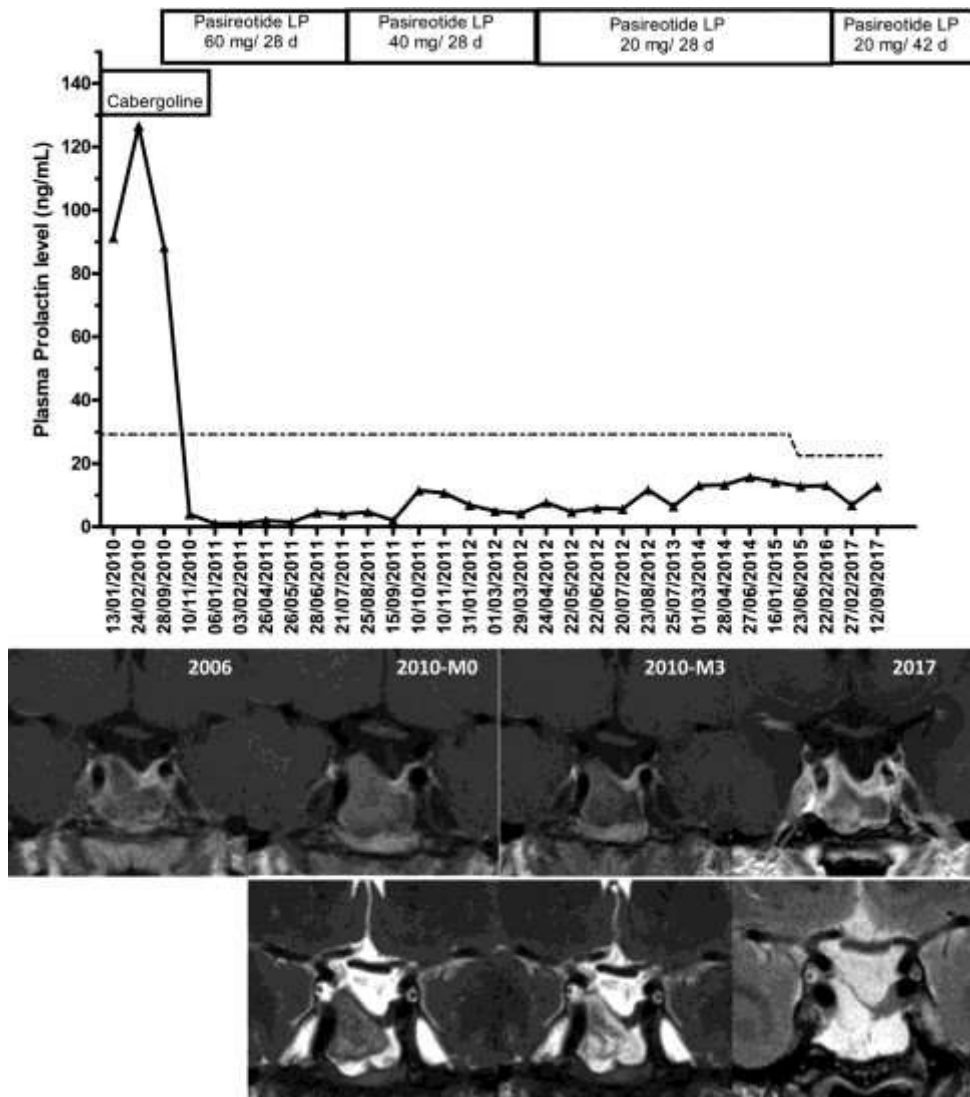


Fig. 2. Prolactin levels and MRI evolution (four years before pasireotide initiation [2006], at pasireotide initiation [2010-M0], at 3 months after pasireotide initiation [2010-M3] and at the last follow-up [2017]).

Cushing’ disease. However, the glucose tolerance disturbance induced by the IGF1 and cortisol excess likely potentiates the hyperglycemia occurring under pasireotide. A lower rate of hyperglycemia events should be expected in patients with prolactinoma.

In acromegalic patients, the relation between SSTR subtypes expression by immunohistochemistry and response to somatostatin analogs is suggested [15,16]. Our results support this hypothesis in prolactinomas for which SSTR5 expression is significant. SSTR subtype expression profile could therefore potentially guide therapeutic choice and allow a personalized therapy for these rare patients.

In conclusion, our case has demonstrated the potential role of pasireotide for treating DA-resistant macroprolactinomas. SSTR expression may guide the choice of therapy. However,

pasireotide being quickly efficient, administration as a short therapeutic test may be sufficient to identify potential responders.

Patient consent

The author confirm that informed consent has been obtained from the patient for publication of the case-report and accompanying images.

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Disclosure of interest

The authors declare that they have no competing interest.

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Confirmation of a new therapeutic option for aggressive or dopamine agonist-resistant prolactin pituitary neuroendocrine tumors

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Abstract

Recent publications suggested that pasireotide could be a good therapeutic option in some dopamine-resistant or aggressive prolactinomas. We discussed the two published cases and describe another case of poorly differentiated plurihormonal PIT-1-positive adenoma with moderate SSTR2 expression and intense SSTR5 expression successfully treated with PAS-LAR 40 mg/month.

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This very interesting case report by Coopmans *et al.*, demonstrating response to pasireotide therapy in an aggressive dopamine-resistant prolactinoma, recapitulates, in most respects, the description of a previous case published by Lasolle *et al.* (1). Both these cases demonstrated the rapid and sustained normalization of prolactin secretion in a macroprolactinoma resistant to high doses of cabergoline (up to 7 mg/week). Prolactin normalized within a few weeks (2 and 1 month), and the effect was maintained for more than 3 and 9 years respectively. Prolactin normalization was associated with a change of the tumor on MRI in both cases, with higher T2 signal intensity with increasing treatment duration suggestive of cystic degeneration and tumor cell necrosis. However, tumor shrinkage was evident only in the case published here (Coopmans *et al.*). This discrepancy could be explained by the tumor location, which was limited to the sphenoid sinus in the first published case (1). Indeed, it is frequently observed in macroprolactinoma that despite major tumor shrinkage of the suprasellar extension on cabergoline treatment, shrinkage is less obvious in the sphenoid component of the tumor due to its anatomical characteristics (2).

Contrary to this case in which cabergoline was maintained at a low dose despite cardiac valvular regurgitation, in the Lasolle *et al.* case (1), cabergoline, being ineffective, was stopped after 3 months of PAS-LAR without an increase in prolactin. Moreover, we were able to reduce the dose of PAS-LAR to 20 mg every 6 weeks associated with slight, clinically non-significant, prolactin increase and without change seen on MRI.

SSTR2 and SSTR5 expression, analyzed by immunohistochemistry, demonstrated variable membranous expression with IRS 2 and 9 for SSTR2a and 4 and 12 for SSTR5 (Lasolle *et al.* and Coopmans *et al.* respectively). SSTR2a being low in one case and 10-months treatment with SRL lanreotide autogel at 120 mg/month, a SSTR2a analog, having no effect on prolactin secretion or tumor size, suggested that PAS-LAR action was mediated by SSTR5 expression. IRS for SSTR5 was moderate or high, suggesting that SSTR5 expression analysis could predict tumor response to PAS-LAR and may be used to select patients who may benefit from this treatment before considering temozolomide treatment.

However, it should be noted that SSTR2a and SSTR5 expression are uncommon in prolactinomas. In our cohort

of 47 macroprolactinomas, operated in our referral center from 2010 to 2019, SSTR2 and SSTR5 expression were present in 3/23 and 3/21 analyzed tumors respectively. One tumor expressed both receptors, while 4 other tumors expressed SSTR2a or SSTR5 only. IRS for SSTR5 were mild (3; $n=1$) to intense (12; $n=2$).

In addition to our previously published case, we recently treated a 55-year-old woman, who was referred to our institution with a diagnosis of giant silent prolactinoma resistant to cabergoline, with PAS-LAR 40mg/month. The initial diagnosis was made in another center in June 2016 on MRI performed to evaluate recent visual field defects and severe headache (Fig. 1). She underwent surgical biopsy which led to the diagnosis of silent prolactinoma. Her prolactin level was normal at diagnosis but according to the pathology report she was treated with cabergoline at a dose of up to 3.5 mg/week. In the absence of a tumor effect, after 6 months of treatment, she was referred to our reference center for the indication of surgical debulking. Surgery via a transcranial approach was performed in December 2016. As expected, post-operative MRI revealed a large residual tumor with persistent optic nerve compression. Pathology demonstrated

a PIT-1 positive pituitary neuroendocrine tumor with low expression of prolactin, scattered β -TSH-positive cells, moderate SSTR2 expression and intense STTR5 expression in 90% of cells (Fig. 2). The WHO 2017 classification recognizes this subtype of pituitary neuroendocrine tumors as a plurihormonal PIT-1-positive adenoma. These tumors are considered by the WHO as high-risk pituitary adenomas due to their clinical aggressive behavior (3). PAS-LAR 40mg/month was proposed to the patient after a multidisciplinary discussion. The treatment was initiated in May 2017 and was well tolerated without digestive or metabolic complaints. Clinical and MRI evaluation after 4 months of treatment demonstrated rapid ophthalmologic improvement and tumor shrinkage, allowing the continuation of PAS-LAR for an additional 3 months. After 7 months, the residual tumor being stable, transsphenoidal endoscopic surgery was proposed to allow a better debulking and raise the possibility of adjuvant radiotherapy. MRI performed 3 months after the second surgery demonstrated good tumor removal of the intrasellar part, but the large extrasellar extension close to the optic chiasm was unchanged. The second pathology report demonstrated high STTR5 expression and thus PAS-

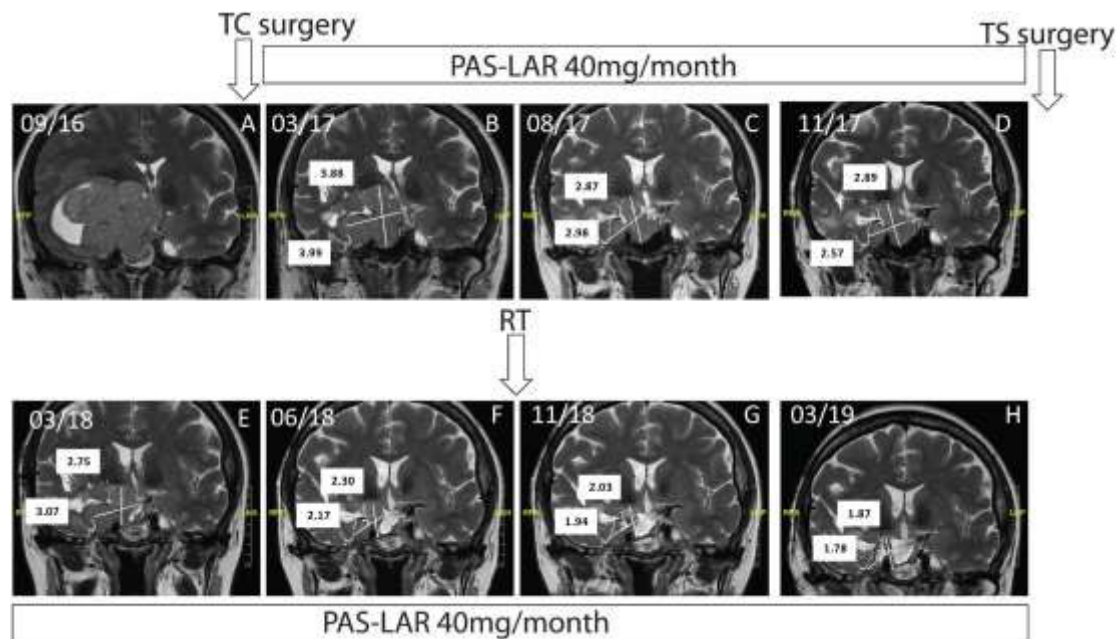


Figure 1 MRI scans during the different surgical and medical therapies. T2-weight MRI scan obtained after 6 months of cabergoline before transcranial (TC) surgery (A), 3 months post-op and pre-PAS-LAR (B), 4 months (C) and 7 months post-PAS-LAR before transsphenoidal (TS) surgery (D), 3 months post-surgery and before re-initiation of PAS-LAR (E), and during 3–9 and 12 months of PAS-LAR (F, G and H) and radiation therapy (RT).

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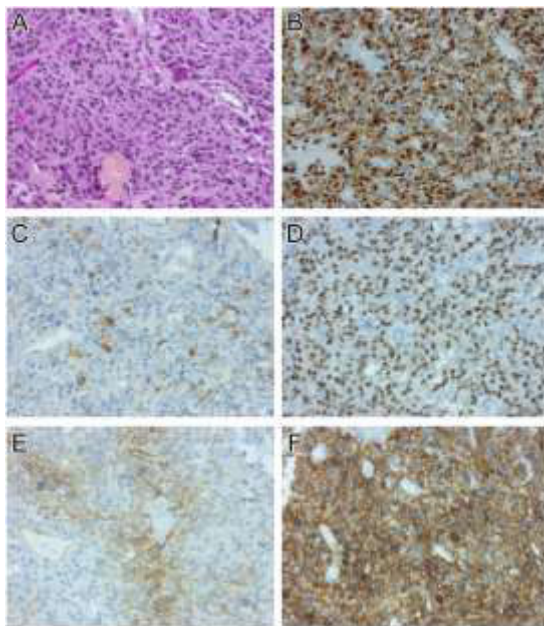


Figure 2

Histopathological features of the tumor at first surgery. (A) The tumor is composed of diffusely arranged cells showing weakly eosinophilic cytoplasm. Nuclei are slightly irregular with a thin chromatin and a prominent eosinophilic nucleolus (Hematoxylin-Phloxine-Saffron staining). (B) Cytokeratin 18 immunopositivity in tumoral cells shows a perinuclear and Golgi distribution. Fibrous bodies are rare. (C) Prolactin immunopositivity present in 8% of tumoral cells. (D) There is a diffuse nuclear immunopositivity of PIT-1 in neoplastic cells. (E) 20% of tumoral cells express SSTR2 with a moderate intensity (IRS = 4). (F) 90% of tumoral cells express SSTR5 with a strong intensity (IRS = 12). (Original magnification $\times 200$); IRS, immunoreactive score. There were no significant features of proliferation observed (no mitotic figures, Ki67 < 3%, P53 negative).

LAR 40mg/month was proposed after multidisciplinary discussion. This second course of PAS-LAR was associated with additional significant tumor shrinkage, after 3 months, allowing the continuation of the treatment. Independent of this tumor shrinkage, as decided after the first multidisciplinary discussion, stereotactic radiotherapy (54Gy) was performed according to the ESE guideline on aggressive pituitary tumors (4). At last follow-up after

12 months of PAS-LAR 40mg/month and 4 months after radiation therapy, the residual tumor size continued to reduce. Of note, T2 signal changes were visible only after 12 months of PAS-LAR.

The encouraging results of these two papers and our personal experience led us to consider PAS-LAR treatment before temozolomide treatment in dopamine-resistant prolactinomas and aggressive pituitary tumors. Indeed, temozolomide can control aggressive prolactinomas in about 40% of cases but long-term control is not guaranteed, and side effects are more frequent compared to PAS-LAR (5). However, no data are available on PAS-LAR for treating prolactin carcinomas with proven metastasis, so in such rare conditions, temozolomide is still the first-line option. Moreover, SSTR5 expression being rare in prolactinoma (<15%), its analysis should be considered before starting PAS-LAR in this indication.

Declaration of interest

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Review

Emerging and Novel Treatments for Pituitary Tumors

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Abstract: A subset of pituitary neuroendocrine tumors (PitNETs) have an aggressive behavior, showing resistance to treatment and/or multiple recurrences in spite of the optimal use of standard therapies (surgery, conventional medical treatments, and radiotherapy). To date, for aggressive PitNETs, temozolomide (TMZ) has been the most used therapeutic option, and has resulted in an improvement in the five-year survival rate in responders. However, given the fact that roughly only one third of patients showed a partial or complete radiological response on the first course of TMZ, and even fewer patients responded to a second course of TMZ, other treatment options are urgently needed. Emerging therapies consist predominantly of peptide receptor radionuclide therapy (20 cases), vascular endothelial growth factor receptor-targeted therapy (12 cases), tyrosine kinase inhibitors (10 cases), mammalian target of rapamycin (mTOR) inhibitors (six cases), and more recently, immune checkpoint inhibitors (one case). Here, we present the available clinical cases published in the literature for each of these treatments. The therapies that currently show the most promise (based on the achievement of partial radiological response in a certain number of cases) are immune checkpoint inhibitors, peptide receptor radionuclide therapy, and vascular endothelial growth factor receptor-targeted therapy. In the future, further improvement of these therapies and the development of other novel therapies, their use in personalized medicine, and a better understanding of combination therapies, will hopefully result in better outcomes for patients bearing aggressive PitNETs.

Keywords: pituitary neuroendocrine tumors (PitNETs); pituitary adenoma; aggressive pituitary tumors; treatment; temozolomide; peptide receptor radionuclide therapy; bevacizumab; tyrosine kinase inhibitors; everolimus; immunotherapy

1. Introduction

Pituitary neuroendocrine tumors (PitNETs) are typically slowly progressing tumors of the anterior pituitary gland, with most cases being easily treated by medical treatment or surgery. However, some of these tumors show an aggressive behavior, being either locally invasive and rapidly growing or showing resistance and/or multiple recurrences in spite of optimal use of standard therapies (surgery, conventional medical treatments, and radiotherapy). In rare cases these tumors may even metastasize [1,2]. Therefore, alternative treatment options are needed for the treatment of aggressive PitNETs, including carcinomas. To date, temozolomide (TMZ), an oral alkylating agent, has been the most used in the clinic [1,3]. Although the use of TMZ in aggressive PitNETs, including carcinomas, has led to a remarkable improvement in the five-year survival rate in responders (both overall and progression-free) [4–6], and established itself as the first-line chemotherapy once standard therapies have failed [1], a significant percentage of patients either do

not respond to TMZ (30% of the patients showed progressive disease (PD) on first-line TMZ in the largest published series [3]), or progress once TMZ is stopped [3]. Moreover, the response to a second course of TMZ was less effective [3], therefore other treatment options are clearly needed. Several new therapies, notably peptide receptor radionuclide therapy (PRRT), vascular endothelial growth factor receptor (VEGF)-targeted therapy, tyrosine kinase (TK) inhibitors, PI3K/Akt/mTOR pathway inhibitors, and recently, immune checkpoint inhibitors, are starting to emerge for the treatment of aggressive PitNETs. In this review, to report the response to these different treatments, and in order to standardize the available published data, we have used, where this information was available, the same criteria used by McCormack 2018 et al. in the recent European Society for Endocrinology (ESE) survey on the treatment for aggressive PitNETs: (1) For the radiological response: Complete response (CR) = no visible tumor, partial response (PR) = >30% tumor regression, stable disease (SD) = <30% tumor regression and < 10% increase in tumor size, and progressive disease (PD) = >10% increase in tumor size or new metastases, and (2) for the biochemical response: CR = hormone level normalization, PR = >20% hormone level reduction, SD = <20% change in the hormone level, and PD = >20% hormone level increase [3].

