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Effects of siRNA-squalene nanoparticles on RET/PTCs junction oncogenes in papillary thyroid carcinoma : from molecular and cellular studies to preclinical investigations

Hafiz Muhammad Ali

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UNIVERSITE PARIS SUD-11
Faculté de Médecine

École Doctorale : Signalisation et Réseaux intégratifs en Biologie
Discipline : Vectorologie et thérapeutiques anti-cancéreuses

THÈSE DE SCIENCES
pour l'obtention du grade de
Docteur de l'Université Paris-Sud XI

**Effets des nanoparticules de siRNA-Squalène sur les
oncogènes de jonction RET/PTCs dans le carcinome
papillaire de la thyroïde :**
études moléculaires, cellulaires et investigations précliniques

Présentée et soutenue par

Hafiz Muhammad ALI

le 22 / 04 / 2014

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UNIVERSITE PARIS SOUTH-11
Faculty of Medicine

Doctoral School : Signaling and integrative networks in biology
Discipline : Vectorology and anti-cancer therapeutics

THESIS OF SCIENCE

submitted to obtain the degree of
Doctor of the University of Paris-South XI

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ABBREVIATIONS

ANTS	1-aminonaphthalene-3, 6, 8-trisulfonic acid
ARA70	Androgen receptor-associated protein 70
AS-ONs	Antisense oligonucleotides
ATA	American Thyroid Association
ATC	Anaplastic (undifferentiated) thyroid carcinoma
BID	Twice daily
C	Control
CBR	Clinical beneficial response
CCDC6	Coiled-coil domain-containing gene 6
CEACAM1	Carcino-embryonic antigen-related cell adhesion molecule-1
Chol	Cholesterol
CLSM	Confocal laser scan microscopic
CPTC	Conventional PTC
CR	Partial response
Ct	Cycle threshold
CTCA	Cancer Treatment Centers of America
DBVs	Designer biomimetic vectors
DMEM	Dulbecco's modified eagle medium
DSBs	Double-strand breaks
DTC	Differentiated TC
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
eIF2 α	Eukaryotic initiation factor 2 α
ERP	Estrogen receptor related protein
FAM	6-CarboxyFluorescein-Aminohexyl Amidite
FCS	Fetal calf serum
FDA	Food and Drug Administration of USA
FISH	Fuorescence in situ hybridization
FNAC	Fine-needle aspiration cytology
FoxP3	Forkhead box P3
FRET-SE	Fluorescence Resonance Energy Transfer-Sensitized Emission
FVPTC	Follicular variant PTC
GALA	Glutamic-Alanine-Leucine-Glutamic acids
GDNF	Glial cell line derived neurotrophic factor
GFR	Growth factor receptor
GnRH	Gonadotrophin-releasing hormone
GPI	Glycosyl-phosphatidyl-inositol
GR	Glucocorticoid-R

HBME-1	Human mesothelial cell marker 1
I ¹³¹	Radioactive iodine 131
IgG	Immunoglobulin G
IκB-α	Nuclear factor-κB
LDL	Low-density lipoprotein
LIMD2	LIM kinase domain containing 2
LM-PCR	Ligation-mediated PCR
LTB	Lymphotoxin beta
MAGE	Melanoma-associated antigen
MAPK	Mitogen-activated protein kinase
MEN	Multiple endocrine neoplasia
MKIs	Multi-kinase inhibitors
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MRT	Magnetic resonance tomography
MTC	Medullary thyroid carcinoma
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NBCS	New born calf serum
NcoA4	Nuclear receptor coactivator-4
NGF	Nervous (central and peripheral) growth factor
NHEJ	Non-homologous end joining
NPs	Nanoparticles
NTRK-1	Neurotrophic receptor-tyrosine kinase-1
ONs	Oligonucleotide
OSE	Ovarian surface epithelial
Pax-8	Paired box-containing gene-8
PBS	Phosphate Buffered Saline
PCCL3	Parental well differentiated rat thyroid cell line cells
pCND	Prophylactic central compartment neck dissection
PD	Progressive disease
PDGFR	Platelet derived growth factor receptor
pDNA	Plasmid DNA
PET	Positron emission tomography scans
PFS	Progression free survival
PI3K-mTOR	Phosphatidylinositol-3 Kinase-mammalian target of rapamycin
PKR	Protein kinase R
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-phosphatidylglycerol
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PR	Partial response

PTC	Papillary thyroid carcinoma
PTMC	Papillary thyroid micro-carcinoma
PTPRC	Protein tyrosine phosphatase receptor type Concentration
PTPRJ	Protein Tyrosine Phosphates Receptor type-J
QD	Once daily
R8-MEND	Octa-arginine peptide-multifunctional envelope-type nano device
RAI	Radioactive iodine
RES	Reticulo-endothelial system
RET	REarranged during Transfection
RFG	Ret fused gene
RISC	RNA induced silencing complex
RNAi	RNA interference
RPL13A	Ribosomal Protein 13A
RT-qPCR	Real Time quantitative Reverse Transcription Polymerase Chain Reaction
SD	Stable disease
SDS	Sodium dodecyl sulphate
shRNAs	Short hairpin RNAs
siRNA	small interfering RNA
SQ	Squalene
T3	Tri-iodothyronine
T4	Tetra-iodothyronine
TCPTC	Tall-cell PTC
TEM	Transmission electron microscopy
Tf-FITC L	Transferrins-fluorescein isothiocyanate liposomes
TG	Thyroglobulin
TH	Thyroid hormone
TKR	Tyrosine kinase receptor
TLR3	Endosomal toll-like receptor-3
TPO	Thyroid peroxidase
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
TSH-R	TSH receptor
TTF-1	Thyroid transcription factor-1
u-PA	Urokinase-type plasminogen activator
VEGFR	Vascular endothelial growth factor receptor
WB	Western blot

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AIM OF THE THESIS

Thyroid cancer is the most common endocrine malignancy and accounts for up to 95% of cancers involving the endocrine system. The rate of thyroid cancer is steadily increasing since the last few decades in most of the developed countries. Among the various types of thyroid disorders, papillary thyroid carcinoma (PTC) is the most commonly found type of thyroid cancer which is present in about 80–90% of thyroid disease cases. PTC is generally developed by gene rearrangements where the REarranged during Transfection (RET) gene is the key player which fuses with 12 other genes under the exposure of ionizing radiations, leading to 13 different onco-fusions (Romei and Elisei 2012). The most common variants are RET/PTC1 and RET/PTC3 formed by the fusion of RET with H4/CCDC6 or ELE1/NcoA4 genes respectively. RET/PTC1 is present in about 60-70% cases of PTC in adults while RET/PTC3 is present in 20-25% but mostly found in young patients with metastatic disease, poor prognosis and where efficient therapies are still missing.

PTC is a differentiated cancer due to retained ability to take up iodine, to secrete thyroglobulin (TG) and be responsive to TSH (Nikiforov and Nikiforova 2011). Thus the prognosis is usually good and about 80% of patients are cured by classical treatment (total thyroidectomy and radio-iodine therapy) but in 20-30% cases, the disease relapse and almost half of them have an aggressive course with distant metastasis. Therefore, for these patients, it is a great need to introduce a new therapy different from the conventional treatment plan. Seeking an efficient alternative treatment, the inhibition of fusion oncogene expressions by small interfering RNA (siRNA) has appeared a great alternative treatment since RET/PTCs fusions are only present in the tumour cells excluding the normal thyroid tissue.

Firstly, a pharmacological approach targeting RET/PTC1 oncogene was conceived in the laboratory, using specifically designed siRNA against this particular rearrangement. The designed siRNA RET/PTC1 was tested *in vitro* in human BHP 10-3 and TPC-1 cell lines harbouring RET/PTC1 fusion oncogene and was found efficient in silencing the gene and protein expressions (Gilbert-Sirieix *et al.* Thyroid, 20(10):1053-65, 2010). But *in vivo* delivery of siRNA is a key challenge and the biological efficacy of the siRNAs is hampered by their short plasmatic half-life due to poor stability in biological fluids and by low intracellular penetration due to highly hydrophilic character. Thus, in order to protect the siRNA from degradation, to improve intracellular penetration and to contribute in hydrophobic character, the siRNA RET/PTC1 was vectorized by “squalenylation” method (For review: Raouane *et al.* 2012, Ali *et al.* 2012). This new non-cationic nano-carrier

squalenoylation system has recently been introduced (Couvreur *et al.* 2006; Couvreur *et al.* 2008) to prepare nanoparticles (NPs) by using a neutral lipid; squalene (SQ) that is biodegradable nano-vector and also considerably used in various pharmaceutical preparations as an excipient. The nanoassemblies of SQ with siRNA RET/PTC1 were prepared and then tested for targeted RET/PTC1 silencing. The NPs were found to inhibit tumour growth and RET/PTC1 gene and protein expressions *in vivo* but interestingly, found to be inefficient *in vitro* (Raouane *et al.* J Med Chem., 54(12):4067-76, 2011).

With the aim to further ameliorate the results, a fusogenic peptide GALA-Cholesterol (GALA-Chol) was added to the siRNA RET/PTC1-SQ NPs. The hypothesis was that GALA-Chol might interact with the cell membrane in the target tissue and would lead to enhanced trans-membrane and intra-cellular penetration of siRNA-SQ nanoparticles up to their destination. In the first part of my PhD project, the nanoparticles of siRNA RET/PTC1-SQ GALA-Chol NPs were tested for their efficiency *in vitro* and *in vivo*. The nanoparticles with GALA-Chol were found to be efficient *in vitro* but not *in vivo* while the inverse results were found for siRNA RET/PTC1-SQ NPs. The nanoparticles without GALA-Chol (siRNA RET/PTC1-SQ NPs) were considered as an efficient therapy for RET/PTC1 fusion oncogene and thus advocated to be used for further pharmacological studies intended for *in vivo* applications (Research Article: Ali *et al.* Thyroid, 2014 24(2): 327-338).

Secondly, we extended this study to RET/PTC3; this junction oncogene was found highly prevalent in young patients after Chernobyl irradiation explosion. In this part of study, we established a novel cell line stably expressing RET/PTC3 and then knockdown of junction oncogene was done by siRNA. When coupled with SQ, the nanoparticles of siRNA RET/PTC3-SQ were found to be efficient and specific of RET/PTC3 junction oncogene *in vivo*. Thus, siRNA RET/PTC3-SQ NPs were accredited as a therapeutic application against RET/PTC3 junction oncogene (Research Article: Ali *et al.* PlosONE, 2014 accepted).

In conclusion, the results obtained during my PhD project highly support the use of siRNAs RET/PTCs-SQ NPs as a new promising treatment for patients affected by PTC expressing RET/PTC1 and RET/PTC3.