2. Temozolomide (TMZ)

TMZ works by methylating the DNA, which further leads to irreversible damage of DNA and cell death. O6-methylguanine-DNA methyltransferase (MGMT), an endogenous DNA repair enzyme that removes added methyl groups, potentially counteracts TMZ cytotoxic effects [1,3,6], and therefore low levels of MGMT expression may predict a better response to TMZ treatment [6]. In glioblastomas, methylation of the *MGMT* promoter leading to gene silencing and lower levels of MGMT expression is a frequent event, and the methylation status of *MGMT* was shown to correlate with the protein expression level (determined by immunohistochemistry) and to be a reliable prognostic marker of response to TMZ [1,6,7]. On the contrary, in PitNETs, the methylation status of *MGMT* gene has not been demonstrated to correlate with the protein expression level (suggesting that other factors may impact *MGMT* gene transcription), and therefore, immunohistochemistry is currently recommended for the assessment of the MGMT status in PitNETs [1,7].

In the largest published series on TMZ, the ESE survey [3], 157 patients with aggressive PitNETs and carcinomas were treated with TMZ as first-line chemotherapy. 37% of the patients showed a radiological response (31% PR, and 6% CR), an additional 33% of the patients showed SD, and finally 30% showed PD. The maximal radiological response occurred within three months in 23% of cases and within six months in 59% of cases. In terms of the biochemical response, 19% showed CR, 34% PR, 27% SD, and 21% PD.

A better response to TMZ was recorded in patients bearing clinically functioning tumors independently of their MGMT status, these tumors being 3.3 times more likely to regress on TMZ than their non-functioning counterparts. Concerning patients' MGMT status, patients with a low MGMT status (defined as <10%) showed a better response than patients with an intermediate MGMT status (10%–50%), and both these categories responded better than those patients having a high MGMT status (>50%). Moreover, CR was recorded only in patients having a low MGMT status. Patients who received concomitant radiotherapy (either the Stupp protocol, i.e., administering six weeks of fractionated radiotherapy concomitantly with TMZ 75 mg/m² daily, followed by TMZ 150–200 mg/m² daily for five days every 28 days for another 6–12 months, or variations of this protocol), also showed a two-fold increase in response rate [3].

A low MGMT status has been shown not only to predict a better response to first-line TMZ [3], but also to predict survival of patients treated with first-line TMZ [7]. Moreover, in the same study [7], five patients having recurrent/progressive disease were given a second course of TMZ, of these one patient with low MGMT status showed PR, another patient showed SD, while both of the patients with high MGMT status (defined as >10%) had PD, leading to the conclusion that some patients having low MGMT status may also respond to administration of a second course of TMZ [7]. This is particularly interesting given the fact that following first-line TMZ, PD is not rare (in the ESE survey, 25% of the patients with CR, 40% of the patients with PR, and 48% of the patients with

SD showed PD [3]) and that, when given a second course of TMZ, only two patients out of 18 had PR (though one received TMZ + bevacizumab), five patients out of 18 had SD (though additional treatment was given in three of them), and the remaining 11 patients had PD [3].

The ESE guidelines on aggressive PitNETs, including carcinomas [1], recommend TMZ either as monotherapy (150–200 mg/m² daily for five days every 28 days) or as the Stupp protocol if patients have not previously reached maximal radiotherapy doses [1]. This treatment should be evaluated after three cycles in order to identify responders, and in responders to first-line TMZ, the treatment should then be continued for six months or more when a continued therapeutic effect is noted [1]. Data on long-term treatment with TMZ are too scarce to recommend its maintenance over a long duration however, clinical observations suggest that TMZ should be maintained as long as it is efficient in controlling tumor progression and hormonal secretion, and it is well tolerated.

The most common side-effects of TMZ in patients with PitNETs were fatigue, cytopenias, and nausea/vomiting, but other side-effects including hearing loss, headache, hypotension, edema, adrenal crisis, and abnormal liver function tests have also been reported [1,3]. The current guidelines recommend prophylactic anti-emetic therapy during five out of 28 days when TMZ is administered, a hematological profile on the 22nd day of the 28-day cycle, and monitoring of hepatic function before each TMZ cycle, halfway through the first cycle, and 2–4 weeks following cessation of the treatment. Moreover, prophylactic antibiotic therapy (pentamidine or trimethoprim-sulfamethoxazole) has been recommended in case of significant lymphopenia or in case of dose-dense regimens, concurrent radiotherapy, or hypercortisolism (due to Cushing's syndrome or oral corticosteroids) [1].

3. Tyrosine Kinase (TK) Inhibitors

TK inhibitors are orally administered drugs that lead to reduced TK phosphorylation of targeted proteins [8]. In PitNETs, the use of TK inhibitors is now emerging, with the main aim of targeting the ErbB family signaling pathway. Of the four members of ErbB receptors: ErbB1 (also known as epidermal growth factor receptor (EGFR) or HER1), ErbB2 (or HER2/Neu), ErbB3 (or HER3), and ErbB4 (or HER4), the first two have been the most studied in PitNETs [9].

The existing preclinical and clinical evidence concerns mainly lactotroph and corticotroph tumors [9–15] and was recently reviewed by Ben-Shlomo and Cooper [9]. Both in vivo and in vitro experiments showed a decrease in cell proliferation/tumor size and/or a decrease in hormonal secretion of lactotroph and corticotroph tumors with TK inhibitor treatment [9].

Considering published cases (Table 1) [3,10,16], of eight cases treated with lapatinib, a dual ErbB1/ErbB2 inhibitor, six of these cases consisted of dopamine-resistant lactotroph tumors not previously treated with TMZ. At six months, these cases showed either SD (four cases) or PD (two cases) in terms of tumor volume, and either PR (three cases) or PD (three cases) in terms of hormonal secretion [10,16]. Four other cases of unknown tumor type, that received either lapatinib, erlotinib (an ErbB1 inhibitor [17]), or sunitib (a multitargeted TK inhibitor [18]) as a second or third line therapy after TMZ, showed PD [3].

TK inhibitor side-effects are mainly related to their simultaneous targeting of normal tissues [19]. The toxicity profile of TK inhibitors varies as a function of their target TKs [18] and of their potency [19]. Side-effects encountered with TK inhibitors include hematological events (anemia, neutropenia, and thrombocytopenia), hypertension, thrombosis/embolism, myocardial infarction, bleeding, skin rash, edema, nausea and vomiting, diarrhea, constipation, muscle cramps, bone alterations, fatigue, fever, neuropathy, and elevation of liver enzymes. Lapatinib is, in general, well-tolerated, its most common adverse effects include diarrhea (that may be dose limiting), moderate fatigue, nausea, and acneiform rash [18].

Although TK inhibitors thus far have been proposed in PitNETs for targeting of the ErbB pathway, TK inhibitors do not only target this pathway. In fact, there are more than 20 currently approved TK inhibitors, some of them with multiple targets, that can be categorized, based on their main target, into inhibitors of EGFR, VEGF receptor (VEGFR), breakpoint cluster region-Abelson

Table 1. Cases of aggressive pituitary neuroendocrine tumors and pituitary carcinomas treated with tyrosine kinase (TK) inhibitors.

Ref.	Sex and age at diagnosis	Tumor type	Carcinoma	Previous treatments	Treatment	Response	Adverse effects
[3]	NA	NA	NA	TMZ*	Lapatinib*	PD*	NA
[3]	NA	NA	NA	TMZ*	Lapatinib*	PD*	NA
[3]	NA	NA	NA	TMZ*	Erlotinib*	PD*	NA
[3]	NA	NA	NA	TMZ*	Sunitib*	PD*	NA
[10, 16]	Female, 42y	Functioning lactotroph	No	BCT, NS, CAB 7 mg weekly	Lapatinib 1250 mg daily + CAB 7 mg weekly for 6 months	TV: SD at 6 months HS: PR at 1 month and 6 months	Alopecia (mild), diarrhea (single self-limited episode), appetite loss (mild), and rash
[10, 16]	Female, 31y	Functioning lactotroph	No	CAB, BCT, NS, BCT, CAB 7 mg weekly	Lapatinib 1250 mg daily + CAB 7 mg weekly for 6 months	TV: SD at 6 months HS: PR at 1 month and 6 months	Alopecia (mild), diarrhea (few self-limiting episodes), and rash
[16]	2 females and 2 males, 19-70y	Functioning lactotroph	1 carcinoma*	NS (1 in 1 patient and 2 in 3 patients), RT in 1 patient, DA in all	Lapatinib 1250 mg daily + DA for 6 months (3 patients) and for 3 months (1 patient)	TV: SD (2 patients at 6 months), and PD (1 patient at 3 months and 1 patient at 6 months)** HS: PR (1 patient at 6 months) and PD (1 patient at 3 months and 2 patients at 6 months)	Reversible elevation of transaminases (grade 1), rash (grade 2), and asymptomatic bradycardia (grade 1)

Reference (Ref.), not available (NA), temozolomide (TMZ), progressive disease (PD), years (y), bromocriptine (BCT), neurosurgery (NS), cabergoline (CAB), tumor volume (TV), stable disease (SD), hormonal secretion (HS), partial response (PR), RT (radiotherapy), DA (dopamine agonist); *no further information available **as assessed by the authors (the percentage by which the tumor increased or decreased not reported)

kinase (Bcr-Abl), and anaplastic lymphoma kinase (ALK) [8]. Given the fact that VEGF has also been shown to be implicated in the pathogenesis of PitNETs [20], and that ALK7 was associated with an increased proliferation in lactotroph tumors [21], a more personalized choice of TK inhibitor may hopefully translate into a better response to this therapy in the future.

4. VEGF-Targeted Therapy

VEGF-targeted therapy is another emerging therapeutic option. Bevacizumab, an anti-VEGF monoclonal antibody, is the main option used in PitNETs, but some TK inhibitors such as sunitinib, also target the VEGFR pathway.

In a series of 148 PitNETs, six of which were carcinomas [22], Lloyd et al. found an increased VEGF expression, by immunohistochemistry, in non-treated somatotroph tumors, silent corticotroph tumors, non-oncocytic null cell tumors, and in carcinomas in comparison to the other subtypes, while VEGF expression was decreased in tumor tissue in comparison to the adjacent non-tumor tissues when the latter was present [22]. In another series of 197 PitNETs [23], 58.9% showed high VEGF expression by immunohistochemistry. This high VEGF expression was more frequently found in gonadotroph (29 cases high versus eight cases low), lactotroph (17 cases high versus 11 cases low), and in non-functioning PitNETs (40 cases high versus 30 cases low), while in corticotroph, somatotroph, and thyrotroph PitNETs, nearly half of the tumors showed high VEGF expression levels and half showed low VEGF expression [23].

To date, bevacizumab has been administered either as monotherapy [24,25] or in different combinations (with TMZ [26], with TMZ and radiotherapy [27] or with pasireotide [28]) in five cases of corticotroph tumors, of which four were carcinomas (Table 2). Four out of the five cases showed SD [24–26,28], while in the case where bevacizumab was associated with TMZ and radiotherapy, CR was obtained [27]. In the ESE survey [3], another seven cases treated with bevacizumab as monotherapy or in combination with TMZ were reported (Table 2), with PR, SD, or PD as the outcome [1,3], but unfortunately there were no details provided regarding the tumor type. However, it is noteworthy that from 18 cases in the ESE survey that presented with PD after the cessation of TMZ and were then administered a second course of TMZ, only two patients showed subsequent PR, of whom one received bevacizumab in combination with TMZ [3].

The side-effects of bevacizumab most commonly include hypertension and proteinuria, however arterial thrombosis, including acute myocardial infarction, venous thromboembolism, hemorrhage, gastrointestinal perforation, and wound healing complications have also been reported [29,30].

Interestingly, dopamine is also an inhibitor of VEGF signaling and has been proposed as a novel anti-angiogenic therapy that acts through the stabilization of abnormal blood vessels and in doing so also improves the efficacy of other drugs [31]. In a recent study [31], Chauvet et al. treated a mouse model of hemorrhagic lactotroph tumor with either bromocriptine alone, axitinib (a VEGFR inhibitor) alone, or with a combination of the two drugs, and found that bromocriptine not only blocked tumor growth, but also induced regression of the abnormal blood supply and normalization of the blood vessel structure. Axitinib also restrained the tumor growth and improved vascular remodeling, while only the combination of bromocriptine and axitinib therapy suppressed intratumoral hemorrhage and was able to restore blood vessel perfusion [31]. This is in line with older experiments that showed increased VEGF expression in models of dopamine-resistant lactotroph tumors: When anti-VEGF treatments were administered, both a decrease in vascularity and a decrease in proliferation/tumor size were observed [32]. Moreover, somatostatin and the somatostatin analog pasireotide were also shown to decrease the VEGF secretion of human non-functioning PitNETs in culture by 15% or more, in 13 out of 25 primary cultures studied [33].

5. mTOR Inhibitors

Upregulation and/or overactivation of the PI3K/Akt/mTOR pathway have been reported in human PitNETs [34–36] and inhibitors of this pathway were shown to have anti-tumoral effects in *in vitro* human PitNET cultures and in *in vitro* and *in vivo* murine models and cell lines [37–41].

Table 2. Cases of aggressive pituitary neuroendocrine tumors and pituitary carcinomas treated with VEGF-targeted therapy.

Ref.	Sex and age at diagnosis	Tumor type	Carcinoma	Previous treatments	Treatment	Response	Adverse effects
[25]	Male, 38y	Silent corticotroph	Yes	4 NS, RT, NS, TMZ, NS, TMZ, surgery for metastases, TMZ, RT for metastases, NS	BVZ 10 mg/kg every 2 weeks for 26 months (ongoing)	TV: SD for 26 months**	NA
[28]	Female, 25y	Functioning corticotroph	Yes	2 NS, bilateral adrenalectomy, NS, RT, SSA, RT, TMZ	BVZ + pasireotide for 6 months	TV: SD at 6 months** HS: PR at 6 months	NA
[24]	Female, 50y	Functioning corticotroph	No	2 NS, RT, NS, bilateral adrenalectomy, 2 NS, LAR+CAB, TMZ	BVZ*	Transient SD** (patient deceased due to complications of another NS)	NA
[27]	Male, 63y	Functioning corticotroph	Yes	NS	BVZ 10 mg/kg every 2 weeks + TMZ 75 mg/m ² daily + RT for 2 months, then TMZ 200 mg/m ² daily for 5 consecutive days out of 28 days for 12 cycles	TV: CR of the pulmonary nodule 8 weeks after starting BVZ+TMZ+RT, with no recurrence for 5 years HS: NA	NA
[3]	NA	NA	NA	No previous TMZ*	BVZ + first-line TMZ*	PR*	NA
[3]	NA	NA	NA	TMZ*	BVZ + second course of TMZ*	PR*	NA
[3]	NA	NA	NA	TMZ*	BVZ + second course of TMZ*	PD*	NA
[3]	NA	NA	NA	TMZ*	BVZ + second course of TMZ*	NA	NA
[1,3]	NA	NA	NA	TMZ*	BVZ as second-line therapy*	PR after 3 months*	NA
[1,3]	NA	NA	NA	TMZ*	BVZ as second-line therapy*	SD*	NA
[1,3]	NA	NA	NA	TMZ*	BVZ as third-line therapy*	PD*	NA
[26]	Male, 50y	Functioning corticotroph adenoma transformed into silent corticotroph carcinoma	Yes	NS, RT, surgery for metastases, RT for metastases, TMZ	BVZ 10–15 mg/kg every 2 weeks (09/2010–11/2012) + TMZ 150–200 mg/m ² daily for 5 consecutive days monthly (09/2010–08/2011)	TV: SD for 8 years**	NA

Vascular endothelial growth factor (VEGF), reference (Ref.), years (y), neurosurgery (NS), radiotherapy (RT), temozolomide (TMZ), bevacizumab (BVZ), tumor volume (TV), stable disease (SD), not available (NA), somatostatin analogue (SSA), hormonal secretion (HS), partial response (PR), lanreotide (LAR), cabergoline (CAB), complete response (CR), progressive disease (PD).

*No further information available, **as assessed by the authors (the percentage by which the tumor increased or decreased not reported and/or hormone levels not reported).

Table 3. Cases of aggressive pituitary neuroendocrine tumors and pituitary carcinomas treated with everolimus (EVE).

Ref	Sex and age at diagnosis	Tumor type	Carcinoma	Previous treatments	Treatment	Response	Adverse effects
[42]	Male, 45y	Functioning corticotroph	Yes	2 NS, RT, bilateral adrenalectomy, RT on metastases, TMZ	EVE 5 mg daily + octreotide 30 mg for 1 month, then EVE 5 mg daily for another 2 months	No effect at 3 months**	NA
[43]	Female, 46y	Functioning corticotroph	Yes	2 NS, bilateral adrenalectomy, NS, RT, OCT, NS, OCT, NS, capecitabine + TMZ, NS	EVE 7.5 mg daily + palliative RT on metastases (01-02/2015), EVE 10 mg daily (02-06/2015), palliative RT on metastases (06-07/2015), EVE 7.5 mg daily + capecitabine 1000 mg/m ² b.i.d. two weeks out of three (07-09/2015)	TV: ~5 months transient stability, then PD** HS: NA	Multifocal herpes zoster (left arm and left eye, requiring enucleation) despite prophylaxis, and neutropenia
[3]	NA	NA	NA	TMZ*	EVE as second- or third- line therapy*	PD*	NA
[3]	NA	NA	NA	TMZ*	EVE as second- or third- line therapy*	PD*	NA
[3]	NA	NA	NA	TMZ*	EVE as second- or third- line therapy*	PD*	NA
[44]	Male, 62y	Functioning lactotroph	No	NS, RT, CAB, NS, CAB 0.75 mg daily	EVE 10 mg daily + CAB 1.5 mg daily	TV: SD at 5 and 12 months HS: PR at ~20 weeks (stable at ~35 weeks)	Altered mental status due to hyperglycemia, transient hypogeusia, and mouth sores

Reference (Ref.), years (y), neurosurgery (NS), radiotherapy (RT), temozolomide (TMZ), not available (NA), octreotide (OCT), twice daily (b.i.d.), tumor volume (TV), progressive disease (PD), hormonal secretion (HS), cabergoline (CAB), stable disease (SD), partial response (PR), *no further information available, **as assessed by the authors (the percentage by which the tumor increased or decreased not reported and/or hormone levels not reported).

Of the PI3K/Akt/mTOR pathway inhibitors, everolimus (EVE), an orally active mTOR inhibitor, is the only one that has been tested in patients, with six cases reported so far (Table 3) [3,42–44]. EVE, by binding to a protein named FKBP12, inhibits mTOR and its downstream signaling cascade, resulting in decreased protein synthesis, reduced cell growth, and cell cycle arrest [37,38]. In *in vitro* models of PitNETs, EVE administration resulted in the inhibition of cell proliferation, reduction of cell viability, and promotion of apoptosis [38,45].

Regarding the cases treated with EVE, in the only case not previously treated with TMZ, the patient achieved stability of tumor volume for 12 months and a decrease in prolactin levels (no data is available subsequent to this point) [44]. However, from the five cases where EVE was tried as a second- or third-line therapy in patients previously treated with TMZ, it was found to be unsuccessful in four cases [3,42], while in one case of a corticotroph carcinoma harboring an mTOR pathway mutation (STK11/F298L), the patient showed transient SD before then showing progression [43].