1.1. Thyroid gland

The thyroid gland or simply, the thyroid is one of the largest endocrine glands in vertebrate anatomy and is a key player in the endocrine system. The thyroid gets its name from the Greek adjective for “shield shaped” due to shape of the related thyroid cartilage.

1.1.1. Anatomical location and structure of the thyroid gland

The thyroid gland is a small bilobed organ located in front of trachea opposite to 5th to 7th cervical and 1st thoracic vertebrae in the neck region, just below the thyroid cartilage. Here it forms the laryngeal prominence or Adam's apple, immediately adjacent to the larynx and trachea (Figure 1A). It has two lobes, present on either side of trachea and connected with a small bit of tissue called isthmus. Together, this creates a shape, vaguely resembles a butterfly (Arora *et al.* 2010, Tahir and Mughal 2012).

The gland is covered in a tough fibrous capsule which protects it from the surrounding organs and tissues. The gland weighs approximately 20-60g in a fully grown adult and can vary in size greatly from person to person. The thyroid gland receives its blood supply from the superior thyroid artery, a branch of the external carotid artery. Blood drainage occurs *via* the superior or inferior thyroid vein by which thyroid pour its secretion in blood; thus called an endocrine gland (Arora *et al.* 2010).

1.1.2. Histological structure of the thyroid gland

The microscopic structure of the thyroid is quite distinctive. The gland is divided by capsular septa into lobules containing thyroid follicles which are lined by thyroid epithelial or follicular cells (Figure 1B). When gland is not producing the secretion, the epithelial cells range from cuboidal to low columnar in shape but when active, the cells become tall columnar. These cells are responsible for the synthesis of thyroid secretion/hormones. Follicles have empty space or lumen, filled with thyroid secretion called colloid; a proteinaceous depot of materials for thyroid hormone (TH) production mostly iodinated thyroglobulin and thus also a reservoir of TH. Colloid is rich in a protein called thyroglobulin. In addition to thyroid epithelial cells, the gland houses another important endocrine cell type nested in spaces among thyroid follicles and is called as parafollicular C cells which secrete the hormone, calcitonin (Arora *et al.* 2010, Tahir and Mughal 2012, Lokanadham and SubhadraDevi 2013).

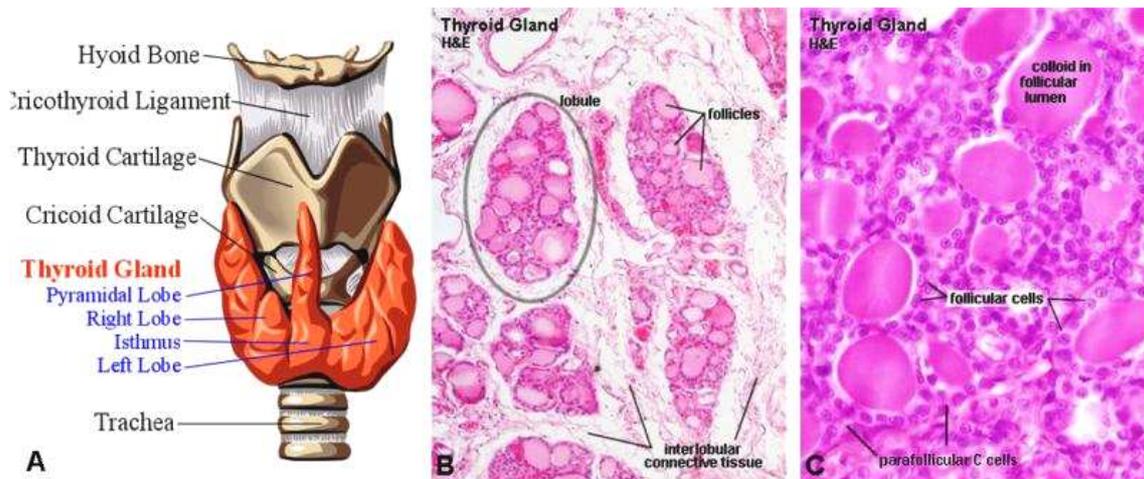


Figure 1: Anatomical and histological structure of thyroid gland. A. Thyroid gland is a small bilobed organ, located in front of trachea just below the thyroid cartilage. B. Histologically, it is divided by interlobular connective tissue into lobules that contain thyroid follicles. C. Follicles are lined by cuboidal thyroid epithelial or follicular cells and filled with colloid. Among the thyroid follicles, parafollicular C cells are dispersed. Ref: A. <http://www.fpnotebook.com/endo/exam/thyrdantmy.htm>
B & C. <http://www.lab.anhb.uwa.edu.au/mb140/corepages/endocrines/endocrin.htm>

1.1.3. Function of the thyroid gland

The gland produces three hormones; tri-iodothyronine (T₃), thyroxine (tetra-iodothyronine; T₄) and calcitonin; vital for the metabolic function of the body (Figure 2). T₄ is relatively inactive and is converted to T₃ in many peripheral organs such as liver, kidney and spleen (Jansen *et al.* 2005). But both are extremely important in the regulation of development, functioning of many body systems and subsequent metabolism and homeostasis of a person. Iodine and tyrosine are required for proper functioning and hormone production by thyroid gland. About twenty five percent of the body's iodide ions are present in the thyroid gland. Dietary iodine deprivation will lead to non-pathological swelling of the “pirate gland” while pathological enlargement is referred as goiter. Calcitonin plays a role in calcium homeostasis, reduces blood calcium levels and inhibits reabsorption of calcium and phosphorus in the renal collecting tubules (Bowen 2006).

Thyroid differentiation and functioning is characterized by the expression of a variety of marker proteins, specifically synthesized by thyroid follicular cells. These include NIS (sodium/iodide symporter; a trans-membrane glycoprotein), TG (thyroglobulin), TPO (thyroid peroxidase) and TSH receptor (De Vita *et al.* 1998, Fenton *et al.* 2001). The expression of these genes depends on their promoters, activated by the binding of thyroid-specific transcription factors like TTF-1 (Thyroid transcription factor-1) and Pax-8 (Paired box-containing gene-8). TG and TPO promoters have the binding sites for both TTF-1 and Pax-8 (De Vita *et al.* 1998), thus these two factors are responsible for activation of these promoters. Thus, by active transport, NIS (sodium/iodide symporter; a trans-membrane glycoprotein)

transports circulating iodine into the follicular cytosol where in the apical plasma membrane, TPO enzyme oxidizes it and iodinates tyrosine residues of the thyroglobulin molecules in lysosomes to produce T₃ and T₄ (T₃ have one iodine atom less than T₄). Thus TH secreted from the gland comprised is about 80-90% T₄ and 10-20% T₃. Required T₃ is produced in various extrathyroidal tissues via 5' deiodination under the effect of types 1 and 2 iodothyronine deiodinases (D1 and D2). The plasma concentrations of free T₄ and T₃ remain constant while T₃ concentration in different tissues varies according the amounts of hormone transported and the activity of the tissue deiodinases (Friesema *et al.* 2005; Jansen *et al.* 2005; Sesmilo *et al.* 2011). The production and release of hormones is regulated by thyroid stimulating hormone (TSH) released from anterior pituitary gland which itself is regulated by thyrotropin releasing hormone (TRH) produced by hypothalamus. Their circulatory level is controlled by negative feedback mechanism within the hypothalamic-thalamic-thyroid axis at both hypothalamus and pituitary levels for suppressing the production of TSH. Calcitonin controls the calcium level in the body circulation by transferring excessive calcium to bones. In case of need, calcium is transferred back to blood stream under the effect of parathormone; a hormone released by para-thyroid gland, present on posterior side of each lobe of thyroid (Porter and Kaplan 2013).

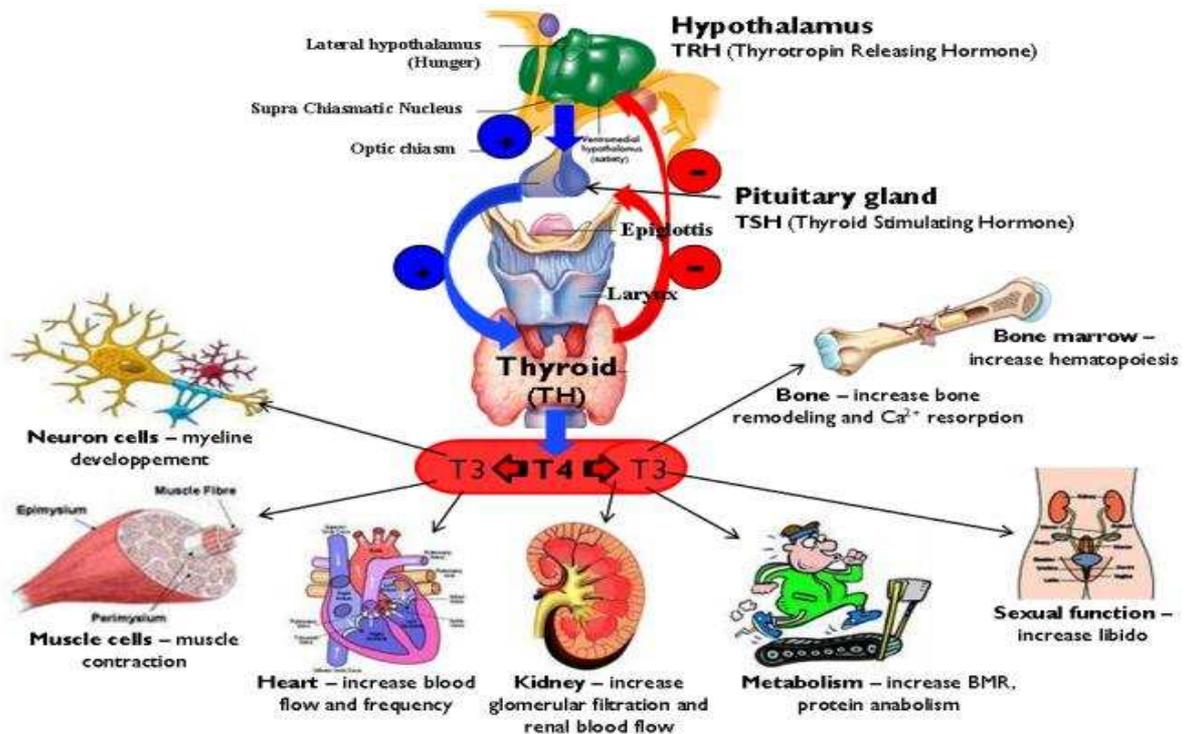


Figure 2 : Hypothalomo-Pituitary-Thyroid axis. Hypothalamus release thyrotropin releasing hormone (TRH) which stimulates the secretion of thyroid stimulating hormone (TSH) from pituitary gland. Under the effect of TSH, thyroid gland releases T₃ and T₄. In the peripheral organs T₄ transforms into T₃; which is responsible for regulation of various physiological and metabolic activities in the body. T₃ level in the circulation is controlled by negative feedback mechansim at both hypothalamus and pituitary levels, for suppressing the production of TSH.