Preclinical studies in primary cultures from human nonfunctioning PitNETs have shown that, when associated with a somatostatin analogue (SSA)—either octreotide or pasireotide—EVE showed increased efficacy, including a change from being non-effective to effective [45,46]; the same was true in the Aft-20 (mouse corticotroph tumor) cell line with the addition of octreotide [46]. However, from the five cases treated unsuccessfully with EVE, one patient with a corticotroph carcinoma did not receive concomitant SSA treatment [43], another patient with a corticotroph carcinoma did not tolerate the SSA and therefore received only one injection [42], and for the remaining three cases [3] there is no information available on the tumor type, nor on whether EVE treatment was associated with an SSA.

In terms of side effects of EVE, the most common are usually mild and include fatigue, rash, stomatitis, diarrhea, myelosuppression, hyperglycemia, and hyperlipidemia [47,48]. However, mTOR inhibitors can also have less common side effects, such as symptomatic non-infectious pneumonitis, which can be life threatening [47].

Preclinical studies in either primary cultures from human PitNETs [40,41], or in murine models/cell lines [39–41] have also shown that the use of PI3K inhibitors, alone or in combination with mTOR inhibitors, hold promise for the treatment of aggressive PitNETs, and it is notable that clinically approved PI3K inhibitors already exist. Furthermore, a dual PI3K/mTOR inhibitor has been tested, either alone or in combination with TMZ, in *in vivo* and *in vitro* murine models and showed a synergistic anti-tumoral effect [49].

6. Immune checkpoint inhibitors

The recent demonstration of PitNETs (especially functioning ones) showing the presence of tumor-infiltrating T lymphocytes [50–52] and expressing programmed death ligand 1 (PD-L1), a potential biomarker of response to immune checkpoint inhibitors therapy [50,51], raised the hope of possible efficacy of immune checkpoint inhibitors in these tumors. Immune checkpoint inhibitors work by blocking inhibitory signals of T cell function/activation, signals that would otherwise allow tumors to evade the immune response. More precisely, these molecules work by blocking CTLA-4 or PD-1 found on T cells, or its ligand, PD-L1, found on tumor cells and antigen-presenting cells. By doing so, the immune checkpoint inhibitors enhance the function of T cells and reactivate anti-tumoral immune responses, thus favoring the destruction of tumor cells [51,53,54].

A single case [55] of a functioning corticotroph carcinoma, previously treated with TMZ and carboplatin, and bearing hypermutations suggestive of being alkylator-induced, has been reported in the literature. The therapeutic sequence prior to the use of the immune checkpoint inhibitors included two neurosurgeries, radiotherapy, two additional neurosurgeries, pasireotide, cabergoline, four cycles of TMZ in combination with capecitabine, bilateral adrenalectomy, two additional cycles of TMZ in combination with capecitabine, two cycles of etoposide and carboplatin, and again radiotherapy for the primary tumor, the immunotherapy being started shortly thereafter. The patient was treated with a combination of two immune checkpoint inhibitors, five cycles of nivolumab (an anti-PD-1 monoclonal antibody, 1 mg/kg every three weeks) in combination with

ipilimumab (an anti-CTLA-4 monoclonal antibody, 3 mg/kg every three weeks). Following the five cycles with the two immune checkpoint inhibitors, the primary tumor volume decreased by 59% and the main liver metastasis volume decreased by 92%, while the levels of plasma ACTH fell from 45,550 to 66 pg/mL. After the five cycles, the patient was kept on maintenance nivolumab, and at the six months follow-up, continued to respond. The reported side effects consisted of a 40 °C fever, mild elevation of transaminases, and supposed hypophysitis (the patient already had hypopituitarism) [55].

The side effects of immune checkpoint inhibitors are immune-related adverse events. They can affect every organ, but generally the skin, the gastrointestinal tract, the liver, and the endocrine system are most affected, the patients potentially developing a rash, pruritus, vitiligo, diarrhea, colitis, hepatitis, hypophysitis, dysthyroidism, primary adrenal insufficiency, and diabetes [53,54]. Concerning the organ systems less frequently affected, side effects include sarcoidosis, inflammatory pneumonia, episcleritis, uveitis, renal insufficiency, aseptic meningitis, transverse myelitis, Guillain–Barre syndrome, red cell aplasia, thrombocytopenia, neutropenia, and acquired hemophilia A [53].

Despite these potential side effects, immune checkpoint inhibitors represent a promising treatment option in aggressive PitNETs, especially if the patients have been previously treated with TMZ, which for the moment is the recommended first-line chemotherapy. Indeed, as we observed for the other therapeutic options, the administration of a first course of TMZ seems to not only render the tumor less responsive to a second course of TMZ, but additionally to most of the other available treatment options. In contrast, TMZ (and other conventional chemotherapies) may induce somatic hypermutations, which in turn will potentially render the tumor more responsive to immunotherapy [55]. Moreover, there are also studies that have shown that the efficacy of PD-1 inhibitors could be enhanced by concurrent radiotherapy [56]. Therefore, combining PD-1 inhibitor therapy with radiotherapy may also prove to be a therapeutic option in PitNETs in the future.

7. Peptide Receptor Radionuclide Therapy (PRRT)

PRRT consists of the use of radiolabeled somatostatin receptor (SSTR) binding molecules in order to target tumors expressing these receptors [57]. The rationale behind the administration of PRRT in PitNETs is the widespread expression of SSTR by different PitNET subtypes (most frequently SSTR2A and SSTR5) [58] and the demonstrated uptake of radiolabeled SSTR analogs on positron emission tomography/computed tomography (PET/CT) [1,59].

To date, the experience with PRRT in aggressive PitNETs, including pituitary carcinomas, is also limited, with only 20 cases being reported in the literature (Table 4) [3,4,60–67]. In terms of tumoral response, from these 20 cases, only three cases demonstrated PR [3,64,67] and three patients had SD [3,61,62], with one having overall SD, but showing CR in some of the nodules [62]. It is interesting to note, however, that patients showed PR or SD in all but one case not previously treated with TMZ [60], in sharp contrast to the patients previously treated with TMZ, in which the disease either progressed, or information on the outcome was lacking.

In terms of side effects, PRRT is mainly toxic for the bone marrow and kidneys. Toxicity for the bone marrow manifests usually as transient anemia, thrombocytopenia, and/or leucopenia, which must be monitored. Concerning the kidney toxicity, solutions of amino acids should be administered to confer nephroprotection [64,68].

Although PRRT seems a promising treatment option, patients with tumors expressing marginal levels of SSTR2 or where this expression is lost with the progression of the tumor, cannot at the moment benefit from this therapy [69]. In order to improve the SSTR2 targeting, Taelman et al. [69] screened for drugs able to upregulate SSTR2 and identified nine epigenetic modifiers capable of upregulating SSTR2 in vivo and in vitro, and having synergistic effects. Their method therefore holds promise for converting ineligible patients to eligible candidates [69]. Moreover, all of the currently approved PRRT represent radiolabeled SSTR analogs. However, in recent years, interest in radiolabeled SSTR antagonists has grown and increasing evidence (consisting of preclinical studies, but also human data) points to the potential superiority of radiolabeled SSTR antagonists [70]. JR11,

an antagonist of SSTR showing selectivity for SSTR2, is now under clinical development, both as an imaging and a therapeutic agent [70].

Table 4. Cases of aggressive pituitary neuroendocrine tumors and pituitary carcinomas treated with peptide receptor radionuclide therapy (PRRT).

Ref.	Sex and age at diagnosis	Tumor type	Carcinoma	Previous treatments	Treatment, number of cycles (cumulative dose/activity)	Response	Adverse effects
[60]	Female, 16y	Functioning corticotroph	Yes	2 NS, RT, 4 NS, RT, NS, bilateral adrenalectomy, NS, RT	90Y-DOTATOC, 2 cycles (200mCi)	NA, but the patient died of elevated intracranial pressure shortly after	NA
[61]	Male, 56y	NIR	No	NS, RT	177Lu-DOTATOC, 3 cycles (600 mCi)	TV: SD**	NA
[62]	Male, 40y	NF*	Yes	NS, RT, NS, surgery for metastasis	177Lu-DOTATATE, 4 cycles (~30 MBq)	TV: CR in some nodules, and overall SD at 40 months**	Transient thrombocytopenia
[62]	Male, 39y	Functioning somato-lactotroph	No	NS, RT, LAN, CAB, 4 NS with gliadel wafers in 2, TMZ, RT	177Lu-DOTATATE, 2 cycles (15.3 Mbq)	TV: PD** (the patient died 2 months after PRRT was started—deterioration of brainstem disease) HS: NA	NA
[62]	Male, 26y	Silent corticotroph	No	2 NS, RT, NS, TMZ, TMZ, NS	177Lu-DOTATATE, 1 cycle	TV: PD**	Severe increase in the facial pain
[63]	Male, 59y	NF*	No	3 NS, RT, SSA, TMZ	177Lu-DOTATATE*	TV: NA, but the patient died in the following months	NA
[63]	Male, 46y	Functioning somatotroph	Yes	6 NS, RT, SSA/PEG, TMZ, palliative RT	90Y-DOTATATE*	No effect**	NA
[64]	Male, 23y	Functioning somatotroph	No	SSA, NS, SSA, RT	90Y-DOTATATE, 4 cycles (400mCi)	TV: PR at 12 months HS: PR at 12 months	Transient anemia and leucopenia
[4]	NA	NA	NA	TMZ*	DOTATOC*	No effect**	NA
[4]	NA	NA	NA	TMZ*	DOTATOC*	Ongoing*	NA
[3]	NA	NA	No	No previous TMZ*	As first-line therapy*	PR*	NA
[3]	NA	NA	No	No previous TMZ*	As first-line therapy*	SD*	NA

[3]	NA	NA	NA	TMZ*	As second- or third-line therapy*	PD*	NA
[3]	NA	NA	NA	TMZ*	As second- or third-line therapy*	PD*	NA
[3]	NA	NA	NA	TMZ*	As second- or third-line therapy*	PD*	NA
[3]	NA	NA	NA	TMZ*	As second- or third-line therapy*	PD*	NA
[3]	NA	NA	NA	TMZ*	As second- or third-line therapy*	PD*	NA
[65-67]	Female, 58y	Functioning lactotroph	No	BCT, NS, CAB, RT	111In-octreotide, 5 cycles (37 GBq) + CAB 0.5 mg daily	TV: PR after the first 2 cycles, further PR after the next 2 cycles, and further PR after the last cycle HS: PR	None
[65, 66]	Male, 54y	Functioning lactotroph	No	CAB, 3 NS, RT, TMZ	177Lu-DOTATOC, 2 cycles (12.6 GBq)	TV: PD HS: Good biochemical control**	None
[65, 66]	Female, 53y	NF*	No	5 NS, RT, TMZ	177Lu-DOTATOC, 5 cycles (29.8 GBq)	TV: PD	None

Reference (Ref.), years (y), neurosurgery (NS), radiotherapy (RT), 90Yttrium-DOTATOC (90Y-DOTATOC), not available (NA), non-immunoreactive (NIR), 177Lutetium-DOTATOC (177Lu-DOTATOC), tumor volume (TV), stable disease (SD), non-functioning (NF), 177Lutetium-DOTATATE (177Lu-DOTATATE), complete response (CR), lanreotide (LAR), cabergoline (CAB), bromocriptine (BCT), temozolomide (TMZ), progressive disease (PD), somatostatin analogues (SSA), pegvisomant (PEG), 90Yttrium-DOTATATE (90Y-DOTATATE), 111Indium-DTPA-octreotide (111In-octreotide), hormonal secretion (HS), partial response (PR); *no further information available, **as assessed by the authors (the percentage by which the tumor increased or decreased not reported and/or hormone levels not reported).

8. Conclusions

In conclusion, in aggressive PitNETs, including carcinomas, after the failure of conventional treatments, TMZ is currently the recommended first-line treatment. Where the patients have not already reached maximal radiotherapy doses, the Stupp protocol, in which TMZ is first associated with fractionated radiotherapy, should be used [1]. Given the fact that roughly one third of patients showed a radiological response to first-line TMZ, one third showed SD, one third showed PD, and even fewer patients responded to a second course of TMZ [3], other treatment options are urgently needed. Therapies currently showing the most promise (based on achieving radiological PR in a certain number of cases) are immune checkpoint inhibitors, PRRT, and VEGF-targeted therapy, while TK inhibitors and everolimus have so far shown, at best, radiological SD. However, too few patients have been treated, and, moreover, the protocols used and therapeutic sequences varied greatly between patients, making it difficult to draw any definitive conclusion. Therefore, there is an absolute need for clinical trials for aggressive PitNETs, including carcinomas. Given the fact that these tumors are rare, it would also be important to have available “basket” studies. In the case of immune checkpoint inhibitors, there is at the moment one such basket clinical trial available on combination treatment with nivolumab and ipilimumab (NCT02834013), but only for pituitary carcinomas. Last, but not least, it is also of crucial importance to validate and standardize predictive markers of response, especially given that there appears to be a trend for a better response to most of the alternative therapies in TMZ-naïve patients, though no definitive conclusion can yet be drawn. In the future, the further development and/or improvement of these novel therapies, their personalized use, and a better understanding of combination therapies, including their correct sequencing and timing, will hopefully result in better outcomes for patients bearing aggressive PitNETs.

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RESEARCH

Pasireotide-LAR in acromegaly patients treated with a combination therapy: a real-life study

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Abstract

Purpose: Little data are available regarding the safety and efficacy of switching to Pasireotide-LAR monotherapy in acromegaly patients with partial resistance to first-generation somatostatin agonists (1gSRL) who require combination treatment with cabergoline or pegvisomant.

Method: In this monocentric prospective study within a tertiary university hospital, 15 consecutive acromegalic adults partially resistant to 1gSRL treated with octreotide LAR or lanreotide SR, and cabergoline ($n = 4$, 3.5 mg/week) or pegvisomant ($n = 11$, median dose 100 mg/week), were switched to Pasireotide-LAR (8 with 40 mg/month; 7 with 60 mg/month). Immunohistochemical expression level of SSTR5 and the granulation pattern of nine somatotroph adenomas were retrospectively determined to test for a correlation with the therapeutic efficacy of Pasireotide-LAR.

Results: Median IGF-1 concentration at the first evaluation (median 3 months) was similar to baseline (1.0 vs 1.1 ULN). 11/15 patients had IGF-1 levels ≤ 1.3 ULN before and after the switch but individual changes were variable. Hyperglycemia was frequent and greater in diabetic patients. 7/15 patients stopped Pasireotide-LAR due to lack of control of IGF-1 or intolerance. 8/15 patients received Pasireotide-LAR for a median of 29 months with IGF-1 levels ≤ 1.3 ULN and acceptable glucose tolerance (median HbA1c 6.1%). Two patients required initiation of oral antidiabetic treatment. The intensity of SSTR5 expression and the granulation pattern of adenomas were of limited value for the prediction of Pasireotide-LAR effectiveness.

Conclusion: Pasireotide-LAR may represent a suitable therapeutic alternative in a subset of acromegalic patients requiring combination therapy involving a 1gSRL

Key Words

- ▶ acromegaly
- ▶ somatostatin analogs
- ▶ pegvisomant
- ▶ cabergoline
- ▶ Pasireotide-LAR

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Introduction

Acromegaly is predominantly caused by a growth hormone (GH)-secreting pituitary adenoma resulting in excessive insulin-like growth factor-I (IGF-I) secretion that is responsible for numerous co-morbidities, reduced

quality of life and increased mortality rate (1). Surgical excision of the pituitary adenoma is the only curative treatment for acromegaly, but its effectiveness is limited depending on the size and extension of the pituitary

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adenoma, as well as the expertise of the surgeon (2). Medical management of acromegaly is used when surgery fails to control the disease or when surgery cannot be performed (3). First-generation long-acting somatostatin analogs (1gSRL) (octreotide LAR and lanreotide Autogel) are the most widely used first-line medical treatments and induce a significant decrease in GH/IGF-1 levels in a majority of patients. IGF-1 normalization is highly variable across studies ranging from 23 to 98% of patients (4). The GH receptor antagonist, pegvisomant (PEG), is a second-line treatment which normalizes IGF-1 plasma levels in 60–70% (5) of patients in follow-up registries and up to 97% in single-center series (6). The dopamine D2-receptor agonist, cabergoline, is an alternative to 1 GSSA, normalizing IGF-1 plasma level in 30% of patients, especially when IGF-1 is moderately increased (7). Combination of 1gSRL and PEG is an increasingly prescribed alternative, in patients with partial resistance to 1gSRL (8) showing high efficacy with the added benefit of lowering the required PEG dose, thus reducing the frequency of injections from a daily to a weekly regimen and lowering treatment costs. Combination of 1gSRL with cabergoline is a less frequently used alternative in these patients.

Pasireotide-LAR is a multireceptor-targeted somatostatin analog with superior clinical efficacy over octreotide-LAR (9) that can control GH and IGF-1 concentrations in patients resistant to 1gSRL (10). Consequently, it is approved for treatment of acromegaly where surgery is not an option (or was not curative) and where it is not controlled by treatment with a 1gSRL. Pasireotide-LAR could therefore be an alternative to 1gSRL+PEG and 1gSRL+cabergoline combination therapies. Two previously published studies have examined this possibility. The largest study used a relatively complex prospective clinical research protocol involving a run-in phase with a 50% reduction dose of PEG prior to the start of Pasireotide-LAR and additional titration of the PEG dosage to achieve IGF-1 control and, when possible, Pasireotide-LAR monotherapy (11). Shimon *et al.* published a study which was more consistent with ‘real-life’ practice and included nine and four patients who were previously treated with either an association of 1gSRL and PEG or cabergoline respectively (12). However, this study was retrospective and multicentric, involving various initiation doses and adjustments of Pasireotide-LAR as well as various laboratories for GH and IGF-1 measurement. Recent recommendations for the use of Pasireotide LAR have been published by one expert group mentioned above (13). However, given the

scarcity of studies, the effectiveness and safety of the switch to Pasireotide-LAR in acromegaly patients partially responsive to 1gSRL and treated with a combination therapy still warrants complementary studies.

We report herein the results of a single-center ‘real life’ study evaluating the effectiveness and safety of switching consecutively 15 acromegalic patients, from a combination therapy including 1gSRL to Pasireotide-LAR therapy. In an effort to identify biomarkers of treatment outcome, we also examined whether the *in vivo* efficacy of Pasireotide-LAR correlated with expression levels of SSTR5 and the granulation pattern of the adenomas.

Materials and methods

Study design

From December 2015 to August 2017, 15 acromegalic patients treated with a medical combination therapy including a 1gSRL and who were seen as part of their usual follow-up were prospectively proposed to be switched to monotherapy with Pasireotide-LAR. A consent to care was obtained in all patients for the change of treatment of acromegaly after full explanation of the purpose and nature of all procedures used. The data were collected under conditions of regular clinical care and were anonymised and protected for the study. The study was approved by the Ethical Committee of the Bordeaux University Hospital.

Prior to the switch, all patients underwent the following standard evaluations of care: a clinical evaluation using a non-validated standardized 20-point score that is used in our department (4 points for asthenia, headache, sweating, arthralgia and swelling, respectively); a centralized IGF-1 assay; pituitary MRI (Coronal, sagittal T1 and T2 sequences, with and without gadolinium); measurement of hemoglobin A1C (HbA1c) and fasting blood glucose (FBG). Patients were considered diabetic if they were being treated for diabetes and/or when FBG was ≥ 126 mg/dL and/or HbA1c $\geq 6.5\%$. Patients were considered glucose intolerant when FBG was ≥ 100 mg/dL and ≤ 125 mg/L and/or HbA1C was > 5.7 and $\leq 6.4\%$. All patients underwent a dietary evaluation and received dietary advice and education about frequent measurement of capillary glycemia during the first 3 months of treatment. Patients were encouraged to attend for outpatient consultation in the event of a major increase in capillary glycemia.

The first evaluation was performed after approximately 3 months of treatment and during the week before the next

planned administration of Pasireotide-LAR. It included the same assessments as the baseline evaluation with the addition of 1–3 measurements of plasma GH. A variation in the clinical score ≥ 2 was considered as significant.

Depending on the results of this evaluation, Pasireotide-LAR was either continued or interrupted. If the treatment was continued, patients were monitored at roughly 3-monthly intervals. A pituitary MRI was scheduled after the third month of treatment. Evaluation of the tumor height on MRI scans, performed before and after the switch, was retrospectively evaluated by a single experienced physician (A.T.) who was blinded to the identity and previous treatment of patients.

Immunohistochemistry

Formalin-fixed paraffin-embedded tumoral tissue was available for nine patients. Immunohistochemical (IHC) analysis was performed on 4 μ m sections using the BenchMark® ULTRA automated immunostainer (Ventana Medical Systems Inc, Tucson, AZ, USA). The primary antibodies used were against SSTR2 (rabbit monoclonal, clone UMB-1, 1:4000, Abcam), SSTR5 (rabbit monoclonal, clone UMB-4, 1:250, Abcam), and cytokeratin 18 (mouse monoclonal, clone DC10, 1:50, Dako). Bound antibodies were detected using a Ventana kit incorporating diaminobenzidine (DAB) as the color reaction (ultraView Universal DAB Detection Kit). Additional amplification was used for the SSTR5 immunostaining.

SSTR5 membranous immuno-positivity was evaluated by a semi-quantitative immunoreactive score (IRS) (14). This score, ranging from 0 to 12, is the product of the proportion of immunoreactive cells (0: 0%; 1:1–10%; 2:11–50%; 3:51–79%; and 4: \geq 80%) and the staining intensity (0: no staining; 1: mild; 2: moderate; and 3: strong). We considered the staining as being negative where IRS was 0 and 1, weakly positive when IRS scores were 2 and 3, moderately positive for IRSs 4–8, and strongly positive for IRSs >8 .

The pattern of cytokeratin 18 immunoexpression was used to classify somatotroph adenomas into sparsely or densely granulated adenomas. Adenomas with a ‘transitional’ distribution were considered as densely granulated adenomas (15).

Histopathological analyses were performed by an experienced pathologist in the field (A.V.).

Statistical analysis

All quantitative data are presented using median, minimum and maximum. Pre- and post-switch quantitative data were

compared using a paired Wilcoxon rank test. Significance was defined as a *P* value of less than 0.05.

Assays

GH and IGF-1 were assayed using LIAISON XL (Diasorin) immunoassay. For GH, coefficient of variation (CV) was 6.7 and 6.3% at 3.69 and 19.3 ng/mL, respectively; for IGF-1, CV was 10.4 and 10.2% at 97.5 and 389 ng/mL, respectively. Results are expressed relative to sex and age upper limits of the normal range (ULN). Normal ranges of IGF-1 were taken from the ‘Variété’ study (16).

Patients

Fifteen patients (10 women, 5 men) aged 50 years (range 27–67) were included. The age at diagnosis was 38 years (16–61), and IGF-1 and basal GH at diagnosis were 3.2 ULN (1.7–5.4) and 18.6 ng/mL (3.2–162), respectively. All but one patient had a macroadenoma. One 29-year-old patient had germinal *AIP* mutation ($n^{\circ}4$, Table 1).

Fourteen of 15 patients underwent surgical debulking of the pituitary adenoma and 13 of these 14 patients were treated with 1gSRL prior to surgery. Additionally, four patients received post-operative radiotherapy 2.8, 5.8, 8.1 and 14.1 years before Pasireotide-LAR initiation. All patients were treated post-operatively with 1gSRL (somatoline autogel (120 mg/month, $n=7$) or octreotide LAR (30 mg/month, $n=7$)). None of the patients showed normalization of IGF-1 levels. The maximal IGF-1 decrease during 1gSRL ranged from 39 to 50% and the median IGF-1 level prior to the combination therapy was 1.7 ULN (1.4–3.5). Consequently, the patients were treated with an association of 1gSRL and cabergoline (3.5 mg/week for four patients) or PEG (100 mg/week, (40–200) for 11 patients) (see Table 1). Pasireotide-LAR was introduced after 39 months (1.5–164) of treatment with the combination therapy. The first injection was performed at the time usually scheduled for the injection of the 1gSRL and patients simultaneously interrupted their intake of cabergoline and injections of PEG.

The median clinical score before the switch was 3/20 (0–12) and median plasma IGF-1 level was 1.0 ULN (0.4–4.1). The IGF-1 level was considered as controlled (≤ 1.3 ULN) in 11 patients, was >1.3 ULN and ≤ 2.0 ULN in 2 patients and >2 ULN in 2 patients. IGF-1 levels in patients receiving a 1gSRL and PEG combination tended to be lower than those in patients receiving 1gSRL and cabergoline in combination: 0.9 (0.4–3.3) vs 1.5 (1.0–4) ULN,

Table 1 Patients characteristics and treatments.

No.	Sex	Age at inclusion	Combination therapy					Pasireotide-LAR first evaluation				Pasireotide-LAR last evaluation			
			Treatment	Duration (months)	RT	IGF-1 (ULN)	GH (ng/mL)	Dose (mg/28 d)	IGF-1 (ULN)	GH (ng/mL)	Status after evaluation	Dose (mg/28 d)	IGF-1 (ULN)	GH (ng/mL)	Status after evaluation
1	F	36.5	Lanreotide 120 mg/28 d Pegvisomant 40 mg/7 d	1.5	N	0.4	NA	60	0.7	1.9	OG	27	1.1	7.26	OG
2	F	65.3	Octreotide 30 mg/35 d Pegvisomant 140 mg/7 d	163.7	Y	0.6	NA	40	1.5	NA	STOP				
3	F	38.1	Octreotide 30 mg/28 d Pegvisomant 60 mg/7 d	21.5	N	0.8	NA	60	0.9	6.1	STOP				
4	M	40.3	Octreotide 30 mg/28 d Pegvisomant 60 mg/7 d	105.6	N	0.9	NA	40	0.5	2.5	OG	40	0.5	2.5	OG
5	H	47.5	Lanreotide 120 mg/28 d Pegvisomant 70 mg/7 d	31	N	0.9	NA	40	0.9	NA	OG	40	1.2	0.9	OG
6	F	43.3	Lanreotide 120 mg/28 d Pegvisomant 80 mg/7 d	47.2	N	0.9	NA	60	1.2	1.0	OG	60	1.1	2.0	OG
7	F	48.9	Octreotide 30 mg/21 d Cabergoline 3.5 mg/7 d	15.7	N	1.0	1.9	40	0.6	0.8	OG	40	0.5	0.5	OG
8	F	57.0	Lanreotide 120 mg/21 d Pegvisomant 100 mg/7 d	67.5	Y	1.0	NA	60	0.6	0.1	OG	60	0.6	0.2	OG
9	F	67.0	Lanreotide 120 mg/28 d Pegvisomant 105 mg/7 d	54.4	N	1.1	NA	40	1.0	0.8	OG	40	1.8	1.1	STOP
10	F	51.3	Lanreotide 120 mg/28 d Pegvisomant 200 mg/7 d	38.8	Y	1.1	NA	40	1.8	NA	OG	60	1.6	1.3	STOP
11	M	50.2	Octreotide 30 mg/28 d Cabergoline 3.5 mg/7 d	42.4	N	1.3	2.8	40	1.3	1.4	OG	60	0.9	NA	OG

12	M	26.5	Octreotide 30 mg/42 d Pegvisomant 100 mg/7 d	92.6	Y	1.7	NA	60	0.9	0.5	OG	60	0.9	0.2	OG
13	M	35.6	Octreotide 30 mg /28 d	19.2	N	1.7	1.0	60	1.7	1.7	STOP	60	1.2	0.9	STOP
14	F	50.6	Cabergoline 3.5 mg/7 d Lanreotide 120 mg/28 d Pegvisomant 120 mg/7 d	1.8	N	3.3	NA	40	1.3	1.8	OG	60	1.2	0.9	STOP
15	F	52.4	Octreotide 30 mg/28 d Cabergoline 3.5 mg/7 d	5.3	N	4.1	7.8	60	3.3	8.9	STOP	60	1.2	0.9	STOP

d, days; NA, non-available; OG, ongoing.

respectively (P value=0.06). As most of the patients were treated with PEG, plasma GH concentrations prior to the switch were not considered (Table 1).

The median size of pituitary adenoma remnants was 9.3 mm (5-17) in 12 patients and it was non-measurable in two patients.

Median HbA1c was 5.8% (5.3–7.2) and median fasting glycemia 100 mg/dL (0.9–1.8). Four patients had normal glucose tolerance, six had pre-diabetes and five patients were diabetic (see Table 2). Among five diabetic patients, two were treated with insulin (n , 9 and 14, Table 1), one with metformin (n , 10, Table 1) and one with a ddp4-inhibitor associated with metformin (n , 2, Table 1).

Results

First clinical evaluation following the switch to Pasireotide-LAR-treatment

The initial monthly dosage was randomly assigned except for diabetic patients who received 40mg/month. Seven patients started with 60mg/month and 8 with 40mg/month. The first evaluation was obtained at 3.0 months (2.0-6.5) after 2 to 6 injections of Pasireotide-LAR (Table 1 and Fig. 1).

Median clinical score was 1.5 (0-3) and was similar to baseline values ($P=0.30$). Most patients had identical scores (variation ≤ 1 point). Four patients improved their score (a decrease of 2–5 points), two of these presented a concomitant IGF-1 decrease and two a stable IGF-1 level. The improvement of the score mainly related to asthenia. One patient had a 2 points worsening of their score contrasting with a significant IGF-1 decrease from 3.3 to 1.27 ULN (n , 14, Table 1).

Median IGF-1 at the first evaluation was 1.0 ULN (0.5–3.3), similar to that measured prior to the switch (1.1 ULN (0.4–4.1); $P=0.49$). Median GH was 1.54 ng/mL (0.14–8.9) ($n=12$). Eleven of 15 patients had acceptable IGF-1 levels ≤ 1.3 ULN (0.5–1.3) using a monthly dose of Pasireotide-LAR of 40 mg in six patients and 60 mg in five patients. Prior to the switch, plasma IGF-1 was ≤ 1.3 in nine patients, >1.3 and ≤ 2 in one patient and >2 in one of these 11 patients, respectively. Three of 15 patients had IGF-1 levels >1.3 ULN and ≤ 2 (1.5–1.8) using a monthly dose of Pasireotide-LAR of 40 mg in two patients and 60 mg in one patient. Prior to the switch, two of these three patients had IGF-1 ≤ 3 ULN and one had IGF-1 >1.3 and ≤ 2 . One of the 15 patients presented an IGF-1 level >2 ULN (3.3) with a monthly dose of Pasireotide-

Table 2 Evolution of glucose tolerance status.

No.	Combination therapy			Pasireotide-LAR first evaluation			Pasireotide-LAR last evaluation			
	FBG (g/L)	HbA1c (%)	Glucose tolerance	FBG (g/L)	HbA1c (%)	Glucose tolerance	Treatment modifications	FBG (g/L)	HbA1c (%)	Treatment modification
1	0.99	5.6	NGT	1.15	5.9	PD			6.1	
2	1.19	7.2	TD	1.08	8.4	TD	insulin 4/d	Stop Pasireotide-LAR		
3	0.97	5.8	PD	1.08	6.5	D		Stop Pasireotide-LAR		
4	0.99	5.3	NGT	0.94	5.4	NGT		0.94	5.4	
5	0.97	6.7	D	1.20	6.2	PD	Dpp4	1.01	6.3	
6	1.03	5.8	PD	1.40	6.6	TD	Metf + dpp4	1.80	7.2	
7	0.99	5.8	PD	1.21	6.5	TD		1.66	6.2	
8	1.15	5.6	PD	1.20	6.2	PD		1.24	6.1	
9	1.78	6.9	TD	0.82	7.4	TD	Dose increase + Dpp4 - sulf	2.30	10.9	Stop Pasireotide-LAR
10	1.4	6.5	TD	NA	7.6	TD			8.6	Stop Pasireotide-LAR
11	0.94	5.6	NGT	1.13	5.9	PD		0.99	6.1	
12	0.90	5.4	NGT	1.03	5.9	PD		Stop Pasireotide-LAR	5.8	
13	1.01	5.5	PD	1.17	6.0	PD		2.34	8.4	Stop Pasireotide-LAR
14	1.42	5.4	TD	1.55	7.1	TD	+ Met			
15	1.06	6.3	PD	1.60	6.3	D		Stop Pasireotide-LAR		

D, diabetes not treated with a specific medication; Dpp4, dpp4 inhibitor; DT, diabetes treated with medication; Met, metformin; NA, not available; NGT, normal glucose tolerance; PD, prediabetes.

LAR of 60mg. His IGF-1 level prior to the switch was 4.1 ULN (Table 1).

Overall and, in comparison with the combination therapy, 7 vs 6 patients had an IGF-1 <1 ULN, four vs five patients had IGF-1 ≥1 ULN and ≤1.3 and 4 vs 4 patients had and IGF-1 >1.3 ULN (Fig. 1). Using the strict criteria of GH ≤1 ng/mL with IGF-1 ≤1 ULN, 3/15 patients were controlled with Pasireotide-LAR. The median IGF-1 level prior to the switch tended to be lower in patients with acceptable IGF-1 control (≤1.3 ULN) than in those with IGF-1 >1.3 ULN during Pasireotide-LAR therapy: 0.9 (0.4–1.7) vs 1.5 (0.6–4.1); P=0.06. Similarly, in patients treated with a combination therapy involving PEG, the PEG weekly dose was lower in patients with acceptable IGF-1 control (≤1.3 ULN) than in those with IGF-1 >1.3: 75 mg (40–105) vs 140 (120–200); P<0.02.

Digestive tolerance was found to be acceptable. No abnormality of hepatic biochemistry occurred. One patient complained of alopecia and one of dizziness.

A significant increase in FBG was observed compared to baseline levels: 117 mg/dL (82–160) vs 101 mg/dL (90–178) respectively, P<0.04. Similar findings were observed for HbA1C: 6.3% (5.4–8.4) vs 5.8% (5.3–7.2), P<0.01. Significant therapeutic modifications in antidiabetic therapy were carried out in the four diabetic patients, including a switch from oral therapy to insulin injections in one. FBG and HbA1C remained stable in the remaining diabetic patient treated with diet only. Four of the five patients with pre-diabetes became diabetic, with a mild increase in HbA1C in two of these, while a pharmacological treatment was required in two patients. Four of the five patients with normal glucose tolerance became pre-diabetic with a mild increase in HbA1C (from 5.6 (5.4–5.6) to 5.9% (5.9–6.0)). Overall, a pharmacological anti-diabetic intervention was given in 6/15 patients, as early as 15 days after the beginning of Pasireotide-LAR treatment (Table 2).

The treatment with Pasireotide-LAR was interrupted at the first evaluation in 4/15 patients in view of a lack of control of IGF-1 in two patients treated with 60mg/month (n, 13 and n, 15; Table 1); dizziness in one patient treated with 40mg/month despite controlled IGF-1 (n, 3; Table 1) and due to association of major hyperglycemia (300mg/dL) requiring initiation of insulin treatment and lack of control of IGF-1 (n, 2; Table 1) in one patient treated with 60mg/month.

Eleven patients initially treated with 40mg/month (n=7) and 60mg/month (n=4) continued to be treated with Pasireotide-LAR. The dosage was increased from 40 to 60mg/month in three patients who had IGF-1 levels 1.3, 1.3 and 1.8 ULN at the first evaluation.



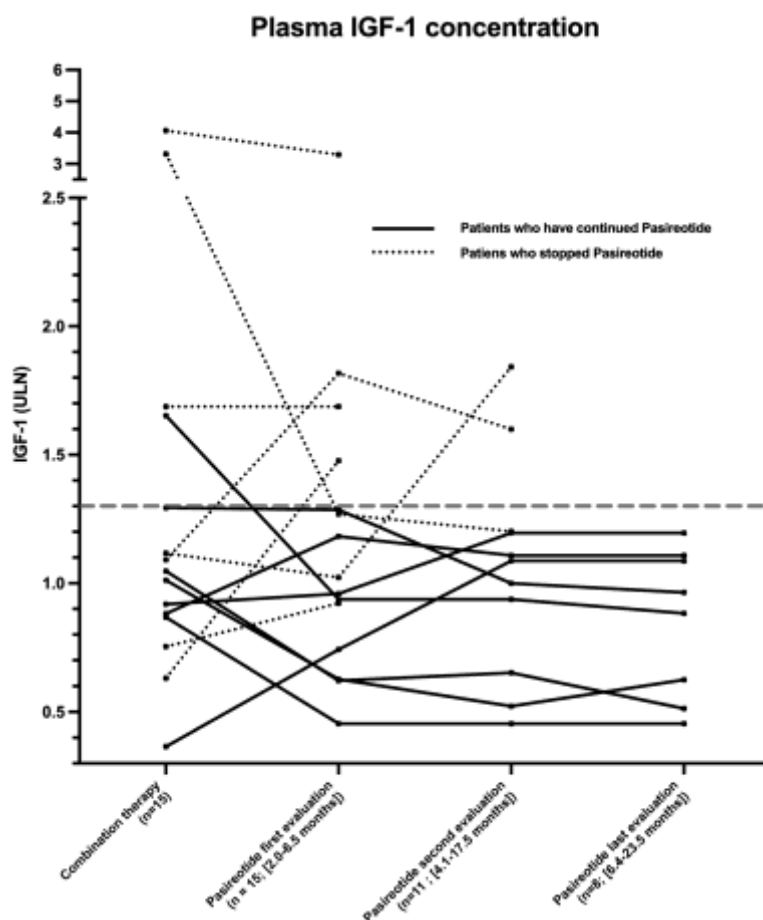


Figure 1 Evolution of IGF-1 results (expressed relatively to the upper limit normal of the assay ULN) for each patient during combination therapy and at subsequent evaluations (ev) following the switch to Pasireotide-LAR. The broken line is set at the upper limit of 'controlled' IGF-1 levels (1.3 ULN).

Final clinical evaluation following the switch to Pasireotide-LAR-treatment

Three of the 11 patients stopped Pasireotide-LAR after 6, 6 and 18 months of treatment: 2 patients (*n*, 10 and 9, Table 1) treated with 40 and 60mg/month had persistently increased IGF-1 levels (1.6 and 1.8 ULN) and had unacceptable hyperglycemia (HbA1c 8.6 and 10.9%) despite intensification of anti-diabetic treatment. Another patient (*n*, 14, Table 1) treated with 60mg/month had a controlled IGF-1 (0.6 ULN compared to 3.3 ULN during the combination therapy) but also developed major hyperglycemia (HbA1c 11%) despite anti-diabetic treatment (Table 1 and Fig. 1).