1.2. Thyroid cancer

These include abnormally increased (hyperthyroidism) or decreased (hypothyroidism) thyroid activity and formation of thyroid nodules; generally benign thyroid neoplasms but may be thyroid cancers. All these disorders may give rise to goiter or enlarged thyroid. Hyperthyroidism or overactive thyroid results in overproduction of the two thyroid hormones and is most commonly caused by the development of Graves' disease; an autoimmune disease wherein produced immunoglobulin G (IgG) antibodies binds to the thyrotropin receptor (TSH receptor) on the follicular cells and stimulates cells to secrete excessive quantities of thyroid hormones. This results in very high level of circulating hormones while negative feedback regulation keeps TSH level low. Thus thyroid dysfunction leads to various disorders including thyroid carcinoma, Graves' disease and Hashimoto's thyroiditis (an autoimmune disorder wherein body's own immune system reacts against the thyroid tissue) (Lokanadham and SubhadraDevi 2013).

1.2.1. Oncogene and oncogenesis

An oncogene is normally silent but can be activated by chromosomal translocations, deletions or mutations and have ability to "transform" normal cells into a condition of uncontrolled replication and growth. Most oncogenes could be closely related to normal growth factors, cell division maintenance or to hormone receptors. If turned on, promote uncontrolled cell growth and cell divisions and depress differentiation. Typically, activation of a single oncogene may not be enough to produce malignancy except accompanied by expression of another oncogene or other genes regulated by that oncogene. Similarly, if gene mutation or duplication occurs, the cell may also progress towards a malignant structural and functional potential. Information on expression of oncogenes in human thyroid tissue is rapidly accumulating and more than 30 oncogenes have been recognized so far in the human genome (Pacini and De Groot 2013).

1.2.2. Incidence and distribution of thyroid cancer

Thyroid cancer is the most common endocrine malignancy and accounts for up to 95% of cancers involving the endocrine system (Keefe *et al.* 2010). Its rate has been steadily increasing over the last few decades in most of the developed countries (Chen *et al.* 2009; Enewold *et al.* 2009; Kilfoy *et al.* 2009; Pellegriti *et al.* 2013), Figure 3A. The annual incidence of thyroid cancer varies considerably among different registries; ranging from 1.2-2.6 in men to 2.0-3.8 in women per 100,000 individuals (Nagataki and Nystrom 2002;

Venencia *et al.* 2002; Zaman *et al.* 2006). Amongst, it is particularly elevated and about two times higher in Iceland and Hawaii than in North European countries, Canada and USA (Pacini and De Groot 2013). American Cancer Society reported a rapid rise in cases of thyroid cancer from 37,200 in 2009 to about 60,220 in 2013 in USA wherein three patients are women out of four, with a mortality rate of about 1.3 times higher than men *i.e.* 1,040 in females vs 810 in males, estimated deaths are based on US mortality data during 1995-2009 by National Center for Health Statistics, Center for Disease Control and Prevention (Siegel *et al.* 2013). From 2005 to 2009, incidence rates have increased by 5.6% per year in men and 7.0% per year in women (American Cancer Society, 2013). In United States, 56,460 and 60,220 estimated new cases of thyroid cancer have been reported in 2012 (Siegel *et al.* 2012) and 2013 (Siegel *et al.* 2013). Thus its level rose from 6th (Enewold *et al.* 2009) to 5th after breast, lung, colorectal and uterus among most common cancers in women (Siegel *et al.* 2013, American Cancer Society, 2013). The variation in the incidence rate and cancer distribution among geographical regions is not only due to racial and cultural habitants and environmental factors like spontaneous background radiation or dietary habits but also different standards of medical proficiencies, health care levels and cancer detection methods play their role (Zhu *et al.* 2006; Pacini and De Groot 2013).

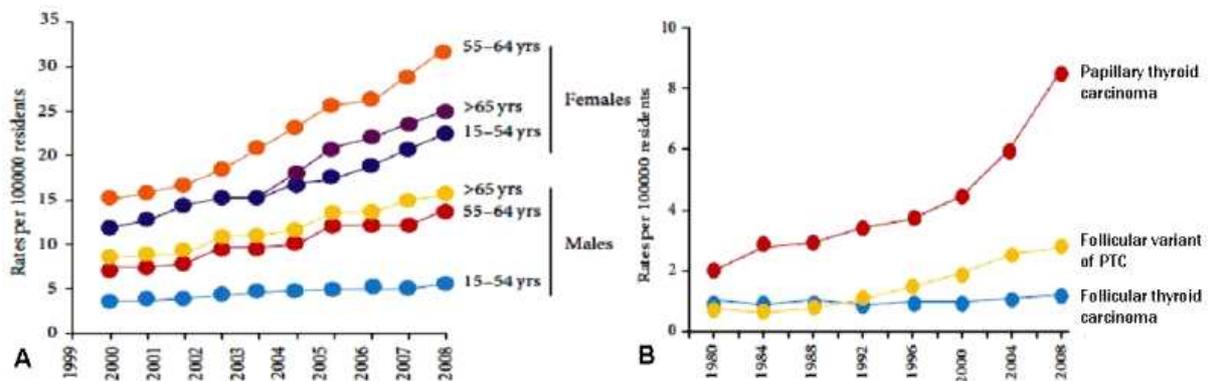


Figure 3: Trend in the incidence of thyroid cancer. A. Trend of the thyroid cancer in North American population from 1999 to 2008 sub-divided in to different age groups by gender. The reported rate is always found higher in women of all age groups than men. B. Trend in the thyroid cancer by histo-type in the United States from 1980 to 2009. The increasing incidence of thyroid cancer is entirely due to increase in incidence of papillary histo-type without much contribution by other types of thyroid carcinoma. Ref: Pellegriti *et al.*, 2013

1.2.3. Forms and types of thyroid cancer

The most frequently occurring forms of thyroid cancer originate from two distinct endocrine cell types: thyroid hormone producing follicular epithelial cells and calcitonin producing parafollicular C-cells. The forms of thyroid carcinoma (TC) derived from follicular epithelial cells include differentiated TC (DTC) [including papillary (PTC; about 70-80%)

and follicular (FTC; 10-20%) TCs] and undifferentiated or anaplastic thyroid carcinoma (ATC; 2-5%). While PTC subtypes include conventional (CPTC), follicular variant (FVPTC), tall-cell (TCPTC) and few other rare variants like columnar, diffuse sclerosing, lymphoma (thyroid lymphocytes) and thyroid sarcoma etc. Hürthle cell thyroid cancer (parathyroid oxyphil cell carcinoma) is a unique subtype of FTC that accounts for 2–3% of thyroid cancer. The thyroid tumour derived from parafollicular C-cells is termed as medullary thyroid carcinoma (MTC) and is only about 3-10% of thyroid cancer (Benvenega 2008; Zhu and Cheng 2009; Chougnet *et al.* 2010; Keefe *et al.* 2010).

Despite their common origin, these cancers have separate biological conformation and also arise through distinct tumor initiation and progression pathways. Papillary type could be differentiated from follicular type in having papillary architecture and characteristic abundant granular cytoplasm with nuclear features including enlargement, oval shape, elongation, overlapping and inclusions (Xing 2013). It has the propensity for lymphatic metastasis. FTC is characterized by hypercellular, microfollicular patterns, lacking nuclear features of PTC with large, mitochondria rich oncocytic cells having dense nuclei and nucleoli. Undifferentiated thyroid carcinoma is characterized by a mixture of spindle, pleomorphic giant and epithelioid cells. DTC have high tendency to metastasize *via* blood stream locally to the cervical lymph nodes (Machens *et al.* 2002; Vasko *et al.* 2005; Costa *et al.* 2009; Pasquali *et al.* 2011; Joo *et al.* 2012; Xing 2013; Xing *et al.* 2013) and in the neck region (Roh 2006) or distally to liver and lungs (Lin *et al.* 2004; Wells *et al.* 2010), unlike FTC which spreads also to bone (Lin *et al.* 1999; Basolo *et al.* 2000). But there are also reports of PTC metastasis rarely to bone (Mishra *et al.* 2002; Kusunoki *et al.* 2003; Batawil 2013), skin (Koller *et al.* 1998; Ronga *et al.* 2007; Luo *et al.* 2008), brain (Imamura *et al.* 2000; Pazaitou-Panayiotou *et al.* 2005; Lecumberri *et al.* 2010), spinal cord (Jeon *et al.* 2011), adrenal glands (Koutkia and Safer 2001; Wagenaar *et al.* 2008; Batawil 2013), pituitary gland (Masiukiewicz *et al.* 1999; Bell *et al.* 2001), sub-mandibular gland (Sarda *et al.* 2004), pancreas (Jobran *et al.* 2000; Siddiqui *et al.* 2006; Borschitz *et al.* 2010), kidneys (Gamboa-Dominguez and Tenorio-Villalvazo 1999), ovaries (Logani *et al.* 2001; Brogioni *et al.* 2007; Xu *et al.* 2011), sphenoidal sinus (Argibay Vazquez *et al.* 2005) and skeletal muscles (Pucci *et al.* 2006; Panoussopoulos *et al.* 2007; Bae *et al.* 2011).

1.2.4. Diagnosis of thyroid cancer

There are no recommended screening tests for the early detection of thyroid cancer (without symptoms) but since symptoms usually develop early, most thyroid cancers are

diagnosed at an initial stage. If it remains asymptomatic during an extended period of time then becomes the usual reason of delayed diagnosis. Screening tests include blood tests to determine levels of thyroid hormones, medical imaging to determine the size and characteristics of the nodule and nearby lymph nodes and biopsy to determine the nature of nodular cells whether are benign or malignant.

As soon as detected or the symptoms start to appear, is revealed as a mass in the neck region which might have local signs including a tight or full feeling in the neck, difficulty in breathing or swallowing, swollen lymph nodes and pain in the throat or neck region. Other clinical signs include fixation to the trachea, damage to recurrent laryngeal or cervical sympathetic nerves. PTC tends to extend very slowly and usually develop in only one lobe of thyroid gland but even often metastase locally to the lymph nodes in the neck region or distally to other parts of the body (Wells *et al.* 2010).

During a physical examination, tumour is palpated as a painful nodule alongwith enlarged cervical lymph nodes. The case is thus reffered to an endocrinologist or a thyroidologist. An ultrasound examination is thus performed to confirm the presence of the nodule, to assess the status of tumour, to distinguish from cystic lesions and to identify calcifications. The ultrasound is also very effective to discover microcarcinomas upto less than 1cm size. Other tests used for the evaluation of thyroid nodules are medical imaging techniques; to determine the size and characteristics of the nodule and of nearby lymph nodes, blood tests; to determine levels of thyroid hormones and biopsy; to determine if the cells in the nodule are benign or malignant. Magnetic resonance imaging (MRI) or tomography (MRT) of the neck region and anti-thyroid antibodies could be done; to differentiate and decide the presence of a functional thyroid disease such as Hashimoto's thyroiditis; a known benign nodular goiter. Calcitonin measurement is equally performed to exclude the presence of MTC. Finally, to arrive at definitive diagnostic conclusion, fine-needle aspiration cytology (FNAC) is performed.

Supplementary diagnostic tools include the use of thallium²⁰¹ chloride, Technetium (^{99m}Tc)-methoxy-iso-butyl-isonitrile or Tc-MIBI, Positron emission tomography (PET) scans (helps to identify metastatic TC), gallium⁶⁷ imaging (helpful to visualize lymphomas) and I-meta-iodo-benzyl-guanidine (MIBG) imaging (useful to detect MTC). TSH-R mRNA measurement after FNA enhances pre- and post-operative detection of cancer to reduce unnecessary surgery and to predict residual/metastatic disease. Cytology on bronchoalveolar

lavage specimen helps to explore any lung metastases. Thus, an appropriate combination of one or more of the cited techniques helps to arrive at a decision about the specific type of thyroid disease.

1.2.5. Treatment of thyroid cancer

Treatment of thyroid cancer depends on the type of cell, size of the tumour and the extent of disease development and spread.

1.2.5.1. Classical treatment of thyroid cancer

The first choice of treatment includes the partial or total thyroidectomy (surgical resection of thyroid having tumour) in nearly all cases. Along with total thyroidectomy, prophylactic central compartment neck dissection (pCND; surgical removal of related lymph nodes) is recommended by American Thyroid Association (ATA) in PTC (Cooper *et al.* 2009) and advocated by others to avoid future loco-regional recurrence (Machens *et al.* 2009; Popadich *et al.* 2011; Cisco *et al.* 2012). Post-surgical radioactive iodine (I^{131}) therapy treatment is also recommended for more advanced disease to destroy any remains of cancerous tissue (Sipos and Mazzaferri 2008). Supplementary hormone therapy with L-thyroxine is then given life long to replace hormones produced by the thyroid gland and to prevent the pituitary from producing excessive TSH and thus to decrease the likelihood of recurrence (American Cancer Society 2013, American Thyroid Association 2013). In case of metastases, patients are prescribed to receive anti-neoplastic therapy that inhibits metastatic growth and cell proliferation and helps in palliating symptoms in progressive disease.

Chemotherapy based on cisplatin or doxorubicin could be supplemented but have proven limited efficacy, however, could be helpful in patients with bone metastasis. Doxorubicin is the most commonly used agent in ATCs with a response rate of approximately 20% and it is the only available treatment that is approved by the U.S. Food and Drug Administration (FDA) for patients of DTC, however is accompanied by severe myelosuppression and cumulative cardio-toxicity (Sun *et al.* 2013). Paclitaxel although have tumour suppressor effects and in a clinical trial of ATC, found to be well tolerated but not showed very promising results (Pushkarev *et al.* 2013; Sosa *et al.* 2013). Other chemotherapeutic agents like bleomycin, etoposide, cisplatin and methotrexate have poor responses and offer no improved survival ratios. Similarly, vincristine and melphalan also show diminutive therapeutic benefits.

1.2.5.2. Demerits and disadvantages of classical treatment

Despite excellent prognosis, about 40% patients experience recurrence within a 40-years period where two-thirds are within the first decade, even after a successful initial classical therapy (Mazzaferrri and Massoll 2002; Tucker 2013). Cervical and ipsilateral lateral lymph node metastases are frequent (Machens *et al.* 2002; Costa *et al.* 2009) with a 40-90% recurrence rate (Grodski *et al.* 2007; Moo *et al.* 2010). Side effects could also not be avoided and sometimes need special attention for their remedy and treatment. These undesirable secondary effects include hypoparathyroidism (due to removal of parathyroid glands), recurrent laryngeal nerve paralysis and vocal cord damage related with temporary or permanent hoarseness or loss of voice, transient or permanent hypocalcemia, seroma, scarring, hematoma (can be life-threatening if it compresses the trachea) and chyle leak are the other possible outcomes of surgical interventions (Kouvaraki *et al.* 2004; Uruno *et al.* 2004; Shindo and Stern 2010; Ahn *et al.* 2013).

Need of post-operative radioiodine therapy, hypocalcemia and overall morbidity are the usual outcomes of pCND (Ahn *et al.* 2013, Kim *et al.* 2004, Lang *et al.* 2013). Radiation therapy rather leads to a high incidence of hypothyroidism in up to 80% cases which requires supplementary thyroid hormone in the form of daily pill(s). Secondary malignancies after therapeutic radioactive iodine therapy are not uncommon, with the most consistent evidence for haematological malignancies, sarcomas and carcinomas (Aman 1999; Teixeira 2006). Additionally, this therapy also cause to develop various resistant genetic phenotypes like FoxP3 (Forkhead box P3, associated with severe immunodeficiency); found over expressed in PTC cases treated with radiation therapy (Ugolini *et al.* 2013). Furthermore, radioiodine therapy cannot be exploited to treat anaplastic/undifferentiated and MTC as these types do not take up iodine (American Cancer Society 2013).

Post-operative chemotherapy although show efficient cytotoxic responses against cancer but is associated with significant toxicity and varying efficacy (Sherman 2010; Crouzeix *et al.* 2012; Marotta *et al.* 2013). It not only works against cancer cells but also against other rapidly dividing healthy cells of the body like in bone marrow, lining of mouth, intestines and the hair follicles. Some drugs can have other specific side effects like doxorubicin (one of the most common drugs used in thyroid cancer) can affect heart function. Other miscellaneous effects include hair loss due to death of hair follicles, immune deficiency due to lose of white blood cells and thus increased chances of secondary infection, easy bruising and resulting extensive bleeding as a result of low blood platelets number. Thus,

although the classical therapy combined with various chemotherapeutic agents is the only practiced approach in the cancer treatment but still there exist the flaws which have to be filled with alternative and more targeted therapies.

1.2.5.3. Inhibition of receptor tyrosine kinase

RET is a principal target among the alternative treatment strategies against thyroid carcinoma (Cote and Gagel 2003). Tyrosine kinase receptor (TKR) represents a useful anti-cancer target and thus several approaches to block the function of this receptor (Fischer *et al.* 2003, Bennisroune *et al.* 2004, Santoro and Carlomagno 2006). Strategies including dominant negative mutants (Gschwind *et al.* 2004), small molecule inhibitors that compete with ATP-binding site to interfere the enzymatic activity of many TKR (Noble *et al.* 2004) and drugs that impede association of adaptor protein have been employed to potentially impair TKR activity (Zinzalla and Thurston 2009). Receptor synthesis can also be blocked either at the level of RNA by interference strategy (Borkhardt 2002) or by direct synthesis inhibition by gene therapy or also by drug-mediated receptor degradation (Cosma *et al.* 1998; Carniti *et al.* 2003). In addition, the blockage of intracellular signal transduction pathways *i.e.* RAF or phosphatidylinositol 3-kinase that are initiated by several tyrosine kinases could potentially block oncogenic function (Bollag *et al.* 2003, Fingar and Blenis 2004). The serine/threonine-protein kinase BRAF^{V600E}; the most frequently altered kinase in differentiated TC, is another promising target for kinase inhibitors (Xing 2005). Anilinoquinazoline ZD6474, a potent vascular endothelial growth factor (VEGF) receptor-2 (flk-1 or KDR) tyrosine kinase inhibitor, was found efficiently block the activity of RET/PTC1 and RET/PTC3 as well as multiple endocrine neoplasia (MEN) 2A- and MEN 2B-related RET in PTC (Carlomagno *et al.* 2002). Two pyrazolo-pyrimidine PP1 and PP2 showed similar effects and blocked RET oncogenic effects at nanomolar (≤ 100 nm) concentration range in PTC (Carlomagno *et al.* 2002; Carlomagno *et al.* 2003) while 2-indolinone derivative RPI-1 also used against RET/PTC1 (Lanzi *et al.* 2000; Lanzi *et al.* 2003) fusion oncogene. PP1 found to induce RET MEN2A and 2B oncoproteins destruction through proteosomal degradation (Carniti *et al.* 2003).

Since the thyroid carcinomas are well vascularized; therefore, many therapies instead of causing direct cytotoxic effects on cancer cells, are targeted against the anti-angiogenic activity *via* the inhibition of TKRs of diverse molecular signaling pathways including various growth factor receptors (GFRs) like vascular endothelial GFR (VEGFR), Epidermal GFR (EGFR) and platelet derived GFR (PDGFR), c-Kit and RET (Table 1). They inhibit

downstream RAF kinase including mutant BRAF and hence prevent tumour growth by antiproliferative, antiangiogenic and proapoptotic effects (Liu *et al.* 2006; Keefe *et al.* 2010; Fallahi *et al.* 2013). The clinical trials of these pharmacological multi-kinase inhibitor (MKI) preparations like sorafenib (Nexavar[®]), sunitinib (Sutent[®]), motesanib (AMG 706), axitinib (Inlyta[®]) and Telatinib (BAY 57-9352) etc for the treatment of DTC are summarized in Table 2.

Table 1: Molecular targets of various pharmacological drugs as tyrosine kinase inhibitors in cancer therapy

Drug	VEGFR1	VEGFR2	VEGFR3	PDGFR	RET	RET/PTC	BRAF	cKIT	EGFR	cMET	FGFR
Axitinib	+	+	+	+	-	-	-	+	-	-	-
Cabozantinib	-	+	-	-	+	-	-	+	-	+	-
Gefitinib	-	-	-	-	-	-	-	-	+	-	-
Imatinib	-	-	-	-	+	-	-	+	-	-	-
Lenvatinib	+	+	+	+	-	-	-	-	-	-	+
Motesanib	+	+	+	+	+	-	-	+	-	-	-
Pazopanib	+	+	+	+	-	-	-	+	-	-	-
Sunitinib	+	+	-	+	+	+	-	+	-	-	-
Vandetanib	-	+	+	-	+	+	-	-	+	-	-
Sorafenib	+	+	+	+	+	+	+	+	-	-	-

VEGFR: Vesiculo-endothelial GFR, PDGFR: Platelet-derived GFR, EGFR: Epidermal GFR, FGFR: Fibroblast GFR, +: Inhibitory effect, -: Non-inhibitory effect. Ref: Regalbuto *et al.*, 2012.