IGF-1 levels following the increase in Pasireotide-LAR dosage in three patients (*n*, 10, 11 and 14; Table 1) changed from 1.8, 1.3 and 1.3 to 1.6, 0.9 and 1.2 ULN, respectively. However, two of these three (*n*, 10 and 14) had to cease treatment due to hyperglycemia (HbA1c increased from 7.6 and 7.1% to 8.6 and 8.4% respectively).

Eight patients were still being treated with Pasireotide-LAR after 29 (17-34) months. The monthly Pasireotide-LAR dosage was 60 mg and 40 mg in five and three patients, respectively. Median IGF-1 was 0.8 ULN (0.5–1.2). Median GH was 0.9 ng/mL (0.2–7.3). Using the strict criteria of GH ≤1 ng/mL plus IGF-1 ≤1 ULN, 3/8 patients were controlled. As compared to the results obtained during combination therapy, five patients vs four had IGF-1 <1.0 ULN, three patients vs three had 1.0 < IGF-1 ≤1.3 and 0 patients vs 1 had IGF-1 >1.3 ULN (Fig. 1).

Median HbA1c in these eight patients was 6.1% (5.4–7.2) and median FBG was 120mg/dL (90–181). Two of these were pre-diabetic prior to the switch and required an antidiabetic treatment with a dpp4-inhibitor (*n*, 6, last HbA1C 7.2%), and metformin plus a ddp4-inhibitor (*n*, 7, last HbA1c 6.2%). The six remaining patients (*n*, 1, 4, 11 and 12 with normal glucose tolerance, *n*, 8 with prediabetes and *n*, 5 with non-treated diabetes prior

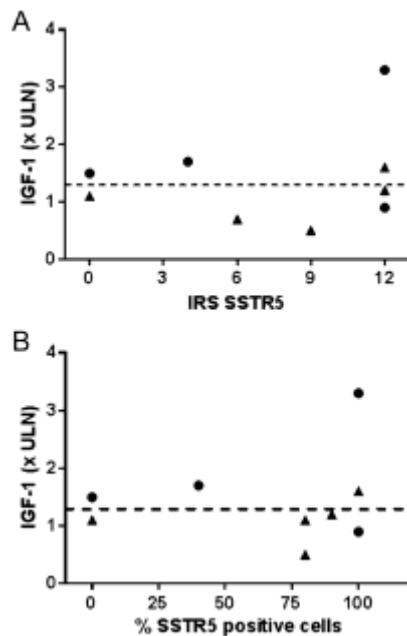


Figure 2 IGF-1 during Pasireotide-LAR treatment (1st evaluation) in relation with SSTR5 expression (A: IRS score, B: number of SSTR5-positive cells) and cytokeratin 18 expression (closed circles: densely granulated, closed triangles: sparsely granulated).

to the switch) achieved acceptable glycemic control (HbA1C=6.1%) with only lifestyle advice.

Pituitary MRI evaluation following the switch

MRI evaluation performed before the switch and after 8 months (4–14 months) of Pasireotide-LAR treatment was available for 11 patients. Tumor residue was non-measurable in two patients, and the median height did not differ between before and after the introduction of Pasireotide-LAR in the nine remaining patients: 9 mm (5–18) vs 8 mm (4–12), respectively ($P=0.44$). At the individual level, one patient presented a significant reduction in tumor size from 6.4 to 3.7 mm during Pasireotide-LAR treatment, while IGF-1 levels were stable. No change in the intensity of the signal was visible in T2-weighted sequences.

Immunohistochemical studies

Retrospective histological analysis of somatotroph adenomas was available for nine patients. All but one had been treated with 1gSRL before surgery. Five tumors were classified as densely granulated (DG) and four as sparsely

granulated (SG). There was no obvious association between the IRS score for SSTR5 and the IGF-1 response to Pasireotide-LAR (Fig. 2). This is illustrated by the IRS in patients with controlled IGF-1 (<1.3 ULN) which was evenly distributed from 0 to the maximal score of 12. In the same vein, one patient with an IGF-1 value of 3.3 ULN during Pasireotide-LAR treatment had a maximal score IRS of 12. Assessment of the percentage of immunoreactive cells for SSTR5 regardless of the intensity of staining, a semi-quantitative and somewhat subjective factor, did not change the results of the analysis. There was a trend toward an association between the IGF-1 response to Pasireotide-LAR and cytokeratin expression (characterizing the granulation phenotype), since three of four patients with sparsely granulated adenomas had controlled IGF-1 versus two of five patients with densely granulated adenomas. Illustrative cases are provided in Fig. 3.

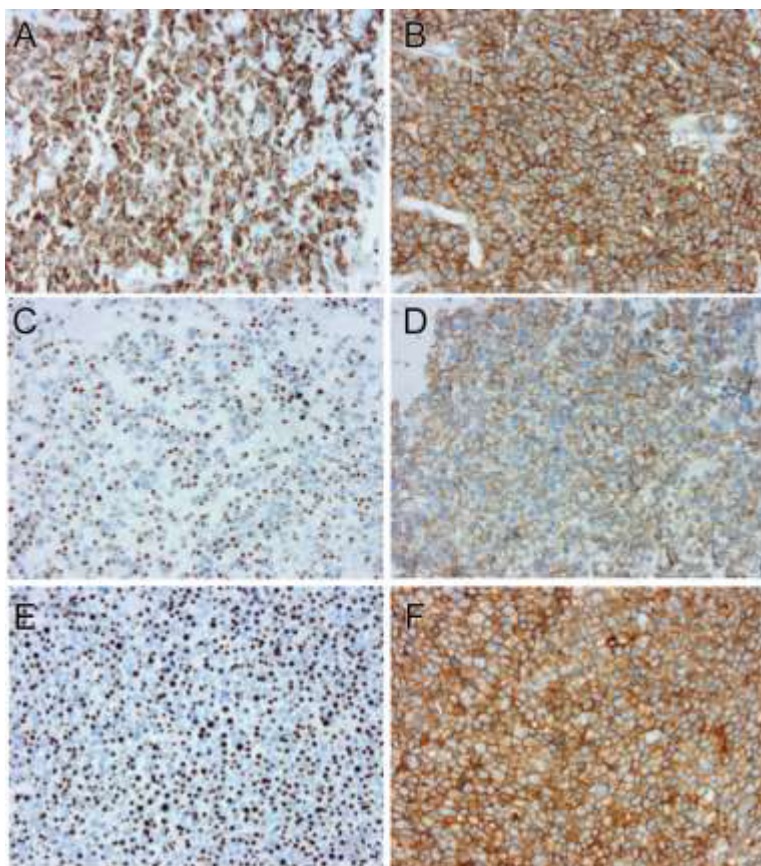
Discussion

Although involving a small cohort, this single-center prospective study is one of few that have evaluated the tolerance and efficacy of the substitution of Pasireotide-LAR to a combination therapy involving a 1gSRL in conditions of 'real-life' clinical practice. Additionally, we have attempted to correlate the response to Pasireotide-LAR treatment with the granulation status and immunohistochemical expression of SSTR5 in previously resected somatotroph adenomas.

Overall, Pasireotide-LAR was able to control IGF-1 at an acceptable level (≤ 1.3 ULN) with an acceptable tolerance in 8/15, while the combination therapy had to be resumed in 7/15 patients due to inefficacy or intolerance. The immunohistochemical expression of SSTR5 was not found to be correlated with IGF-1 control.

In our study, the overall number of patients controlled with Pasireotide-LAR and median IGF-1 levels were comparable to that obtained with combination therapy. The changes in clinical scores were mild, unsurprisingly, since baseline scores were rather low, as expected since 13/15 acromegalic patients had roughly controlled IGF-1. However, there were marked individual differences in the IGF-1 response to the two therapeutic strategies: while 9/15 patients had similar results in terms of IGF-1 control, three showed improvement during Pasireotide-LAR therapy while three showed an increase.

The individual response to Pasireotide-LAR was therefore heterogeneous, similar to a previous study of a small cohort of patients treated with 1gSRL and PEG

**Figure 3**

Examples of granulation and SSTR5 immunorepresentation in 3 patients and correlation with the IGF-1 response to Pasireotide-LAR. Cytokeratin 18 immunohistochemistry (A, C and E, original magnification $\times 200$). Densely granulated adenomas show perinuclear distribution of cyokeratin 18. Conversely, 'fibrous bodies' are typical of sparsely granulated adenomas. SSTR5 immunohistochemistry (B, D and F, original magnification $\times 200$). Immunorepresentation of SSTR5 is expressed using ImmunoReactive Score (IRS). (A and B) Densely granulated adenoma with a strong and diffuse immunorepresentation of SSTR5 (IRS = 12) and poor response to Pasireotide-LAR (IGF-1 = 3.3 ULN). (C and D) Sparsely granulated adenoma with a moderate immunorepresentation of SSTR5 (IRS = 6) and good response to Pasireotide-LAR (IGF-1 = 0.7 ULN). (E and F) Sparsely granulated adenoma with a strong and diffuse immunorepresentation of SSTR5 (IRS = 12) and uncontrolled IGF-1 with Pasireotide-LAR (IGF-1 ULN = 1.8).

(Shimon *et al.* (12)). In our study, it was impossible to predict, using clinical data, the individual response to the switch to enable the selection of the best candidates. This heterogeneity may be partially explained by an acromegalic population with large variations in sensitivity to 1gSRL. Interestingly, it seems that patients receiving the lower dosage of PEG were more likely to respond to Pasireotide-LAR, possibly reflecting better 'baseline' sensitivity to 1gSRL. This finding is similar to that of Muhammad *et al* (11) where the best IGF-1 control was obtained in patients that remained controlled prior to the switch despite a 50% reduction in weekly PEG dosage.

In our study, patients with normalization of IGF-1 at the first evaluation was lower than the 73.3% rate reported in Muhammad *et al* (11), though their study did not specify the individual sensitivity to 1gSRL. Our results accord more closely with the 67% rate of control obtained in nine patients previously receiving a combination therapy studied by Shimon *et al* (12). Sampling fluctuations across small cohorts as well as differences in study design, IGF-1 assays and normative data cohorts, may explain these

variations but still indicate that a subset of acromegalic patients may benefit from a switch to Pasireotide-LAR. The small size of our cohort hampers the study of the Pasireotide-LAR dose/response relationship but it should be noted that some 'sensitive' individuals had IGF-1 levels within the lowest half of the normal range on a dose of 40mg/month and may benefit from a reduced dosage to both improve metabolic tolerance of the drug while achieving acceptable control of the disease (12).

As was noted in previous trials (9, 10, 11, 12, 17), impaired glycaemic tolerance resulting from inhibition of insulin and incretin secretion (18), was the main drawback of Pasireotide-LAR treatment. The proportion of diabetic patients increased at the first evaluation, and introduction/intensification of antidiabetic treatment was required in 6/15 patients. Importantly, and as noted in the Paola study (10), diabetic patients, mainly those requiring pharmacological treatment and, to lesser degree patients with prediabetes, were more susceptible to the hyperglycaemic effect of Pasireotide-LAR. Of note, patients required intensification of antidiabetic treatments

regardless of the evolution of IGF-1 levels. This confirms that Pasireotide-LAR should be prescribed with caution in diabetic patients and should be associated with frequent monitoring of FBG in order to adapt and reconsider treatment. Importantly, none of the patients experienced acute ketoacidosis and, as reported previously, glucose tolerance was re-established after withdrawal of the drug (9).

Finally, 8/15 patients benefited from Pasireotide-LAR treatment with acceptable sustained control of IGF-1 at the expense of requiring an antidiabetic treatment in 2/15. The remaining six patients had slight increases in HbA1c levels, the long-term impact of which is uncertain. Elsewhere, we have to evaluate glucose tolerance in patients who are well controlled with Pasireotide-LAR over the long-term since normalization of IGF-1 results in a decrease of lean mass and an increase in fat mass, both of which play an important role in glucose tolerance (19).

Contrary to PEG, Pasireotide-LAR may help to control tumor size and further reduction in adenoma volume has been noted in patients resistant to 1gSRL (10, 20). We assessed the ability of Pasireotide-LAR to induce further shrinkage in our patients using tumor height measurements, a procedure that may be imprecise in comparison to tumor volume estimations. However, almost all patients in our study had been previously operated and had small-sized remnants which were sometimes difficult to precisely delineate, which may have masked small but definite shrinkage effects and that may render the antitumoral objective meaningless. We did not observe a shift in T2-hyperintensity in MRI scans, reflecting cystic degeneration or tumor cell necrosis that has recently been described in approximately 30% of patients after a switch to Pasireotide-LAR (20).

In an effort to identify biomarkers of treatment outcome and individualize the pharmacological treatment using a personalized approach (21), several studies have examined whether the *in vivo* efficacy of somatostatin analogs correlates with somatostatin receptor expression. Most of studies examined the correlation between immunohistochemical expression of SSTR2 using various scoring systems and the response to 1gSRL (21, 22, 23, 24, 25). Overall, some correlation was found, with the absence of membranous expression having a high negative predictive value (21, 22). The study of SSTR2 expression would have been irrelevant in our series since most patients were treated with 1gSRL before surgery, which has been shown to modify SSTR2 expression via receptor internalization (14).

Studies of histological factors predictive of the response to Pasireotide-LAR are rare. The expression of

SSTR5 is intuitively a good candidate since, contrary to 1 GSSA, Pasireotide is an affine ligand of the SSTR5 and since *in vitro* studies have shown that cultured adenomas with lower SSTR2/SSTR5 mRNA ratios responded better to Pasireotide (compared with octreotide) (23). To date, only two studies have been published. In a small cohort of 11 patients (24), a predictive role of membranous SSTR5 expression was suggested. Conversely, a recent study (26) in nine patients switched from a combination of 1gSRL+PEG to Pasireotide-LAR found the response positively correlated to SSTR2 IRS but not to SSTR5 IRS. We found no correlation between the SSTR5 IRS score, or the percentage of SSTR5-immunopositive cells, and the control of IGF-1 levels. Importantly, pre-surgical treatment with 1gSRL has been shown to have no effect on SSTR5 protein expression in somatotroph adenomas (23). The retrospective study design, the limited number of adenomas studied and the cellular heterogeneity of adenomas that were only partly removed may account for these discrepancies. Elsewhere, it is well accepted that a number of molecular characteristics are involved in the response to 1 GSSA (3) which is likely to be the same for the response to Pasireotide. We found a trend toward a better response of sparsely granulated adenomas compared to densely granulated ones, irrespective of SSTR5 expression. Although this trend comes from a limited number of patients, Iacovazzo *et al* also reported that differences in the response to Pasireotide correlated to cytokeratin expression levels (24). Elsewhere, the association between cytokeratin and SSTR5 expression remains controversial. As in our cohort, this was found to be negative in two large series using IHC analysis (27) and SSTR5 mRNA quantification (28), while Mayr *et al.* found membranous expression of SSTR5 exclusively in sparsely granulated adenomas (29). Further studies involving a larger number of patients with well-characterized *in vivo* responses to Pasireotide-LAR (ideally including patients fully resistant to 1gSRL and variable responses to Pasireotide) and using standardized molecular tools to study multiple factors involved in the response to somatostatin analogs are needed to assess the value of this 'personalized' medicine approach in current clinical practice.

In conclusion, in our 'real-life' study, Pasireotide-LAR controlled IGF-1 levels over the long term with acceptable glucose tolerance in approximately half of the patients previously treated with a combination therapy. Close monitoring of blood glucose is important for all patients and should be reinforced in diabetic patients treated with oral hypoglycemic agents. Complementing recently published expert recommendations (13),

our results suggest that Pasireotide-LAR monotherapy can be a suitable alternative to combination therapies involving 1gSRL and cabergoline or PEG for the control of IGF-1 in a subset of acromegalic patients partly resistant to 1 GSSA. The intensity of SSTR5 expression and the granulation pattern of adenomas were of limited value for the prediction of Pasireotide-LAR effectiveness. Therefore, the identification of good candidates for Pasireotide-LAR remains to be determined and the classical trial-and-error approach to acromegaly treatment in each individual is still necessary. However, non-diabetic patients using relatively low PEGV dosages in association to 1gSRL are probably good candidates for such a switch.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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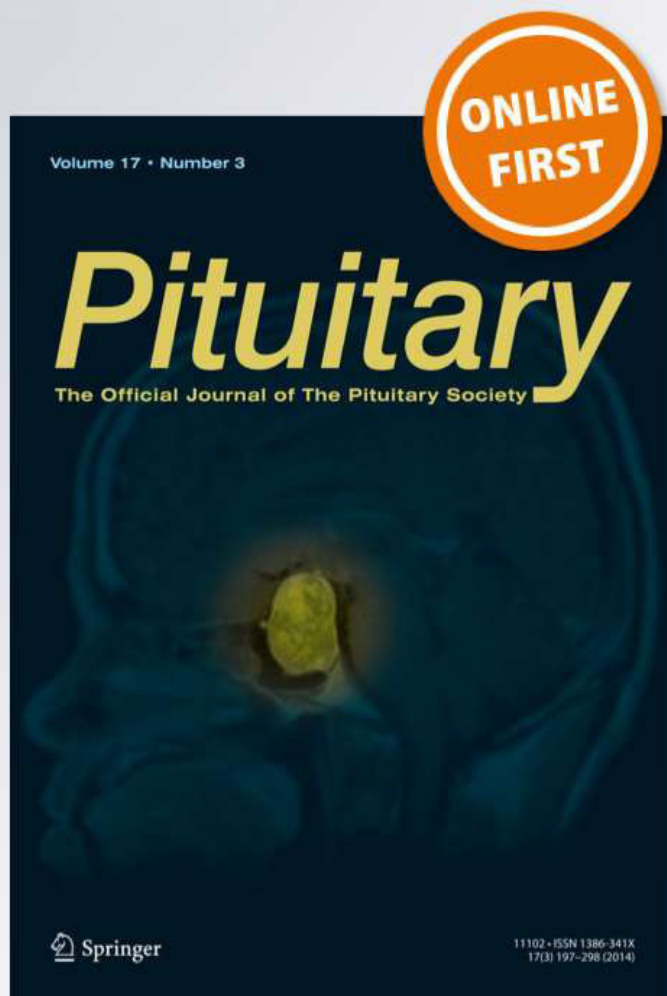
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Aggressive prolactinomas: how to manage?

Hélène Lasolle^{1,2,3} · Mirela Diana Ilie⁴ · Gérald Raverot^{1,2,3}

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Abstract

Purpose Aggressive prolactinomas are defined as radiologically invasive tumors which cannot be cured by surgery, and that have an unusually rapid rate of tumor growth despite dopamine agonist treatment and surgery. In some cases, metastasis occurs, defining prolactin carcinoma which is the second most frequent pituitary carcinoma.

Methods A literature search was performed to review the available data on the treatment of aggressive pituitary prolactinomas or carcinomas.

Results When optimal standard therapies (high dose cabergoline, surgery and radiotherapy) failed, temozolomide, an alkylating drug, is currently the best option, allowing to control tumor growth in about 50% of treated prolactinomas and improving overall survival of these patients. However, long-term complete response occurs in a limited subgroup of tumors. Alternative drugs could be discussed in a subset of aggressive prolactinomas either before temozolomide (pasireotide, peptide receptor radionuclide therapy...) or after temozolomide failure.