Sorafenib (BAY 43-9006, Nexavar, Onyx Pharmaceuticals, Emeryville, CA) is one of the MKIs preventing auto-phosphorylation of various TKRs and showed efficient inhibitory *in vitro* and *in vivo* results on tumour growth in multiple models (Liu *et al.* 2006; Wilhelm *et al.* 2006; Chang *et al.* 2007; Wilhelm *et al.* 2008). It has already been approved by U.S. FDA in 2005 for advanced renal cell carcinoma and in 2007 for hepatocellular carcinoma in adults (Wilhelm *et al.* 2008) and is used for thyroid cancer therapy in clinics (see Table-2). It is not yet approved by the FDA for treatment of thyroid cancer in children but has showed efficient results in an un-avoidable pediatric case (Iyer *et al.* 2014). Other agents are in phase I and phase II clinical trials but a phase III clinical trial of Soarfenib for the treatment of thyroid carcinoma is in progress by Bayer Healthcare in collaboration with Onyx Pharmaceuticals against locally advanced and metastatic radioactive iodine (RAI)-refractory DTC (Brose *et al.* 2011), www.clinicaltrials.gov: NCT00984282).

Table 2: Various clinical trials completed or on-going in papillary thyroid carcinoma

Active compound	Trade name	Research/clinical study (n° of patients)	Daily dose	Total drug therapy	Results	Indications	References
Sunitinib (derivative of indolinone)	SU11248	Case report (1)	50 mg QD	65 weeks		Metastatic PTC	(Kaldrymides <i>et al.</i> 2010)
	SU11248	Phase II (31)	50 mg QD	---	PR=13%, SD=68%, PD=10%	Advanced TCs	(Cohen <i>et al.</i> 2008a)
Motesanib diphosphate	AMG 706	Phase I (71)	50, 100, 175 mg QD	Until unacceptable toxicity or disease progression	---	DTC and MTC	(Rosen <i>et al.</i> 2007)
	Amgen	Phase II (93)	125 mg QD	48 weeks	PR=14%, Median PFS = 40weeks	DTC locally advanced or metastatic	(Sherman <i>et al.</i> 2008)
Axitinib	AG-013736	Phase I (36)	5-30 mg BID	Until disease progression, unacceptable toxicity or consent withdrawal	Max. tolerated dose= 5mg, BID	Advanced Solid Tumors; thyroid=5	(Rugo <i>et al.</i> 2005)
	AG-013736	Phase II (60)	5-10 mg BID	-do-	PR=30%, Median PFS =18months	Advanced TCs (PTC, FTC, MTC)	(Cohen <i>et al.</i> 2008b)
Telatinib	BAY 57-9352	Phase I (53)	20 mg QD to 1,500mg BID	-do-	Recommended dose= 900mg BID	Advanced or Metastatic Solid Tumors	(Eskens <i>et al.</i> 2009)
Sorafenib tosylate	BAY 43-9006, Nexavar; Onyx Pharmaceuticals, Emeryville, CA and Bayer Healthcare, Pharmaceuticals Wayne, NJ	Phase II (26)	--do-	---	PR=18%, SD=82%	DTC=14 & metastatic MTC=12	(Ahmed <i>et al.</i> 2008)
		Phase II (30)	-do-	16 weeks (up to 90 weeks)	PR=32%, SD=68%, PD=0%, mPFS= 79 weeks	Advanced DTCs, ATC and MTC	(Gupta-Abramson <i>et al.</i> 2008)
		Phase II (46)	-do-	---	PR=13%, SD=74%, PD=13%, mPFS= 4-16 weeks	Advanced DTCs, ATC	(Kloos <i>et al.</i> 2009)
		Phase II (31)	-do-	26 weeks	CBR=59%, PR=25%, SD=34%, PD=22%	Advanced TCs	(Hoftijzer <i>et al.</i> 2009)
		Hospital study (15)	Sorafenib = -do- Sunitinib = 50 mg	---	CBR =80% PR=20%, SD=60%, PD=20%	DTC (PTC=8 + FTC=7)	(Cabanillas <i>et al.</i> 2010)
		Pilot study (8)	-do-	Until progressive disease or intolerable toxicity	mPFS= 40.1 weeks PR=50%, OS=55weeks	DTC (PTC=7 + FTC=1)	(Luo <i>et al.</i> 2014)
		Phase III (417)	-do-	30 months	On going NCT009842 82 (Bayer Schering)	DTC (PTC, FTC) and ATC	(Brose <i>et al.</i> 2011)- Ongoing
		Case report (1)	-do-	13 weeks	50 % nodular size reduction	PTC	(Iyer <i>et al.</i> 2014)

N.B: RTKI = Receptor tyrosine kinase inhibitor, GFR = growth factor receptor, VEGFRs = vascular endothelial GFR 1, 2 and 3, PDGFR = platelet-derived GFR, EGFR = Epidermal GFR, Multi-kinase inhibitors (MKIs) = inhibitor of VEGFRs + PDGFR + Flt-3 + c-kit and RET, c-kit = stem cell factor receptor.

CBR = Clinical beneficial response = PR + SD, PFS = progression-free survival, PD = progressive disease,

CR = partial response, PR = partial response, SD = stable disease, QD = once daily, BID = twice daily.

NB: All the drugs are MKIs except Sunitinib which is reported as only TKR inhibitor by the authors.

1.2.5.4. Immuno-therapy in thyroid cancer

Monoclonal antibodies are used with proven clinical efficacy against several TKRs such as VEGFR, EGFR and PDGFR (Imai and Takaoka 2006). Anti-RET antibodies are also reported but have not yet been used in treatment (Yano *et al.* 2000; Salvatore *et al.* 2002). Antibodies to stimulate the immune system are another possible effective therapeutic tool (Putzer and Drost 2004). Specific immuno-therapy could be employed against the tumour associated antigens like cell surface receptors which are over expressed in cancer cells and thus recognized by the immune system.

Amongst are MAGE (melanoma-associated antigen) gene family members encode tumor-specific antigens, MAGE-1 (Ruschenburg *et al.* 1999) and MAGE-3 (Milkovic *et al.* 2006), CD10 (Tomoda *et al.* 2003; Yegen *et al.* 2009), CXC motif chemokine 12 / stromal cell-derived factor-1 α & β (CXCL12/SDF-1 α & β) (Castellone *et al.* 2004), LIM-only (kinase) domain containing 2 (LIMD2), protein tyrosine phosphatase receptor type C (PTPRC, also known as CD45), lymphotoxin beta (LTB), cytokeratin 19 (Cerutti *et al.* 2007; Kaczka *et al.* 2013), thyroglobulin (Tuttle *et al.* 2007; Lin 2008; Kaczka *et al.* 2013), human mesothelial cell marker 1 (HBME-1) (Papotti *et al.* 2005; Nasr *et al.* 2006), human type I CK19 (Rorive *et al.* 2002), galectin-3 (Papotti *et al.* 2005; Than *et al.* 2008) & -7 (Rorive *et al.* 2002; Than *et al.* 2008), survivin (Ito *et al.* 2003), fibronectin (Hesse *et al.* 2005), EGFR (Ness *et al.* 1996), ERP (estrogen receptor related protein), p21-RAS (Akslen and Varhaug 1995), Fascin (Chen *et al.* 2008) and telomerase reverse transcriptase (Saji *et al.* 1999; Umbricht *et al.* 2004; Wang *et al.* 2005; Takano *et al.* 2007) are found over expressed in PTC and FTC. Members of matrix metalloproteinases (MMPs) family, MMP-1 (Buergy *et al.* 2009), MMP-2 (Tan *et al.* 2008), MMP-7 (Cho Mar *et al.* 2006; Ito *et al.* 2006), MMP-9 (Buergy *et al.* 2009), MMP-11 (Ito *et al.* 2006) and their interacting molecules CD147 (Tan *et al.* 2008) and urokinase-type plasminogen activator; u-PA (Sid *et al.* 2008) are also found to more frequently expressed in thyroid malignancies.

Due to lack of established tumour antigens, no promising vaccination regimen has yet been indicated as a standard anticancer therapy against these tumour associated antigens (Papewalis *et al.* 2010). Since some are already successfully tested in other malignancies and could also be potential candidates for immunotherapy in thyroid carcinomas.

1.3. Papillary thyroid carcinoma

Papillary thyroid carcinoma (PTC) also called as papillary thyroid cancers or papillary thyroid adenocarcinomas is the most common thyroid malignancy and accounts for 80–85% of thyroid disorders (Moses *et al.* 2010; LiVolsi 2011; Vivero *et al.* 2013).

1.3.1. Incidence of papillary thyroid carcinoma

Over the last few decades, the incidence of thyroid carcinomas has statistically increased (Siegel *et al.* 2013). This increasing incidence is solely due to increase in the incidence of papillary histotype (Leenhardt *et al.* 2004b, Pellegriti *et al.* 2013). While, there is no significant contribution of follicular, medullary or anaplastic histotypes (Pellegriti *et al.* 2013) Figure 3B. It is also assumed that this increased incidence is due to advancement in the detection technologies. Radiological examinations, ultrasonography and FNA biopsies have now enabled to identify the cases that gone previously undetected and also facilitate to diagnose at an early stage of development (CTCA 2013). By using these techniques, it is now possible to detect PTCs measuring even upto 1mm; usually defined as papillary thyroid micro-carcinoma (PTMC; of size 10mm or less) (Ito *et al.* 2014; Iyer *et al.* 2014). Thus over the years 1988-2010, the diagnosis of PTC by FNA cytology (FNAC) increased from 0% (0/73) in 1988 to 2.0% (17/861) in 2010 with a peak of 2.7% (26/976) in 2006 in the province of Messina, Italy (Benvenga 2013; Latina *et al.* 2013; Rizzo *et al.* 2013). It might be possible that an early diagnosis of small sub-centimeter tumours is the reason for an overall increase in the incidence of thyroid carcinoma. It is reported that commonly at thyroid cancer referral centers; nearly 60%–80% of thyroid carcinomas detected nowadays are PTMC, of less than one cm in size (Leenhardt *et al.* 2004a; Leenhardt *et al.* 2004b). But although the sensitivity of ultrasound and development of technology have contributed to the increase in the diagnosis of small sub-centimeter tumours (Davies and Welch 2006) but there are reports of an overall increase in the detection of tumours of all sizes over time (Kent *et al.* 2007; Chen *et al.* 2009; Enewold *et al.* 2009; Yu *et al.* 2010; Pacini *et al.* 2012).