Conclusion Despite the significant improvement obtained with the use of temozolomide, a need for alternative drugs persists since a majority of these tumors are resistant or will recur during the follow-up. Patients suffering from such a rare condition should have access to clinical trials available for other types of rare cancers, such as tyrosine kinase inhibitors or immunotherapy.

Keywords Pituitary tumor · Prolactinoma · Temozolomide · Pasireotide · Aggressive pituitary tumor · Pituitary carcinoma

Introduction

Prolactinomas are the most common pituitary tumor representing around 50% of all pituitary tumors. Typical prolactinomas present as microadenomas diagnosed in women of reproductive age. A dopamine agonist (DA) is the first line treatment and usually allows hormonal and tumoral control, while being safe and well tolerated. However, some

prolactinomas, especially in men and those in a genetic setting are more aggressive and require treatment with other therapeutic modalities to be controlled.

These aggressive prolactinomas are not well-defined in the literature and some confusion exists with considerations of tumor size, tumor invasion or resistance to dopamine agonists being used as a synonym for aggressive pituitary tumor.

According to the ESE guidelines, an aggressive prolactinoma is defined either as a radiologically invasive tumor which cannot be cured by surgery, and that has an unusually rapid rate of tumor growth, or as clinically relevant tumor growth despite optimal standard therapies [1]. This definition underlines that in the absence of tumor progression, prolactinomas which fail to be hormonally controlled by dopamine agonist treatment should not be considered as aggressive pituitary tumors, unlike the definition of resistant prolactinomas. In contrast, the diagnosis of prolactinoma should be questioned if the tumor continues to grow despite normalization of the prolactin level. In such conditions, surgery should be considered in order to allow

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pathology analysis that would help guide the best therapeutic option.

Resistant prolactinomas are classically defined, according to the Endocrine Society, as showing a failure to normalize prolactin levels or failure to obtain a significant reduction of tumor size on the standard dose of DA [2]. It is important to note that these definitions do not include intolerant patients.

The definition of the optimal standard dose of dopamine agonists is open to discussion. For most authors, a significant reduction corresponds to a 50% reduction of tumor size with the standard dose of DA being up to 2 mg per week cabergoline [2, 3]. This definition takes into consideration cabergoline treatment, which is accepted as the most efficient DA in terms of normalization of PRL levels and tumor shrinkage since cabergoline treatment normalized PRL levels in more than 80% of treated women, compared to less than 60% with bromocriptine treatment, in a large randomized study [4, 5]. DA treatment at the maximal dose should be tested at least 3–6 months before suspecting resistance.

Most resistant prolactinomas are macroadenomas (> 80%) and resistance occurs more often in invasive tumors (tenfold increased risk), in men. The tumors in men display a clinically more aggressive course, for example having more frequent recurrence after surgery, tumor progression under treatment (DA and radiotherapy [6–8]) and more frequently developing into carcinomas. Indeed, among the 41 aggressive or pituitary carcinoma included in the ESE survey 75% were in men [9]. Moreover, MEN1 and AIP mutations, present in about 14% of children and adolescents, are independent predictors of DA resistance [10]. However, only rare cases of pituitary carcinomas associated with MEN1 mutations have been described [11, 12].

Hence, most giant prolactinomas are controlled by dopamine agonist treatment and should not all be considered as aggressive prolactinomas. In a recent series, 11 out of the 18 giant prolactinomas larger than 60 mm studied achieved PRL normalization within a median interval of 20 months [13].

The mechanisms underlying resistance are not fully understood and may be the consequence of a reduction in D2 receptor expression or alterations in the downstream signaling cascade [14]. Numerous factors associated with prolactinoma aggressiveness have been suggested [15, 16] including overexpression of growth factors [vascular endothelial growth factor (VEGF) [17] and epidermal growth factor (EGF)] [18], genes regulating invasion, differentiation and proliferation [8], expression of adhesion molecules (reduced expression of the E-cadherin/catenin complex) [19], higher expression of matrix metalloproteinase 9 [20] and genome instability (loss of the whole Chr11 and gain in the 1q arm were more common in aggressive tumors [21] and trisomy of Chr12 with overexpression of HMGA2 has also been described [22]). Moreover, in both men and women low

expression of estrogen receptor alpha was statistically correlated with tumor size and invasion, pathological markers, surgical cure, DA resistance, and tumor progression [7].

Pituitary carcinomas, defined by the presence of distant metastasis, are rare (0.2% of pituitary tumors) and are associated with a poor prognosis (less than 1-year survival). Prolactin pituitary carcinomas are the second most frequent pituitary carcinomas after ACTH secreting carcinomas, with fifteen out of 40 pituitary carcinomas published in the ESE survey being PRL carcinomas [9]. Screening for metastatic disease should be performed in cases where there is either a discordant increase in prolactin without significant local tumor growth or site-specific symptoms indicating potential metastasis (neck/back pain or neurological complaints) [1]. The central nervous system is generally the first site of metastasis, followed by lymphatic chains of the neck and systemic localization (liver, bone and lung) [23].

In this article we will review the therapeutic options for cases of aggressive prolactin secreting pituitary tumors, in which multimodal therapy is usually required. The most common treatments that are currently used are pituitary surgery, radiotherapy and temozolomide (TMZ) but other drug treatments are emerging.

Surgery

Surgery is the treatment of choice in the case of resistant prolactinomas in order to allow rapid relief of the symptoms that are due to the tumor mass. Moreover, it has been reported that, in expert hands, remission was achieved in 22 out of 61 patients (36%) who demonstrated resistance to dopamine agonist medication [24]. Even if the rate of remission is lower (< 10%) in patients with aggressive prolactinoma, the surgical approach allows the control of neurological symptoms and significantly decreases PRL levels and the required dose of DA [25, 26].

As has been proposed by the ESE guidelines, surgery should be discussed with an expert surgeon in the case of neurological symptoms (severe headaches, acute loss of vision, chiasmal compression) even in patients who have undergone multiple prior surgeries [1].

Radiotherapy

Radiotherapy is a classic option in cases of pituitary tumors that are growing despite medical treatment and surgery. Effects on tumor progression appear to be satisfactory even if no prolonged randomized studies have evaluated it. Effects on prolactin secretion are less evident and may require many years to be obtained. In retrospective studies, hormonal control in prolactinomas is achieved in around 50% of cases and

tumor control in around 90% of cases [27–29], however the course of the tumor before radiotherapy is rarely described. Fractionated external beam radiotherapy and stereotactic radiosurgery both seem to be equally effective. The choice should be made by a specialist, depending on the age of the patient, tumor volume/shape and distance from the optic chiasm. Although the side effects (hypopituitarism, risk of brain tumor and meningioma, optic nerve lesion, vascular events...) should limit the use of radiotherapy in indolent pituitary tumors, the risk–benefit balance is totally different in cases of growing aggressive pituitary tumors [29].

Temozolomide

TMZ is an alkylating chemotherapy drug with lipophilic properties and the capacity to cross the blood–brain barrier. It is a well-known drug in neuro-oncology for the treatment of glioblastoma [30]. TMZ is given by oral route in cycles, at a dose of 150–200 mg/m² for 5 consecutive days in each 28 days in the standard regimen. An alternative protocol based on its radio-sensitizing properties, consists of TMZ first given at a daily dose of 75 mg/m² for 6 weeks in combination with radiotherapy, followed by 6–12 months of 'standard' therapy.

Its first successful use in treatment of pituitary tumors was reported in 2006 [31], followed by the publication of a few successful case-reports. Since that time, a few retrospective studies have been published and have reported around 160 cases of all subtypes of pituitary tumors [32, 33]. Prolactinomas is the second most common tumor type treated with TMZ after corticotroph pituitary tumors, representing around 30% of published cases [32]. More recently, a European Society of Endocrinology Survey reported on 166 patients treated with TMZ, some patients having been previously published, with a total of 40 prolactinomas (25 aggressive pituitary tumors and 15 carcinomas) [9]. A significant tumor reduction was observed in around 40% of patients with aggressive pituitary tumor or carcinoma, with complete response being rare [1, 9]. The rate of response to TMZ in prolactinomas was quite good compared to the global response rate, with significant tumor reduction in more than 50% of treated prolactinomas while 20% of patients showed disease progression [9, 33]. There was no difference in response between men and women [9].

In cases that respond, TMZ has shown a significant improvement in overall survival [34] and the 5 year overall survival was estimated to be 57% in all patients treated with TMZ in a recent systematic review [32]. In our series, the estimated median survival in prolactinomas was 42 months versus 40 months in others subtypes (Fig. 1) [34].

When considering all pituitary tumor subtypes, patients with functional tumors and those receiving associated

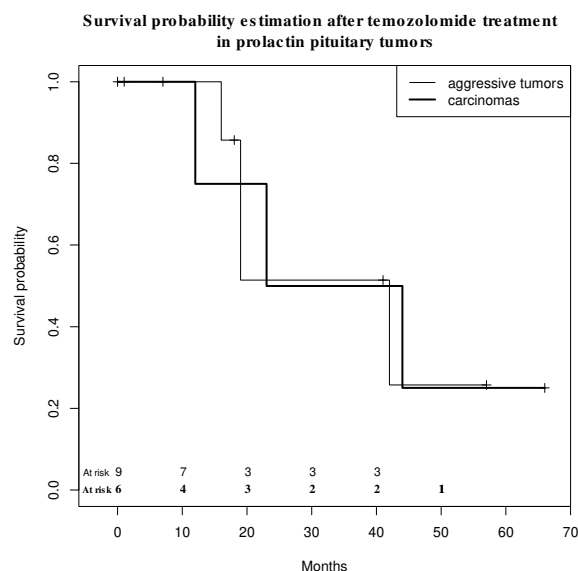


Fig. 1 Survival probability estimation after temozolomide treatment in prolactin pituitary tumors. The survival curve was obtained from data published in Lasolle et al. [24], including 6 PRL carcinomas and 9 aggressive PRL pituitary tumors with a median follow-up 19 months (0–66 months)

radiotherapy seem to be the better responders [9]. However, most of the patients treated with association of TMZ and radiotherapy protocol had no radiation therapy before TMZ use (as illustrated in Fig. 2), as opposed to other patients being treated with TMZ in whom radiation therapy had failed to control tumor growth. This could introduce some bias, with a better response in the first group of patients that could be explained by radiation therapy itself and a worse prognosis in the second group due to selecting the more aggressive pituitary tumors that were resistant to radiation therapy.

There is no standardized duration of treatment with TMZ. Most patients have been treated between 6 and 12 months, as is the recommended duration in glioblastoma treatment [32]. However, long term treatment, in case of response, appears to be beneficial as the overall survival in these patients was high (greater than 90% at 5 years) [32]. Moreover, most of the cases with second courses of TMZ treatment after regrowth failed [9, 34]. It must also be underlined that, even if short term tolerance of TMZ is quite good, hematological toxicity and the risk of myelodysplastic syndrome is a concern for long term treatment of young patients [35]. The risk/benefit therefore must be carefully evaluated.

A need for the identification of predictive factors of response remains. As in glioblastomas, the role of MGMT expression (an enzyme which repairs methylated bases) and methylation of its promoter have been studied in

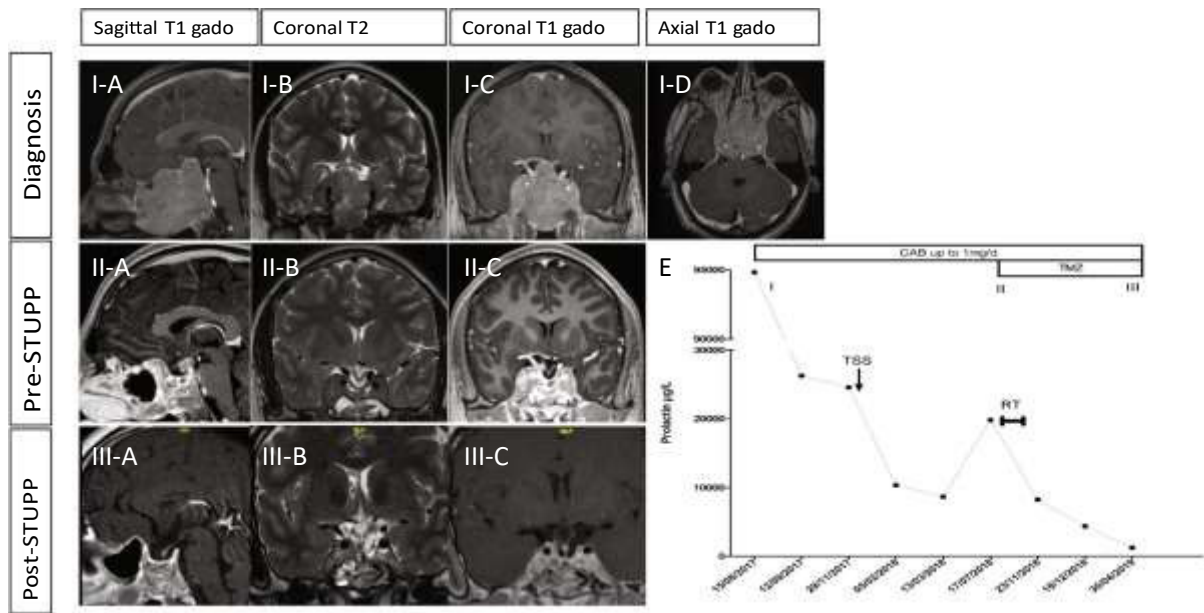


Fig. 2 Illustration of an intensive therapeutic strategy to control an aggressive PRL pituitary tumor in a young man. Bilateral exophthalmia in an 18-year-old man revealed a giant macroprolactinoma extending into the optic canal and invasive for the bones of the skull base as seen on the MRI [sagittal T1 gado (a) Coronal T2 (b), Coronal T1 gado (c), and axial T1 gado (d)] at diagnosis (i). The prolactin level was 94,800 µg/L (e). The patient was treated with increasing dose of cabergoline (CAB), up to 1 mg/day. Due to the limited effect on tumor size and prolactin secretion, transsphenoidal surgery (TSS) was proposed after 4 months of treatment. The pathology report confirmed a grade 2b PRL pituitary tumors (ki67: 9%, mitosis $n=5$, p53 positive). Post-TSS, PRL dropped from 24,000 to 10,300 µg/L,

an additional decrease occurred on cabergoline (1 mg/day) down to 8600 µg/L (e). However, 9 months later PRL levels increased despite cabergoline, and MRI demonstrated large residual tumor in both cavernous sinus and in the suprasellar/retrochiasmatic (II). In response to resistance to treatment, radiation therapy (RT) combined with TMZ treatment (according to the STUPP protocol) was initiated. TMZ was first given at a daily dose of 75 mg/m² for 6 weeks in combination with radiotherapy, followed by six cycles of TMZ at a dose of 200 mg/m²/day. As illustrated in panels (III) MRI, performed after six cycles of TMZ, demonstrated significant tumor shrinkage associated with a decrease in PRL to 1240 µg/L at last follow-up (e)

retrospective cohorts. The results are discordant and limited by the absence of technical standardization, however, lower MGMT expression evaluated by IHC seems to be associated with a better response [1, 9]. A role for mismatch repair proteins, especially MSH6, has also been suggested [36, 37].

In sensitive patients, the first signs of reduction in tumor volume and hormonal levels are observed in the first 3–6 months [9]. Additionally, ESE guidelines advise to test 3–6 cycles of treatment to determine the individual response, regardless of the MGMT status. In our practice, TMZ is maintained as long as a tumor and hormonal effect is observed and according to the clinical and biochemical tolerance. However, after 24 cycles of TMZ, we tend to decrease the dose (half dose) as long as hormonal levels and the tumor is controlled. In the setting of prolactinomas, prolactin levels may help in treatment monitoring.

Few patients have been treated with an association of TMZ with another drug, such as capecitabine, bevacizumab, thalidomide or BCNU, and this low number of patients does not permit solid conclusions to be drawn [9].

Other cytotoxic drugs

Other cytotoxic chemotherapies have been tested with no evidence of success. The association of CCNU and 5-FU has been evaluated in 4 prolactinomas (3 aggressive pituitary tumors, one carcinoma). Reduction of tumor size was noted in only one patient, who eventually died from an another disease after 6 month of treatment, while the other 3 patients showed no significant response [38]. In this study, carboplatin chemotherapy led to moderate hormonal and tumoral response in one aggressive prolactinoma but had to be discontinued due to hematological toxicity. The association of CCNU, procarbazine and etoposide led to tumor stabilization in an aggressive prolactinoma during 12 months, which however, then grew and led to the patient's death [39]. In the Lasolle et al. study, two prolactin carcinomas were treated with carboplatin-etoposide and 5FU-oxaliplatin with no response observed [34]. In the Pernicone et al. study, 3 prolactin carcinomas were treated with a combination of cisplatin, procarbazine, lomustine and vincristine, again with no response [40].

Targeted therapeutics have been more recently tested in aggressive prolactinomas and carcinomas.

Pasireotide

Prolactinomas have been shown, using imaging and histological studies, to express different subtypes of somatostatin receptors (SSTR). Both immunohistochemistry and transcriptional analysis have shown the expression of subtypes 1, 2 and 5 [41]. However, the use of first generation somatostatin analogs have been disappointing and only one successful case of octreotide treatment has been reported in a resistant prolactinoma [42].

The more recent somatostatin analog, pasireotide, which targets multiple somatostatin receptors, has been recently reported to have an effect on prolactin secretion and tumor proliferation, in two patients. One patient had a long history of a DA resistant macroprolactinoma which required 2 surgeries, and showed rapid normalization of prolactin levels with pasireotide treatment [43]. MRI showed necrotic transformation of the lesion and the effect was maintained 7 years after pasireotide treatment and allowed withdrawal of cabergoline. The second patient underwent 2 surgeries, plus conventional radiotherapy and gamma-knife radiosurgery for a resistant macroprolactinoma. Pasireotide normalized prolactin levels within 2 months and a reduction in tumor volume of 75% was seen at 23 months [44]. No side-effect and especially, no diabetes mellitus appeared in these patients. Both patients presented with high SSTR5 expression in the tumor on IHC examination.

In addition to these 2 reported cases of aggressive functional prolactinomas, another case of a 55-year-old woman with a giant silent plurihormonal PIT-1-positive pituitary tumor, with clinically significant residual tumor despite 2 pituitary surgeries, demonstrated tumor shrinkage and visual field improvement after 4 months treatment with pasireotide (LAR 40 mg/month) [45].

As shown by these promising results, pasireotide can be tested in patients with aggressive prolactinomas before proposing chemotherapy agents such as TMZ, especially in those presenting with high SSTR5 expression. It should be underlined that SSTR5 expression is rare, since it was present in only 3 out of 21 macroprolactinomas in our series [45].

Peptide receptor radionuclide therapy

Peptide receptor radionuclide therapy (PRRT) has been proposed for aggressive pituitary tumors based on the description of SSTR expression in prolactinomas, and the uptake by most pituitary lesions of radiolabeled somatostatin analogs seen by imaging (^{111}In -Pentetreotide, ^{68}Ga -DOTATATE imaging...) [46].

Six patients treated with PRRT for aggressive prolactinomas were identified in the literature. Three treated with ^{111}In -DTPA-Octreotide [47, 48], one with ^{68}Ga -DOTATATE [37], two with ^{177}Lu -DOTATOC [24, 38]. Two showed efficacy of the ^{111}In -STPA-Octreotide treatment: the first one showed a 76% reduction in tumor volume after five cycles of treatment (no data reported concerning follow-up) [47] and the second showed a 95% reduction in tumor volume and 91% decrease in plasma prolactin levels, with an effect that was maintained 7 years later [48]. The other patients rapidly stopped the treatment because of progression or died a few months after commencing treatment. No significant adverse effects were reported. One additional patient, with an aggressive somato-prolactin tumor, was unsuccessfully treated [49].

Further studies are needed to better evaluate the candidates for this treatment, to characterize SSTR expression, as well as the optimal time to start the treatment and timing of its administration.

mTOR inhibitors

Evidence of activation of the PI3K/AKT/mTOR pathway in pituitary tumor, especially in prolactinomas [50], and the observed *in vitro* effects of mTOR inhibitors on cell proliferation [51], led to everolimus being tried in the treatment of aggressive pituitary tumors and pituitary carcinomas. The first reports, in two ACTH secreting tumors, showed no significant effect [52, 53]. Moreover, everolimus treatment failed in one aggressive PRL-secreting pituitary tumors and in one mixed GH-PRL pituitary tumors after failure of TMZ treatment [34]. More recently, a case where everolimus treatment was effective on an aggressive PRL pituitary tumors, naive in terms of TMZ treatment, was published. This study showed a significant but moderate reduction in tumor size, and a slight diminution of PRL plasma levels after 12 months treatment [50]. The mild side effects observed in this case consisted of diabetes mellitus, which required insulin glargine and dpp4 inhibitor treatment.

Tyrosine kinase inhibitors

EGFR Tyrosine kinase inhibitors (TKI) have been tested in corticotroph and prolactin Experiments using TKI in rat and human primary cell cultures showed both an antitumoral and antisecretory effect of the dual TKI that inhibits ErbB1 and ErbB2, lapatinib, being more potent than gefitinib [54]. Cooper et al. reported on 2 patients treated with lapatinib for aggressive prolactinomas [18]. Recently, they have described an additional 4 patients including one carcinoma [55]. After 6 months of treatment, one patient showed a 22% reduction in tumor volume, 3 had stable tumor volume and 2 progressed. Prolactin levels decreased, by 42 to 78% in 3

patients, but rose in 3 patients. We have reported the use of labinib in a patient with a PRL secreting carcinoma where no effect was seen and the patient rapidly died [34]. We should note that crizotinib, an ALK TKI, was then tried in this patient but failed.

VEGF targeted therapy

Anti-angiogenic effects of anti-VEGF agents underlie the mechanism of their anti-tumoral effects. VEGF expression has been found in pituitary tumors using IHC [56]. In this context, bevacizumab, an anti-VEGF monoclonal antibody, has been used in a few corticotroph pituitary tumors and showed prolonged stabilization in some patients [57, 58]. Some patients treated with bevacizumab were described in the ESE survey and showed a partial response, however no details were provided regarding the tumor type [1]. Recently, anti-VEGF TKI treatment produced, in combination with DA, a clear reduction in vascularization of the tumor and restriction of tumor growth in a mouse model with an induced prolactinoma, with a synergistic action that induced normalization of the vascularization. These results point to the potential for studies in human lactotroph pituitary tumors to be carried out [59].

Immunotherapy

A single case of a secreting corticotroph carcinoma, treated with an association of anti-PD1 nivolumab and anti-CTLA4 ipilimumab, has been reported in the literature, with an impressive response being observed. The patient showed a 59% reduction in pituitary tumor volume and 92% reduction of liver metastasis volume, as well as showing a significant hormonal response [60].

IHC studies of pituitary adenomas have confirmed expression of PD-L1 in most functioning pituitary tumors, and especially in prolactinomas [61]; as have mRNA studies [62]. Immune checkpoint inhibitors could thus represent a promising therapeutic target in aggressive prolactinomas.

Conclusion

Although aggressive prolactin pituitary tumors are rare, they represent a therapeutic challenge. The first therapeutic option is to use the highest tolerated dose of cabergoline (up to 1 mg/day), and propose debulking surgery which could then favor DA action. A few months of pasireotide treatment challenge seems to be a safe option as, if it shows efficacy, may avoid more aggressive treatments with potential side effects.

In case of a growing tumor despite DA or pasireotide treatment, radiotherapy should be proposed, and association

of radiotherapy to TMZ treatment should be discussed with a multidisciplinary team in some cases (depending on residual tumor size, pathological markers etc...). Identification of distant metastasis is a clear indication for TMZ treatment; however, the time at which to initiate TMZ treatment in locally aggressive pituitary tumors is still a matter for debate.

Compliance with ethical standards

Conflict of interest Gérald Raverot has received research Grants from Novartis Pharma and IPSEN; speaker honorarium from Novartis Pharma and IPSEN, consultant for Pfizer, Ipsen and Novartis. Mirela Diana Ilie and Hélène Lasolle declare they have no conflict of interest.

Research involving human participants and animals rights This article does not contain any studies with human participants or animals performed by any of the authors.

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RESEARCH

SST5 expression and USP8 mutation in functioning and silent corticotroph pituitary tumors

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Abstract

Objective: Somatostatin receptor type 5 (SST5) is inconsistently expressed by corticotroph tumors, with higher expression found in corticotropinomas having ubiquitin-specific protease 8 (*USP8*) mutations. Aims were to study the correlation between characteristics of corticotropinomas and SST5 expression/*USP8* mutation status and to describe the response to pasireotide in five patients.

Design: Retrospective cohort study.

Methods: Clinico-biochemical, radiological and pathological data of 62 patients, operated for a functioning or silent corticotropinoma between 2013 and 2017, were collected. SST5 expression was measured by immunohistochemistry (clone UMB-4, Abcam, IRS > 1 being considered positive), and Sanger sequencing was performed on 50 tumors to screen for *USP8* mutations.

Results: SST5 expression was positive in 26/62 pituitary tumors. A moderate or strong IRS was found in 15/58 corticotropinomas and in 13/35 functioning corticotropinomas. Among functioning tumors, those expressing SST5 were more frequent in women (22/24 vs 9/15, $P = 0.04$) and had a lower grade ($P = 0.04$) compared to others. *USP8* mutations were identified in 13/50 pituitary tumors and were more frequent in functioning compared to silent tumors (11/30 vs 2/20, $P = 0.05$). SST5 expression was more frequent in *USP8*mut vs *USP8*wt tumors (10/11 vs 7/19, $P = 0.007$). Among treated patients, normal urinary free cortisol levels were obtained in three patients (IRS 0, 2 and 6), while a four-fold decrease was observed in one patient (IRS 4).

Conclusion: SST5 expression appears to be associated with functioning, *USP8*mut and lower grade corticotropinomas. A correlation between SST5 expression or *USP8*mut and response to pasireotide remains to be confirmed.

Key Words

- ▶ SST5
- ▶ corticotroph pituitary tumors
- ▶ USP8
- ▶ pasireotide

Endocrine Connections
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Introduction

Cushing's disease is a rare disorder defined as chronic hypercortisolism due to a corticotropin-secreting pituitary tumor (corticotroph tumor) (1). Chronic cortisol excess is responsible for multisystem morbidity, contributing to increased mortality and altered quality of life (2, 3). In some cases, corticotroph tumors are discovered during examination or analyses in patients showing no clinical signs of Cushing's disease and these are termed silent corticotroph tumors. Diagnosis of such tumors arises because of symptoms related to tumor mass, symptoms of hyperprolactinemia or pituitary deficiency, and they are often classified as non-functioning pituitary tumors (4, 5). Treatment of Cushing's disease is obligatory in order to control cortisol excess and to prevent morbi-mortality. Transsphenoidal surgical resection is the first-line treatment with a remission rate of approximately 75% when the surgery is performed by an expert pituitary surgeon (6, 7, 8). However, recurrence occurs in 15 to 66% of patients and some patients either refuse or do not benefit from the surgery (7). Alternative treatments include additional pituitary surgery, radiation therapy or medical treatment (9, 10). Medical treatments can be divided into the use of drugs that block cortisol production (anticortisol drugs) or block cortisol action (glucocorticoid receptor blockers), which do not control pituitary tumor growth and secretion, and those treatments that target the corticotroph tumor (cabergoline or pasireotide). Pasireotide, a second-generation somatostatin analog, acting on somatostatin receptor (SST) subtype 1,2,3 and 5, is the only approved pituitary-targeting drug for the treatment of Cushing's disease (11, 12, 13, 14, 15). Its efficacy is variable. In phase III studies, 24-h urinary free cortisol (24-h UFC) was reported to be normalized in 20 to 40% of patients after 6 months of treatment; however, it was associated with numerous side-effects including hyperglycemia and diabetes mellitus (13, 14). Predictive markers of response to pasireotide are, however, lacking as has been already reported to be the case in somatotroph tumors, though an association between the expression of somatostatin receptor subtypes, in particular SST5, and response to treatment has been suggested (16). Most published studies have quantified SST5 mRNA expression in corticotroph tumors using quantitative RT-PCR and reported its expression in 80 to 100% of tumors (12, 17, 18, 19). However, using immunohistochemistry (IHC), SST5 expression was only found in 20 to 55% of corticotroph tumors (20, 21, 22). Somatic driver mutations in the ubiquitin-specific protease 8

(*USP8*) gene, identified in 35% to 60% of corticotroph tumors (21, 23, 24, 25, 26, 27, 28), lead to an increase in the deubiquitinase activity of *USP8* and to an increase of ACTH secretion by the tumor (28). SST5 expression has been found to be higher in *USP8*mut corticotroph tumors (21). The increased SST5 expression in *USP8* mutated tumors suggests a positive response to pasireotide in those tumors. The prognosis associated with those mutations is controversial, as they seem to be associated with a greater likelihood of surgical remission (23) as well as a higher risk of recurrence of Cushing's disease (24).

The goal of the present study was to characterize SST5 IHC expression and *USP8* mutations in a large cohort of functioning and silent corticotroph pituitary tumors and to correlate its expression with clinical and biochemical characteristics of patients at diagnosis. Additionally, SST5 expression and response to pasireotide was studied in five patients.

Materials and methods

Patients and assessments

Between January 2013 and December 2017, 76 patients underwent surgery for a corticotroph tumor at the neurosurgical service of University Lyon Hospitals (Hospices Civils de Lyon) carried out by one expert neurosurgeon (E J). Six patients operated for recurrence, without access to initial pathology, were excluded from the study, and eight patients were excluded because of insufficient material being obtained to perform SST5 IHC. Initial clinical presentation data, including sex and age at surgery, preoperative hormonal assessment and pituitary MRI, were collected. Five patients were pre-treated with anticortisol drugs before their surgery, either by ketoconazole ($n=4$) or mitotane ($n=1$). Corticotroph tumors were reported as silent when no clinical or biochemical signs of Cushing's disease were present prior to surgery. Preoperative MRI were reviewed by a pituitary specialist radiologist (V L), and the tumor size, defined as the largest diameter observed on MRI, and cavernous sinus invasion (according to Knosp's classification (29)) were both noted.

The response to pasireotide was studied retrospectively in five treated patients operated for a corticotroph tumor at Lyon Hospital between 2010 and 2018. Patients with combined medical treatment of hypercortisolism were excluded. Initial 24-h-UFC levels were compared with those during and at the end of the treatment, and clinical improvement as well as any side-effects were reported.

The study was approved by the ethics committee of Hospices Civils de Lyon. Consent was obtained from each patient or subject after full explanation of the purpose and nature of all procedures used. Patient information was recorded in a local database (PITUICARE-Lyon, registered with the French data protection agency CNIL, 16-021, and clinicaltrials.org, NCT 02854228).

Biochemistry

Cortisol and ACTH measurements were performed using automated immunoassays (Roche Diagnostics). For cortisol assays, inter-assay CVs were 1.9% at the 109 nmol/L level, 2.8% at the 333 nmol/L level and 2.8% at the 741 nmol/L level. For ACTH, inter-assay CVs were 9.2 % at the 20 ng/L level, 2.1% at the 52 ng/L level and 2.3% at the 925 ng/L level. 24-h UFC was measured with a direct competitive immunoassay (Abbott Diagnostics). Inter-assay CVs were 3.6%, 3.9%, 2.2% and 3.2% at concentrations of 124.0 nmol/L, 273.2 nmol/L, 465.6 nmol/L and 1035.1 nmol/L, respectively. Data for 24-h UFC were missing in 19 cases, including 15 silent corticotroph pituitary tumors. In accordance with the French Society of Endocrinology guidelines, biochemical exploration of the corticotroph axis was performed in clinically non-functioning pituitary tumors to screen for corticotroph deficiency and to exclude potential silent corticotroph tumors (30).

Pathology

All tumors ($n=62$) were stained for ACTH, SST5, Ki67 and p53 using IHC and were assessed by a single experienced pathologist (A V) who was blinded to the clinical data. Immunohistochemical analysis was performed on 4- μ m paraffin sections using the BenchMark® ULTRA automated immunostainer (Ventana Medical Systems Inc, Tucson, AZ, USA) and the following antibodies and dilutions: anti-ACTH (clone AH26, 1:300, Diagnostic Biosystems, Pleasanton, CA, USA), anti-SST5 (clone UMB-4, 1:250, Abcam), anti-Ki67 (clone MIB1, 1:100, Dako) and anti-p53 (clone D07, 1:100, Dako). SST5 immunohistochemistry required an additional amplification step (amplification kit, Ventana Medical Systems). To evaluate the expression of SST5, only membrane immunopositivity was considered (inter-endothelial junctional immunopositivity served as an internal positive control). SST5 immunopositivity was scored according to the IRS scoring system (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). As defined by IRS classification, the staining was considered to be negative for IRS 0 and 1

and positive for IRS>1. Four tumors were considered SST5-positive despite the IRS being not evaluable, as the size of the removed tumor was insufficient to precisely assess the percentage of positive cells. The percentage of ACTH-positive cells and granular and strongly basophilic staining of the cytoplasm were analyzed as indicators of corticotroph tumor differentiation. Proliferation markers (mitotic activity, immuno-expression of p53 and Ki67) were also analyzed. A clinico-pathological classification was applied according to Trouillas *et al.* (29). This classification takes into account cavernous and/or sphenoid sinus invasion and the presence of proliferative markers (presence of at least two out of the three following features: more than two mitotic figures, positive expression of p53 or Ki67-positive cells superior or equal to 3%). In six patients, grading was not feasible because of the small size of the tumoral tissue.

Molecular analysis

USP8 analysis was performed in 50 tumors, while analysis could not be conducted in 12 tumors because of the lack of tumor tissue. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue sections using the Maxwell RSC FFPE DNA Kit (AS1450). The DNA sequence was amplified by PCR with the CORE 10 (Mpbio) NH4(SO4)2 Kit (MP Biomedicals, Irvine, CA, USA) using the forward primer 5'-CACCCCTCCAACCTCATAAAG-3' and reverse primer 5'-GTTCTAGGAGTTAAGATAAACATAC-3'. Sanger sequencing of PCR products was performed on the ABI 3500 Dx Analyzer (Applied Biosystems) after having employed the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Results were interpreted with Seqscape Version3 software. In seven complicated cases with results that were difficult to interpret, TOPO cloning was performed after PCR amplification using the TOPO® TA Cloning® Kit (Invitrogen). PCR, sequencing and analyses were carried out following the same protocols.

Statistical analysis

Data are presented as median and ranges (minimum and maximum). The association between SST5 expression (negative or positive) or USP8 mutations (wild type or mutant) and patient/tumor characteristics were tested using Fisher's exact test for qualitative data and Wilcoxon rank test for quantitative data. Statistical analyses were performed using R software version 3.3.0 (www.r-project.org/). *P*-values <0.05 were considered as significant.

Results

Cohort description

The study population consisted of 62 (44F/18M) patients who were evaluated retrospectively. Patient characteristics are described in Table 1. The median tumor size was 11.5 mm with three tumors that were not visible on MRI which were operated after inferior petrosal sinus sampling. Overall, 26/62 tumors were SST5-positive. Eight different USP8 mutations were detected in 13 out of the 50 tumors, and one mutation (Ser719del) was found in 7 of the 13 patients (Supplementary Table 2). Of the 62 patients in our cohort, 23 with neither clinical nor biochemical hypercortisolism were diagnosed postoperatively as having silent corticotroph tumors (Table 1).

Patients with silent corticotroph tumors were older than those with functioning corticotroph tumors (P -value=0.03). Although 24-h UFC and 08:00h cortisol were lower in silent tumors (both P -values <0.001), 08:00h ACTH levels were similar at diagnosis (P -value=0.41). While 26/39 functioning corticotroph tumors were microadenomas, silent corticotroph pituitary tumors were mostly macroadenomas (only one was a microadenoma), with a larger diameter (both P -value <0.001), and were more often invasive and positive for p53 (both P -values=0.05). Silent corticotroph tumors were less differentiated, with a lower percentage of ACTH expression (P -value <0.001, Fig. 1). SST5 expression and USP8 mutations were less frequent in silent tumors compared to functioning corticotroph tumors (P -value <0.001 and 0.05).

Table 1 Clinical, radiological and pathological features of 62 patients operated for corticotroph tumors, categorized by functional characteristics.

	All patients <i>n</i> = 62	Functioning <i>n</i> = 39	Silent <i>n</i> = 23	<i>P</i> -value ^a
Sex ratio F/M	44/18	31/8	13/10	0.08
Age at surgery (years), median (range)	44 (11–75)	40 (11–73)	50 (22–75)	0.03
Cortisol 08:00 h (nmol/L), median (range)	496 (227–1191)	531 (297–1191)	376 (227–808)	<0.001
	NA = 5	NA = 2	NA = 3	
ACTH 08:00 h (ng/L), median (range)	50 (15–204)	55 (15–204)	45 (16–126)	0.41
	NA = 10	NA = 3	NA = 7	
24-h UFC (nmol/24 h), median (range)	377 (49–8657)	620 (102–8657)	96 (49–207)	<0.001
	NA = 19	NA = 4	NA = 15	
Macro adenomas	35	13	22	<0.001
Size on MRI (mm), median (range)	11.5 (0–70)	7 (0–50)	23.8 (7–70)	<0.001
	NA = 1		NA = 1	
Invasive corticotroph tumor	20	9	11	0.05
SST5 positive	26	24	2	<0.001
IRS categories				
Negative	36	15	21	0.001
Mild	7	7	0	
Moderate	12	10	2	
Strongly positive	3	3	0	
	NA = 4	NA = 4		
ACTH expression (%), median (range)	100 (5–100)	100 (30–100)	93 (5–100)	<0.001
	NA = 1		NA = 1	
Ki67 ≥3%	12	10	2	0.1
	NA = 6	NA = 5	NA = 1	
Mitosis > 2	5	4	1	0.64
	NA = 4	NA = 4		
Positive P53 expression	12	4	8	0.05
	NA = 5	NA = 5		
Grade	1a = 34	1a = 23	1a = 11	0.18
	1b = 4	1b = 3	1b = 1	
	2a = 17	2a = 7	2a = 10	
	2b = 1	2b = 1	2b = 0	
	NA = 6	NA = 5	NA = 1	
USP8 mutations	13	11	2	0.05
	NA = 12	NA = 9	NA = 3	

^aCalculated using Fisher exact test and Wilcoxon rank test. 24-h FC, 24-h urinary free cortisol; NA, data not available.