1.3.2. Prognosis of papillary thyroid carcinoma

Prognosis of PTC is generally good despite recurrences in 10–20% of patients (Lang *et al.* 2007; Hay *et al.* 2008). Prognosis depends on the patient's age of disease exposure (Busaidy and Cabanillas 2012), tumour stage, histological type, initial/start-up treatment (Zaman *et al.* 2006) and stage of diagnosis (Saez 2011). Age and gender are also important prognostic factors of thyroid carcinoma (Treseler and Clark 1997; Xing *et al.* 2013; Ito *et al.* 2014). The frequency of PTC is high in women especially during their reproductive (Machens *et al.* 2006; Veiga *et al.* 2013) and pregnant age (Walker *et al.* 2005) while outcome is worse in men of old age (60s or 70s) (Ronckers *et al.* 2005; CTCA 2013). Different investigators suggested the correlation of PTC with reproductive and dietary factors in women but the underlying molecular factors account for the gender and age disparity in incidence and outcome are still unknown (Horn-Ross *et al.* 2001; Iribarren *et al.* 2001; Horn-Ross *et al.* 2002; Sakoda and Horn-Ross 2002; CTCA 2013).

1.3.3. Risk factors and causative agents of PTC

Risk factors include being a female, having a history of goiter (enlarged thyroid) or thyroid nodules, a family history of thyroid cancer and radiation therapy related with a medical treatment. Radiation exposure as a result of radioactive fallout from atomic weapons testing and nuclear power plant accidents at Chernobyl was also linked with increased risk of thyroid cancer, especially in children. Certain genetic syndromes that transfer abnormal hereditary genes to next generation are also a risk in the disease development (American Thyroid Association 2013). About 25% of Familial MTC, MEN 2A & B are inherited while less than 5% of PTC or FTC are thought to be inherited (Cancer.Net 2013). Similarly, there are also reports of PTC incidence alongwith a family history of the disease (Gorson 1992). Iodine deficiency is another causative factor that causes thyroid disorders, not only follicular but also PTC. This condition is especially present in areas of the world where diet is devoid or deficient of iodine. A diet low in iodine further increases the risk of PTC, if the person is previously exposed to radioactivity (American Cancer Society 2013).

1.3.3.1. Role of ionizing or therapeutic radiation exposure

Exposure to radiations is a proven risk factor for thyroid cancer. Source of such radiations could be medical treatment or radiation fallouts from power plant accidents or nuclear weapons. Over the last few decades, there have been significant increase in the use of medical diagnostic radiations, especially pediatric computed tomography (CT) scans which have done in millions in the United States (Amis *et al.* 2007; Mettler *et al.* 2008), thus leading

to increased risk of thyroid cancer in later life (Baker and Bhatti 2006; How and Tabah 2007). Radiation therapy of head or neck diseases as well as in cancers like lymphoma, Wilms' tumor (nephroblastoma) and neuroblastoma is a risk for thyroid cancer in children. But the risk depends on the dose of radiation and the age of child and in general, the risk increases with larger dose and at younger age of therapy. Earlier than 1960s, children were sometimes treated with low doses of radiations for acne, fungus infections of the scalp (ringworm) or enlarged tonsils or adenoids. These radiations also lead to genetic alterations like RET/PTC translocation and BRAF mutation, as found in radiation-induced PTC, associated with therapeutic radiation therapy at childhood (Boaventura *et al.* 2013; Xing 2013). Childhood exposure to radiations during imaging by X-Rays and CT scans also increases the risk of thyroid or other cancers. Later on, the people who had these treatments were found to be at higher risk of thyroid cancer. Irrespective as an outcome of radiation exposure, thyroid cancer is also induced in rodents by using radiations as an experimental method (Pacini and De Groot 2013).

Several studies have reported an increased possibility and appearance of thyroid cancer after radioactive fallouts from nuclear weapons or power plant accidents. For the reason, after accidental radiation exposure events, thousands of people developed thyroid malignancies (Williams 2002; Jacob *et al.* 2006; Jacob *et al.* 2006; Nikiforov 2006). This cancer was found much common in children lived near the Chernobyl nuclear power plant in Ukraine; the site of nuclear plant accident on 26 April 1986 where millions of people including young and adult residents of contaminated areas in Belarus, Russia and the Ukraine, even workers involved in the cleaning of the contamination, were exposed to emitted 370×10^{16} - 740×10^{16} Bq of fission products containing huge amounts of radioactive iodine isotopes (mainly ^{131}I) (Di Cristofaro *et al.* 2005). Second largest radioactive catastrophe of the history was an energy accident at Fukushima Daiichi nuclear power plant in Japan on 11 March 2011 after tsunami. Despite of children, the adults exposed to the effects of the accident also were found to have higher rates of thyroid cancer. Similarly to these events, some radioactive fall outs also occurred over certain regions of the United States after nuclear weapons were tested in western states during 1950s. Since, these exposures were of much lower intensity than that of Chernobyl, thus risk of thyroid cancer was not been proven to be so high by these low level incidents (American Cancer Society 2013).

Recurrence and thyroid cancer related deaths were found to be similar in radiation-exposed and sporadic carcinomas (Rubino *et al.* 2002; Naing *et al.* 2009). The risk of

malignancy remains elevated for decades after exposure and is proportional to the radiation dose exposed to the thyroid gland (Schneider *et al.* 1993; Sigurdson *et al.* 2005; Sigurdson and Jones 2005). But more importantly these radiation exposed cancers are found to be papillary carcinomas (Acharya *et al.* 2003).

1.3.3.2. Age effect in PTC development

The developing thyroid gland of children is especially vulnerable to the carcinogenic action of ionizing radiation and exposure increase the susceptibility to develop thyroid disease. In young people, thyroid carcinoma has typically an aggressive initial clinical presentation (Sassolas *et al.* 2013). At young age, thyroid gland is more susceptible to cancerous transformation (mostly of malignant nature) (Acharya *et al.* 2003) under the effect of environmental (Ron *et al.* 1995; Jacob *et al.* 2006; Jacob *et al.* 2006; Nikiforov 2006) or therapeutic (Tucker *et al.* 1991; Bounacer *et al.* 1997; Bounacer *et al.* 1997; de Vathaire *et al.* 1999; Rabes 2001; Boaventura *et al.* 2013; Mathews *et al.* 2013) radiation exposure. Thus, young age predisposes to the formation of gene translocations and recombinations in thyroid gland. High proliferation rate of thyroid cells is responsible for the increased sensitivity of children's thyroid gland to chromosomal recombinations (Saad *et al.* 2006). The risk decreases with an advancement in adulthood period at radiation exposure with little apparent risk after 20 years of age (Ron *et al.* 1995). This might be due to operating cellular repair mechanisms to counteract the exposure damage during this stage of life (Ron *et al.* 1995; Jacob *et al.* 2006).

It has long been reported that in humans, external radiations increase the risk of development of benign and malignant thyroid tumors at early and younger age. In this context, a pooled analysis was performed to summarise the results of seven independent studies observing the effects of therapeutic (to treat enlarged tonsils and thymus, cervical cancer and other disease conditions) or atomic radiations exposure during childhood. It was concluded from the analysis that thyroid gland at childhood has one of the highest cancer risk coefficient among the body organs. About 88% cases of thyroid cancer diagnosed at a later age were attributed to exposure of only one Gy radiations before 15 years of age (Ron *et al.* 1995). Similarly, after the accident, TC cases were increased in children in Belarus with a latency period as short as 4 years and the highest rate was found in the younger age groups of 0-4 years and 5-9 years of age at the time of exposure (Bleuer *et al.* 1997a, Bleuer *et al.* 1997b). Similarly, the incidence of benign and malignant TC was dramatic increased to about 80 times in the children born or conceived around the time of the accident in Chernobyl and

surrounding areas (Tronko *et al.* 1999). The age factor of the individual expressing PTC is also importantly correlated with the reported genetic translocations. BRAF^{V600E} mutation is commonly present at old age with an aggressive behaviour while RET/PTCs rearrangements are mostly found in children and young adults in PTC (Nikiforov 2002, Xing 2005, Moses *et al.* 2010). Aggressive behaviour of these PTCs is associated with an elevated recurrence but have no influence on the mortality rate (Sassolas *et al.* 2013).

1.3.3.3. Sex effect in PTC development

It was observed that pooled relative excess and absolute risk was higher in females than males reflecting higher rate of natural occurrence of thyroid cancer (Ron *et al.* 1995). The average annual number of new cases of thyroid cancer was 248 and 722 in males and females respectively in 1993-1997, with a rate of about 0.5 and 1.4% among all cancer cases in the Nordic (Denmark, Finland, Iceland, Norway and Sweden) countries (Moller *et al.* 2002). It is the second most frequent cancer in Italy in women; which are below than 45 years of age (Dal Maso *et al.* 2011). Similarly, the incidence rate was found higher in Norwegian females than males *i.e.* 5.1 vs 1.51 per 100,000 persons per year in 2004 (The Cancer Registry of Norway 2006). The higher rates were also found in women in a global statistics data *i.e.* 103,589 in females vs 37,424 in males (Parkin *et al.* 2005). In the USA, 45,310 new cases have been reported in 2013 (Siegel *et al.* 2013) compared to 43,210 in 2012 (Siegel *et al.* 2012). In Europe, females (40,700) affected with thyroid cancer are more than males (12,300) (Ferlay *et al.* 2013). Its level is increased from 6th (Enewold *et al.* 2009) to 5th in the cancers in women but is not in the top 10 cancers found in men (Siegel *et al.* 2013, American Cancer Society 2013). Higher incidence was observed in 2001 (4.2: males, 12: females per 100,000 persons per year (National Cancer Institute 2005). Although, there are no many deaths with thyroid cancer but the reported rate is always higher in women than men *i.e.* 1,040 vs 810 in U.S., 4,270 vs 2,070 in Europe and 160 vs 230 in France during 2012 (Ferlay *et al.* 2013, Siegel *et al.* 2013). Females excess absolute risk ratio was found to be 1.4 times higher than males in the areas of Ukraine and Belarus affected by the Chernobyl accident (Jacob *et al.* 2004). Although the appearance of thyroid cancer is high in females but the size of DTC tumours was found significantly larger in males than in females (papillary: 26.0 mm vs 19.3 mm, follicular: 54.9 mm vs 35.1 mm and medullary: 27.9 mm vs 20.8 mm). Similarly, lymph node and distal metastase was also found higher in males (Machens *et al.* 2006).

1.3.3.4. Miscellaneous factors involved in PTC progression

Higher levels of chemical exposure are associated with increased cellular proliferation, potentially resulting in an increased opportunity for mutations and the development of cancer. Environmental chemicals such as bisphenol A, perfluorinated chemicals and dioxins have been positively correlated with secretion of TSH, T3 and T4 (Rochester 2013; Wen *et al.* 2013). Other factors have been reported to raise thyroid cancer risk especially of papillary and follicular types are increase in body mass index and in height (Engeland *et al.* 2006; Guignard *et al.* 2007), various menstrual and variable reproductive patterns (Negri *et al.* 1999; Brindel *et al.* 2008), use of fertility drugs like clomiphene and progesterone (Hannibal *et al.* 2008) or oral contraceptives and hormone replacement therapy for menopause (La Vecchia *et al.* 1999), insulin resistance syndrome (since insulin is a thyroid GF and stimulates thyrocytes proliferation) (Rezzonico *et al.* 2008), history of benign nodules/adenomas and goiter (Franceschi *et al.* 1999), ingestion of nitrate-contaminated drinking water (Tajtakova *et al.* 2006; Ward *et al.* 2008) and immigration to countries with high thyroid cancer incidence (Nasseri 2008). Intake of cruciferous and other vegetables (Bosetti *et al.* 2002) or fish and shell-fish consumption (Bosetti *et al.* 2001) decreases thyroid carcinoma risk. Alcohol consumption and tobacco smoking (Guignard *et al.* 2007), red meat intake (Tavani *et al.* 2000), use of gonadotrophins, human chorionic gonadotrophin (hCG) or gonadotrophin-releasing hormone (GnRH) (Hannibal *et al.* 2008) were found to be not related with PTC development.

1.3.4. Genetic rearrangements occurring in PTC

The ionizing radiations might render the individuals more susceptible to thyroid malignancies, thus lead to development of various genetic rearrangements and the resulting cancers. Genetic alterations activate the mitogen signal transduction; MAPK (mitogen-activated protein kinase) pathway and are present in about 80% cases of thyroid carcinoma (Moses *et al.* 2010). These include rearrangements in tyrosine kinase receptors (RET/PTC, NTRK1), small GTP-binding signalling proteins (RAS, RAF) or PAX8/PPAR (Pax-8/peroxisome proliferator-activated receptor) gamma rearrangements (Cheung *et al.* 2003; Kimura *et al.* 2003; Melillo *et al.* 2005; Carta *et al.* 2006; Castro *et al.* 2006; Kebebew *et al.* 2007; Sapio *et al.* 2007). The T1799 mutation (nucleotide transversion) in β -type RAF (BRAF) kinase substitutes a valine to glutamic acid in codon 600 (V600E) thus BRAF^{V600E} causes constitutive activation of the serine/threonine kinase and its corresponding signalling pathways (Cohen *et al.* 2003; Fukushima *et al.* 2003; Kimura *et al.* 2003; Xu *et al.* 2003;

Fluge *et al.* 2006). It is the most common genetic alteration found in approximately 45% cases of conventional and anaplastic PTCs (Xing 2005; Xing *et al.* 2013) or 29–69% cases of PTC with aggressive behaviour including extrathyroidal extension (Riesco-Eizaguirre *et al.* 2006; Xing 2010). But BRAF mutation is less common in children and also in the cases developed after radiation exposure (American Cancer Society 2013). It was found that BRAF^{V600E} develops aggressive PTC in transgenic mice (Knauf *et al.* 2005; Charles *et al.* 2011; Lee *et al.* 2011). But in human PTC tumours show intra-tumoral heterogeneity in the BRAF genotype, a minority of cells have BRAF^{V600E} while most of the cells harbour wild-type BRAF (Guerra *et al.* 2012). It has been demonstrated by quantitative assay that conventional PTCs often consist of a mixture of wild-type and mutant BRAFs (Guerra *et al.* 2012; Guerra *et al.* 2012). Previously, it was thought that BRAF^{V600E} is second to occur after PTC tumorigenesis (Vasko *et al.* 2005) but it is not yet clear that whether BRAF^{V600E} is an early event to initiate PTC tumorigenesis or this nucleotide transversion occurs following the development of a thyroid tumour (Xing 2012). Another alteration of the same gene is BRAF-AKAP9 rearrangement [inv(7)(q21–22q34)] (Ciampi *et al.* 2005).

Point mutations of RAS genes are an early event in the development of follicular adenomas and carcinomas and are found in undifferentiated thyroid cancers (Lemoine *et al.* 1988; Namba *et al.* 1990; Suarez *et al.* 1990). RAS proto-oncogenes include HRAS, NRAS, KRAS, are most common in follicular thyroid neoplasms but also found in benign and other malignant follicular thyroid neoplasms (Vasko *et al.* 2003).

Additionally, follicular carcinomas also have a high prevalence of PAX8-PPAR γ rearrangements, generated by the cytogenetically detectable translocation t(2;3)(q13;p25) leading to an in-frame fusion of the PAX8 gene (encodes a paired domain transcription factor, required for thyroid development) with PPAR γ gene (Kroll *et al.* 2000). Functional analysis of this fusion product suggests that PAX8-PPAR γ may serve in part as a dominant negative antagonist of wild-type PPAR γ (Kroll *et al.* 2000; Nikiforova *et al.* 2003). Similarly, NTRK1/NGF [neurotrophic receptor-tyrosine kinase-1/nervous (central and peripheral) growth factor] is oncogenic rearrangement in chromosomal 1 of NTRK1 gene with other activating genes *i.e.* with non-muscle tropomyosin gene [NTRK1-TPM3 inv(1)(1q21-q21.2)] (Butti *et al.* 1995), with translocated promoter region gene [NTRK1-TPR inv(1)(1q21-q25)] (Greco *et al.* 1992) or with TRK Fused Gene [a novel gene on chromosome 3q11–12, NTRK1-TFG] (Greco *et al.* 1995).

1.3.5. Role of gene positioning in PTC progression

Recurrent chromosomal rearrangements are commonly found in human malignancies such as leukemia, lymphoma, prostate and thyroid cancers. Usually the proximity of the two partner genes participating in the rearrangement is the basis for gene translocation and fusion. This concept of loci proximity, to cause chromosomal rearrangements under the effect of ionizing radiations; as they are very close to each other in the nucleus, was first given by Savage in 1996 (Savage 1996). The proximity between recombinogenic loci was then firstly demonstrated in 2000 by using Fluorescence In Situ Hybridization (FISH) three-dimensional microscopy to map the positions of the RET and H4 loci involved in the formation of RET/PTC translocation within interphase nuclei (Nikiforova *et al.* 2000). Thus, spatial contiguity of RET and the partner genes in the nucleus is a structural basis for the formation of RET/PTC rearrangements (Nikiforova *et al.* 2000). In humans, all three genes *i.e.* RET, NcoA4 and H4 are located on the same chromosome (10q) (Gandhi and Nikiforov 2011). In normal human thyrocytes, at least one pair of RET and H4 was found to be juxtaposed (touching or overlapping each other in at least one optical section) in 35% (93/263) of cells. Compared to RET and H4 genes, which are relatively far apart (30 Mb), the distance between RET and ELE1 is only 0.5 Mb which further increases the chances of gene translocation and resulting fusion oncogene products (Nikiforova *et al.* 2000).

The distance between rearrangement-prone loci was also studied by FISH and other techniques by various authors in miscellaneous cells models; thyroid: RET & NcoA4 (Gandhi *et al.* 2006) and NTRK1 & TPR (Roccatò *et al.* 2005), prostate: TMPRSS2 & ERG and TMPRSS2 & ETV1 (Lin *et al.* 2009; Mani *et al.* 2009), leukemia: PDGFR β and H4/D10S170 (Kulkarni *et al.* 2000; Schwaller *et al.* 2001) and lymphomas: MYC, BCL and immunoglobulin genes (Roix *et al.* 2003). Later, it was evidenced by using FRET-SE (Fluorescence Resonance Energy Transfer-Sensitized Emission) analysis that genes participating in chromosomal rearrangements are positioned non-randomly close to each other in nuclei of interphase cells (Gandhi *et al.* 2012). In human thyroid TPC-1 cells harbouring RET/PTC1 junction oncogene, FRET-SE analysis revealed energy transfer in 100 out of 105 (95.24%) nuclei and confirmed that both genes participating in this fusion are located in the same chromosome in a close proximity (<10nm) with a FRET frequency of $5.31 \pm 0.66\%$. Similar results were observed for rearrangement gene pairs of RET & NcoA4 ($3.57 \pm 0.58\%$), BRAF & AKAP9 ($1.77 \pm 0.41\%$) and NTRK1 & TPR ($2.07 \pm 0.45\%$) (Gandhi *et al.* 2012). The results were further counter verified by assessing FRET between chromosomal loci that are

located on separate chromosomes and do not participate in oncogenic fusion. Thus by using probes for RET and BRAF, visual analysis showed that RET and BRAF were positioned at variable distances far away from each other and were colocalized in less than 1% of the 1000 analyzed nuclei (Gandhi *et al.* 2012). The proximity of RET and partner genes thus provide a structural basis for generation of RET/PTCs rearrangements (Gandhi *et al.* 2006; Gandhi *et al.* 2012) by allowing a single radiation track to produce a double-strand break in each gene at the same site in the nucleus (Nikiforova *et al.* 2000).

1.4. RET proto-oncogene

RET gene is the key player of thyroid tumorigenesis and is located on the chromosome 10q11.2 at peri-centromeric region and spans 21 exons in humans (Takahashi *et al.*, 1985) while it is present on chromosome 6F1 in mouse and on 4q42 in rat (Gandhi and Nikiforov 2011). It encodes for a receptor tyrosine kinase that serves as a common receptor for the glial cell line derived neurotrophic factor (GDNF) family of ligands [(four members: GDNF, neurturin (NTN), artemin (ART) and persephin (PSP)] (Santoro *et al.* 2002; Putzer and Drost 2004; Santoro *et al.* 2004). Each ligand binds to RET in conjunction with a cell surface co-receptor of the GDNF family receptor- α (GFR α family members; GFR- α 1-4). The co-receptor consists of two to three globular protein domains linked to the cell membrane by a glycosyl-phosphatidyl-inositol (GPI) anchor (Manie *et al.* 2001; Airaksinen and Saarma 2002; Runeberg-Roos and Saarma 2007). Each ligand first interacts with a GFR α co-receptor to form a heterodimeric complex which then interacts with receptor cadherin homology domains and recruit RET into lipid raft membrane subdomains (Figure 4, 6) (Mulligan 2014). Ligand to receptor complex brings together two RET molecules into cholesterol rich membranes sub-domains (or lipid rafts). Thereby triggering auto-phosphorylation (P) and dimerization for ultimate RET activation via intracellular signalling, through intracellular TK domain that contains at least 12 auto-phosphorylation sites (Liu *et al.* 1996; Salvatore *et al.* 2000; Couplier *et al.* 2002; Kawamoto *et al.* 2004).

1.4.1. Structure of RET

RET is a single-pass trans-membrane protein and have four cadherin-like domains in the extracellular (EC) region (which have a calcium binding site among them that leads to the stabilization of the EC region), a cysteine rich region (adjacent to the cell membrane that promotes dimerization after ligand binding, contains Cys 609, C611, C618, C620, C630 and C634), trans- and juxta-membrane (containing serine-696 PKA phosphorylation site) domains and an intracellular region which encompasses two tyrosine-kinase sub-domains

that are implicated in the activation of several intracellular signal transduction pathways (Manie *et al.* 2001, Airaksinen and Saarma 2002, Santoro *et al.* 2002, Santoro *et al.* 2004a, Santoro and Carlomagno 2006, Wells and Santoro 2009, Medves and Demoulin 2012) (Figure 4). The RET gene is responsible for three major proteins which arise through alternative splicing of the 3' terminus, leading to the expression of proteins that differ in number of their last amino acids *i.e.* n=9 (RET9), 43 (RET43) and 51 (RET51) (Manie *et al.* 2001; Santoro *et al.* 2004b; Wells and Santoro 2009). The initial/basic structure of various post-translational products is common until they differ in the amino acid sequences immediately downstream from glycine 1063 (Santoro *et al.* 2004b).