Silent corticotroph tumors

Out of the 23 silent tumors, only two were SST5 positive, both with a moderate IRS. *USP8* analyses were performed in 20 tumors and two patients had mutations, both of these being Ser719del (Fig. 2 and Supplementary Table 2). Only one patient simultaneously showed positive SST5 expression and a *USP8* mutation. Among these three patients, with either *USP8* mutation or a positive SST5 expression or both, all were women, aged 16 to 75 years old. All tumors were grade 1a macroadenomas, with sizes varying from 11.5 to 21 mm.

Due to the small number of patients that were SST5-positive or showed *USP8* mutations, no comparisons could be made based on SST5 expression or *USP8* mutations.

Functioning corticotroph tumors

SST5 expression

Markers associated with SST5 expression were studied in the 39 functioning corticotroph tumors (Table 2). Among these, 24 of the patients were SST5-positive and 15 were SST5-negative. Positive SST5 expression was more frequent in women (22/24 vs 9/15, P -value=0.04). Although the grade was found to be lower in SST5-positive tumors (P -value=0.04), invasion was not statistically different for all functioning tumors but a difference was found in the subgroup of macroadenomas. Indeed, SST5-positive macroadenomas were less invasive than SST5-negative macroadenomas (4/24 vs 5/15, P =0.03). *USP8* mutations were more frequent in SST5-positive tumors (10/17 vs 1/13, P -value=0.007). The only significant difference between SST5-positive and SST5-negative functioning microadenomas was patient age, as patients with SST5-positive tumors were significantly older

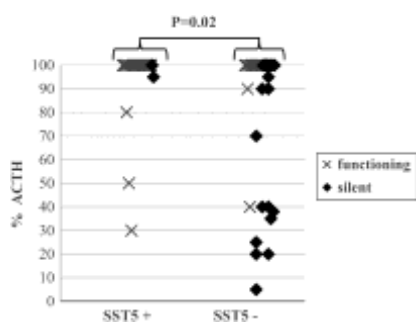


Figure 1 ACTH expression in SST5 positive and negative corticotroph tumors according to the functional status. P -value calculated using Wilcoxon rank test.

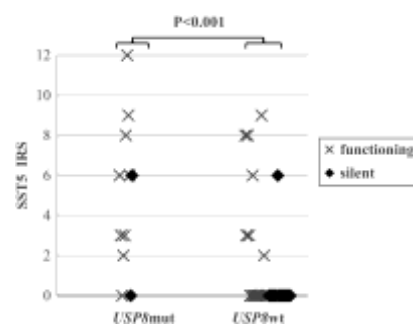


Figure 2 SST5 expression in *USP8*mut or *USP8*wt corticotroph tumors. mut, mutant; wt, wild-type. P -value calculated using Wilcoxon rank test.

(46 vs 29 years, P -value=0.01). Among patients pre-treated by anticortisolic drugs, only one patient pre-treated with ketoconazole had a significant SST5 expression (IRS 6).

USP8 mutations

Among the 20 functioning tumors in which *USP8* sequencing was performed, 11 had *USP8* mutations (Table 3). *USP8*mut corticotroph tumors were more often SST5 positive (10/11 vs 7/19, P -value=0.007) and had a higher IRS (P -value=0.04) compared to *USP8*wt, while no other significant differences were found for other characteristics.

Response to pasireotide treatment

The evolution of 24-h UFC of five patients treated by pasireotide, as well as their IHC results, are presented in Fig. 3. SST5 expression was negative in patient A (IRS 0), while it was positive in four cases with an IRS of 2, 4, 6 and 8 respectively (in patients B, C, D and E). Patients B, D and E were treated due to recurrence of Cushing's disease, occurring between 2 and 6 years after surgery, while patients A and C were treated prior to their pituitary surgery. In all patients, treatment was introduced at a dose of 0.6 mg twice a day. In one patient, the dosage was increased but then rapidly reduced because of digestive side-effects (diarrhea). At the end of treatment, three (A, B and D) out of five patients had a normal 24-h UFC, while a significant change (four-fold decrease) in 24-h UFC was observed in patient C. Clinical improvement of Cushing's symptoms during pasireotide treatment was observed in all cases. Side-effects were frequent with all patients suffering from digestive disorders, while disturbed blood glucose in patients with pre-existing diabetes was observed in four patients and diabetes was newly diagnosed in one patient.

Table 2 Clinical, radiological and pathological features in functioning corticotroph tumors based on SST5 expression ($n = 39$).

	SST5 + <i>n</i> = 24	SST5 – <i>n</i> = 15	P-value ^a
Sex ratio F/M	22/2	9/6	0.04
Age (years), median (range)	43 (22–73)	33 (11–67)	0.09
Cortisol 08:00 h (nmol/L), median (range)	559 (319–1191) NA = 1	513 (297–978) NA = 1	0.25
ACTH 08:00 h (ng/L), median (range)	59 (16–136) NA = 2	46 (15–204) NA = 1	0.49
24-h UFC (nmol/24 h), median (range)	620 (102–8657) NA = 3	479 (145–2300) NA = 1	0.78
Macroadenomas	8	5	1
Size on MRI (mm), median (range)	7 (0–22)	7 (0–50)	0.65
Invasive corticotroph tumor	4	5	0.27
ACTH expression (%), median (range)	100 (30–100)	100 (40–100)	1
Ki67 $\geq 3\%$	6 NA = 2	4 NA = 3	0.71
Mitosis >2	3 NA = 1	1 NA = 3	1
Positive P53 expression	1 NA = 2	3 NA = 3	0.12
Grade	1a = 18 1b = 1 2a = 2 2b = 1 NA = 2	1a = 5 1b = 2 2a = 5 2b = 0 NA = 3	0.04
USP8 mutations	10 NA = 7	1 NA = 2	0.007

^aCalculated using Fisher exact test and Wilcoxon rank test.
24-h UFC, 24-h urinary free cortisol; NA, data not available.

The reasons for the interruption of treatment were: digestive side-effects and no decrease in tumor volume in patient A, poor observance and difficulty of monitoring for patient B, pituitary surgery in patient C, hepatic cytolysis (five-fold increase in transaminases) and the appearance of gallstones in patient D and bilateral adrenalectomy in patient E.

Discussion

In our cohort of 62 patients, more than one-third of corticotroph pituitary tumors were silent tumors, clinically classified as nonfunctioning pituitary tumors but reclassified after pathology analysis as corticotroph tumors. Overall, USP8 mutations were found in 13/50 tumors and SST5 was expressed in 26/62 tumors, results which are in accordance with previous findings (20, 21, 22, 23, 24, 25, 26, 27, 28). Compared to silent tumors, functioning tumors more frequently showed USP8 mutations and positive SST5 expression, with about one-third expressing SST5 receptor with an IRS either moderate or strong. Our results demonstrated an

association between the presence of USP8 mutation and SST5 expression among functioning tumors. Moreover, functioning corticotroph tumors expressing SST5 were more frequent in women and had a significantly lower grade on clinicopathological classification, suggestive of a better prognosis.

The value of SST5 expression studied by IHC in predicting the response to pasireotide is unknown in corticotroph tumors. In somatotroph pituitary tumors, pasireotide has been shown to decrease GH secretion *in vivo* and *in vitro* and to reduce tumor volume (31, 32, 33), with an efficacy which seems to be correlated to SST5 expression (16). In lactotroph pituitary tumors, pasireotide was recently reported to be effective in a few cases of tumors that were resistant to dopamine agonists and expressed SST5, producing biochemical control and either stabilization or reduction in tumor volume (34, 35, 36). One limiting factor in the evaluation of the role of SST5 is the interpretation of its expression using IHC. The intensity of SST5 expression can be evaluated using different scoring systems, which sometimes take into account cytoplasmic-positivity (16, 22, 37). We chose to consider only membrane immunopositivity and,

Table 3 Clinical, radiological and pathological features in functioning corticotroph tumors based on the presence of *USP8* mutations (*n* = 30).

	<i>USP8</i> mut <i>n</i> = 11	<i>USP8</i> wt <i>n</i> = 19	<i>P</i> -value ^a
Sex ratio F/M	10/1	14/5	0.37
Age (years), median (range)	41 (22–73)	37 (11–67)	0.64
Cortisol 08:00 h (nmol/L), median (range)	547 (366–922)	531 (297–1191)	0.76
	NA = 1	NA = 1	
ACTH 08:00 h (ng/L), median (range)	55 (16–119)	54 (15–204)	0.84
	NA = 1	NA = 2	
24-h UFC (nmol/24 h), median (range)	214 (10–8657)	465 (145–2300)	0.26
	NA = 1	NA = 2	
Macroadenomas	5	7	0.71
Size on MRI (mm), median (range)	8 (7–22)	7 (0–50)	0.18
Invasive corticotroph tumor	2	5	1
SST5 positive	10	7	0.007
IRS categories			
Negative	1	12	0.04
Mild	3	3	
Moderate	3	3	
Strongly positive	2	1	
	NA = 2		
ACTH expression (%), median (range)	100 (30–100)	100 (40–100)	0.54
Ki67 ≥3%	3	5	1
		NA = 2	
Mitosis >2	3	1	0.27
		NA = 2	
Positive P53 expression	2	1	0.54
		NA = 2	
Grade	1a = 7	1a = 12	0.09
	1b = 2	1b = 0	
	2a = 1	2a = 5	
	2b = 1	2b = 0	
		NA = 2	

^aCalculated using Fisher exact test and Wilcoxon rank test.

24-h UFC, 24-h urinary free cortisol; NA, data not available; mut, mutant; wt, wild-type.

after comparison of SST5-positive and -negative tumors, to use the IRS scoring system in order to more precisely evaluate SST5 expression. This methodology has been employed in previous studies to assess SST expression in pituitary tumors (21, 38, 39), which allowed a more reliable comparison between studies.

In functioning corticotroph tumors, pasireotide is indicated in order to decrease hypercortisolism. *In vitro* studies on corticotroph tumor cells have found a decrease in ACTH release following use of pasireotide, which seems to be mediated by SST5 (12, 17, 40, 41, 42). Indeed, SST5 is the most highly expressed somatostatin receptor in corticotroph tumors (12, 20, 42), and it has been shown in previous studies that octreotide, which targets SST2, is less effective than pasireotide in decreasing cortisol levels and that SST2 is downregulated in the context of exposure to high glucocorticoid levels (41, 42). The response rate to pasireotide treatment in Cushing’s disease obtained

in previously published clinical trials was between 20 to 40% (13, 14), which is in agreement with the number of patients with functioning corticotroph tumors expressing SST5 with an IRS score of moderate or strong in our cohort, suggesting that only one-third of functioning corticotroph pituitary tumors are likely to be sensitive to pasireotide treatment. In our study, functioning tumors that were SST5-positive had a lower grade and probably a lower risk of recurrence, suggesting that these tumors are less likely to require pasireotide treatment. However, it has been previously reported that *USP8*mut tumors had a higher risk of recurrence (24). The correlation between the expression of SST5 in corticotroph tumors and the efficacy of pasireotide is lacking. Unfortunately, only five patients operated for a corticotroph tumor in Lyon between 2010 and 2018 were treated with pasireotide monotherapy, without combined medical treatment of their hypercortisolism. Four patients expressed SST5,

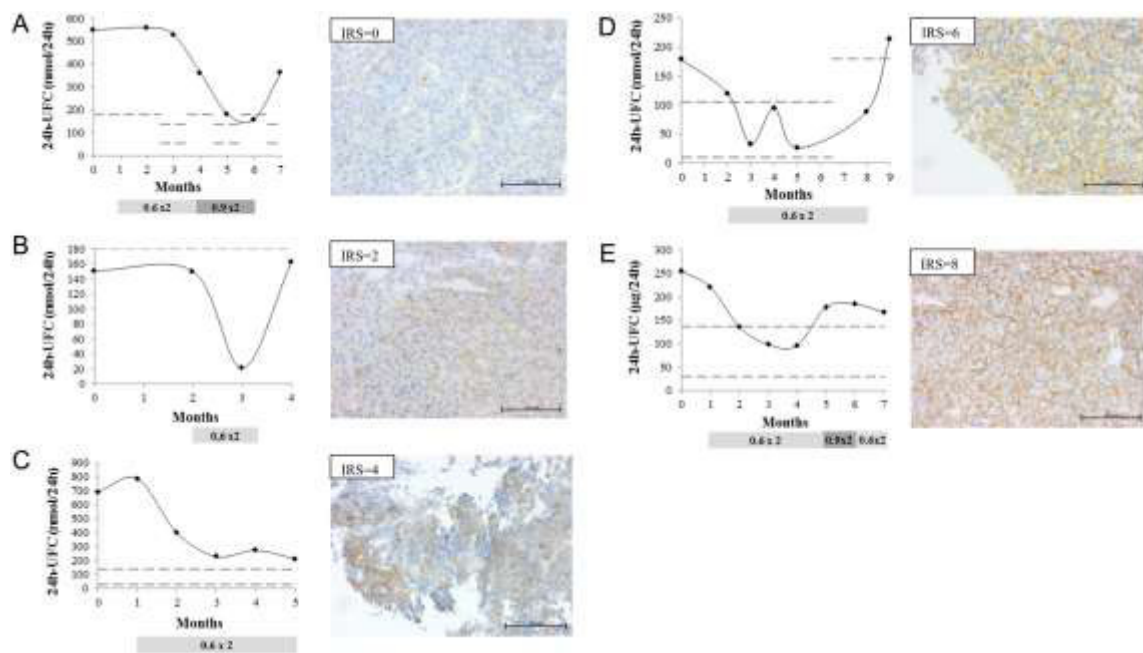


Figure 3 Evolution of 24-h UFC after introduction of pasireotide in patients A to E (left) and SST5 immunohistochemistry (original magnification $\times 200$) (right). 24-h UFC, 24-h urinary free cortisol; IRS, immunoreactive score. Dotted lines on graphs represent 24-h-UFC norm, and daily dosage of pasireotide is represented below each graph, expressed in mg. Photographs were taken in the most immunoreactive area and are not representative of the whole tumor.

one patient showed mild expression and three patients had moderate expression. Though an improvement of hypercortisolism and of Cushing's symptoms was observed in most of the patients, including those that were SST5-negative, the small number of patients and the difference in duration of treatment do not allow us to reach firm conclusions. Pasireotide was used in preoperative treatment, showing a good response in two patients, with an IRS of 0 and 4. It has been reported that preoperative octreotide treatment can result in decreased SST2 expression. SST trafficking is modulated by pasireotide in a different manner than its modulation by octreotide (40, 43). However, as the effect of preoperative treatments on SST5 is not yet well understood, it is not possible to exclude a decrease in SST5 expression following pasireotide treatment.

In previous *in vitro* and *in vivo* studies, pasireotide has also been associated with an anti-tumoral effect (12, 44, 45, 46). A recent publication reporting the use of long-acting pasireotide demonstrated that median tumor volume decreased by 17.8% and 16.3%, respectively, after 12 months of treatment with pasireotide at 10 or 30 mg/month in patients with Cushing's disease (14).

However, the role of SST5 in this anti-tumoral effect is controversial (12, 44). If the anti-tumor effect of pasireotide is partly mediated via SST5, it suggests that silent corticotroph pituitary tumors may not be good candidates for pasireotide treatment, since only 2 out of the 22 patients in our cohort with silent tumors expressed SST5. These results are in agreement with the lower level of SST5 mRNA found in silent corticotroph tumors in comparison to functioning corticotroph tumors that was reported in a previous study (19). On the contrary, SST5 expression was present in 8 of the 13 patients with a functioning macro-corticotroph tumor in our cohort, suggesting that those tumors are more likely to respond to pasireotide. This result is interesting, since macroadenomas associated with Cushing's disease present both a lower rate of remission as well as a higher rate of recurrence (47, 48).

In published studies consisting of 11 and 20 patients with silent corticotroph tumors, somatic mutations of *USP8* were found in 0% to 20% of those tumors (25, 49). In contrast, *USP8* mutations were found in 24 to 62% of functioning corticotroph tumors (21, 23, 24, 25, 26, 27, 28), with a higher level of SST5 expression in

*USP8*mut tumors (21), which is consistent with the results of our study. However, unlike previous findings, mutations in our cohort were not statistically associated with female gender (21, 23, 25, 50) and no differences were found for the age at diagnosis or the tumor size (21, 24, 25). The *USP8* gene encodes a protein with a deubiquitinase activity, which inhibits the degradation of the EGF receptor (EGFR). The activity of *USP8* is regulated by the 14-3-3 protein, and all known mutations are found in exon 14, within or close to the 14-3-3 protein binding motif (715–720). Among the mutations found in our cohort, five were previously known mutations (Ser719del, Pro720Arg, Ser718Cys, Ser718Pro and Pro720-Gln724del) (21, 25, 51, 52, 53). Novel mutations were found in two patients, one adjacent to the binding motif (Asp721Glu) in one patient and two mutations in the other patient (Ser719_Gln724delinsLeu and Asn741Asp). The presence of double *USP8* mutations in the same patient has already been reported (28, 54). In our case, apart from the mutation affecting the 14-3-3 protein binding motif (Ser719_Gln724delinsLeu), the functional impact of the other mutation is difficult to assess. Previous studies have found a greater likelihood of surgical remission (23) as well as a higher risk of recurrence of Cushing's disease (24) in *USP8*mut tumors, while in our study no difference was found between functioning tumors with or without mutations in terms of tumor grade. IHC for *SSTR5* is not routinely performed during histopathological analysis of pituitary tumors. The presence of *USP8* mutations, frequently associated with positive *SSTR5* expression, could be used as a marker to predict *SSTR5* expression and then response to pasireotide treatment. However, such molecular analyses are more costly to perform than immunohistochemistry.

This study has several limits, such as its retrospective nature and the presence of missing data. The number of patients studied, which may seem small, can be explained by the rarity of corticotroph pituitary tumor. Response to pasireotide could be studied only on a small number of patients.

In conclusion, *SSTR5* expression and *USP8* mutations are more frequently found in functioning corticotroph tumors. We did not identify clinical predictive marker of *SSTR5* expression, except for the association with functioning tumors and patient gender. The value of *SSTR5* expression as a theranostic marker of response to pasireotide by corticotroph tumors should be the subject of future studies on a larger prospective cohort and may represent a new avenue for personalized therapy.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EC-20-0035>.

Declaration of interest

Gérald Raverot received investigator-initiated research grants from Novartis and Ipsen, speaker's fees from Ipsen Pharma, Novartis Pharma, Pfizer Inc. and consulting fees from Novartis. Alexandre Vasiljevic received research grants from Novartis. Emmanuel Jouanneau received speaker's fees from Novartis Pharma and Baxter. Françoise Borson-Chazot received speaker's fees from Novartis Pharma, Novo Nordisk and consulting fees from Novartis, Ipsen, Pfizer Inc, Novo Nordisk and ESAI. Hélène Lasolle received speaker's fees from Novartis Pharma. The other authors have nothing to disclose.

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