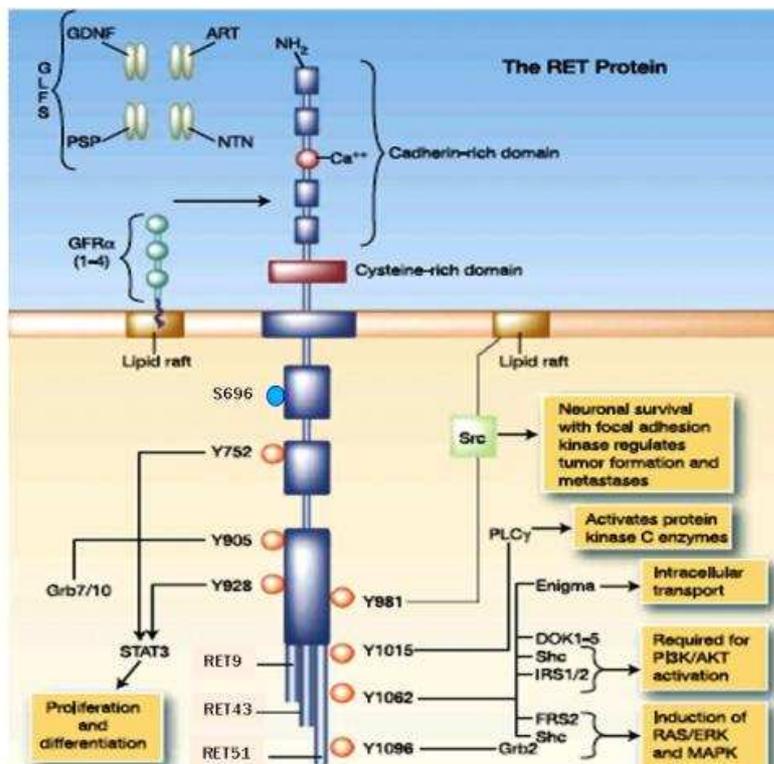


Figure 4. Schematic drawing of the RET protein with the four extracellular cadherin-like domains, cysteine-rich box adjacent to the plasma membrane, intracellular juxta-membrane domain and the split tyrosine kinase domain. The two alternatively spliced forms, RET9 and RET51 are indicated. RET is activated by complexes of GFLs with glycosyl phosphatidyl inositol anchored GFR co-receptors that are distributed within lipid rafts. The network of RET-mediated signaling events is also summarized.

Major auto-phosphorylation sites are shown with their direct targets:

Y905: auto-catalytic residue, binding site for GRB7/10 adaptors, Y1015 binds phospholipase C, Y981 binds SRC, Y1096 (unique to alternatively spliced RET51 isoform) binds GRB2 and Y1062 binds several proteins including Src homology 2 domain containing transforming protein 1 (SHC), SHC transforming protein 3, insulin receptor substrate 1/2, fibroblast growth factor receptor substrate 2, docking protein 1/4/5 and Enigma. S696: serine-696 PKA phosphorylation site, 804: a structural determinant for RET sensitivity to ZD6474 is shown.

Ref: Wells and Santoro 2009.

DOK: docking protein, FRS: FGFR substrate, GDNF: glial-derived neurotrophic factor, GFLs: glial-derived neurotrophic factor family ligands, GFRα: glial-derived neurotrophic factor family receptor; GRB, GFR bound protein, IRS: insulin receptor substrate, PLC: phospholipase C, SHC: Src homology 2 domain containing transforming protein 1, SHC C: SHC transforming protein 3, SRC: protein kinase SRC, ART: Artemin, NTN: Neurturin, PSP: Perseptin

1.4.2. Functions of RET

RET has a role in the regulation of cell growth, differentiation, survival and migration (Tallini and Asa 2001). RET receptor is expressed predominantly in tissues of neuroendocrine origin. RET is required for growth and development of uro-genital system and enteric lymphoid and neuronal tissue. Gene-knockout studies also revealed that RET signalling is essential for kidney development as well as in the maintenance and regeneration of various neurons of the enteric nervous system (Schuchardt *et al.* 1994; Sanchez *et al.*

1996). It is also expressed in interstitial and circulatory immune cells (B and T-cells, monocytes and natural killer cells) (Mulligan 2014). In mice, RET is essential for development of sympathetic, parasympathetic and enteric nervous systems and kidneys (Pachnis *et al.* 1993; Schuchardt *et al.* 1994). Increasing (gain-of-function) or decreasing (loss-of-function) RET expression lead to various abnormalities including thyroid, small-cell lung, breast, melanoma, renal, prostate and head and neck cancers (Mulligan 2014). RET was first reported as frequently activated in human PTC (Fusco *et al.* 1987) and was found also to be involved in lymph node metastases (Grieco *et al.* 1990).

1.4.3. RET recombination and activation

The RET transforming gene was first isolated by Takahashi *et al.* following transfection of NIH/3T3 cells with DNA of human T cell lymphoma (Takahashi *et al.* 1985). Firstly, four aberrant transcripts of 4-7kb were reported, hybridizing only kinase domain encoding probes and not to those of transmembrane and extracellular domains of proto-RET in TPC-1 human PTC cell line (Ishizaka *et al.* 1989). RET was later found activated on recombination between two unlinked human DNA segments (Takahashi and Cooper 1987): the RET proto-oncogene (Takahashi *et al.* 1989) encoding a putative tyrosine kinase receptor and a gene coding for a putative zinc finger protein; rfp (Takahashi 1988; Takahashi *et al.* 1988). Subsequently, the RET gene was found active by rearrangements on transfection in human gastric (Koda 1988) and colon (Ishizaka *et al.* 1989) carcinomas DNAs. Similarly, RET rearrangements were detected as active recombinations in all the transfected samples and metastasis tumours *in vivo*, except in the original transfected DNA in the NIH/3T3 cell line (Grieco *et al.* 1990).

1.4.3.1. Radiation exposure and RET recombination

There exist a direct link between radiation exposure and PTC development following RET/PTC rearrangements. Development of RET/PTC is an early event in the process of thyroid carcinogenesis and thus plays a critical role in the generation of papillary carcinoma (Viglietto *et al.* 1995). RET gene is a primary element affected by ionizing radiations which are direct inducers to activate rearrangements of this gene. There are several pieces of evidence to support this argument. RET/PTCs rearrangements were frequently observed in the thyroid carcinomas post-radiation exposure in the areas of atomic accidents (Di Cristofaro *et al.* 2005; Nikiforov 2006; Unger *et al.* 2006). Irradiations even also induce formation of RET/PTC fusions *in vitro* and *in vivo* (Ito *et al.* 1993; Mizuno *et al.* 2000; Caudill *et al.* 2005; Machens *et al.* 2006; Gandhi *et al.* 2010). Exposure of cell lines not expressing RET/PTCs

rearrangements [8505C (human undifferentiated thyroid carcinoma cells), Daudi (Burkitt lymphoma cell line), G-401 (Wilms' tumor or nephroblastoma cell line) and HT1080 (fibrosarcoma cell line)] and fetal thyroid explants in scid mice to 50 Gy X-ray ionizing radiations results in induction and stable expression of RET/PTCs in the models (Ito *et al.* 1993; Mizuno *et al.* 1997; Mizuno *et al.* 2000). The involvement or the presence of RET rearrangements in PTC was firstly confirmed by RT-PCR (Reverse transcription PCR), XL-PCR (Xtra long-PCR), Southern blot and direct DNA sequencing techniques in 1997. In the study, 39 post-radiation thyroid tumors (19 PTCs and 20 follicular adenomas from patients received radiotherapy for benign or malignant disorders) and 39 spontaneous tumours (20 PTCs and 19 follicular adenomas) were analysed. RET rearrangements were found to be present in 84% PTCs (16/19) and 45% follicular adenomas (9/20). Among spontaneous tumors, only 15% PTCs (3/20) presented RET rearrangements. It was concluded that RET play crucial role in activating rearrangements and tumour development after therapeutic or accidental ionizing irradiations and that rearrangements are even present in follicular adenomas after radiation exposure (Bounacer *et al.* 1997).

1.4.3.2. Mechanism of RET recombination

The genetic hallmarks of PTC are chromosomal inversions or translocations that cause recombination of the intra-cellular kinase-encoding RET domain with heterologous genes, thus causing dimerization of the domain and generating RET/PTC chimeric oncogenes and resulting chimeric oncoproteins Santoro *et al.* 2004a; Santoro *et al.* 2004b) (Santoro *et al.* 2002; Santoro *et al.* 2004; Santoro *et al.* 2004). In normal thyroid follicular cells, there is no or very low RET expression and thus RET activation usually occurs on chromosomal rearrangements with other genes (Menicali *et al.* 2012). On radiation exposure, RET is activated in thyroid on generation of oncofusions with partner genes via intra-chromosomal inversions or inter-chromosomal translocations where ionizing radiations lead to fusion of 3' portion (intracellular RET tyrosine kinase) of RET gene with 5' end (N-termini) of other genes (Figure 5) (Rabes 2001; Santoro *et al.* 2004; Romei and Elisei 2012). The radiations are the main causative factor for RET genetic recombinations and lead to topoisomerase I or II-mediated breakage in both DNA strands (Weterings and Chen 2008, Dillon *et al.* 2013, Xing 2013) at chromosomal fragile sites *i.e.* genome regions highly susceptible to DNA damage/breakage and chromosomal translocations (Burrow *et al.* 2009; Menicali *et al.* 2012) in all developed oncogenic rearrangements. These DNA breaks are usually repaired by two pathways; either by homologous recombinations or by non-homologous end joining (NHEJ;

(Rothkamm *et al.* 2001; Shrivastav *et al.* 2008) depending on the proximity of the two broken ends (Yates and Morgan 1993; Rothkamm *et al.* 2001).

The dysfunction of these normal repair mechanisms leads to development of chromosomal translocations (Gasparini *et al.* 2007). It has recently been showed by using FISH and ligation-mediated PCR (LM-PCR) analysis that RET and its fusion partners CCDC6 and NcoA4 genes share common FRA10C and FRA10G fragile sites (Burrow *et al.* 2009, Gandhi *et al.* 2010) and thus undergo genetic recombinations on DNA breakage after exposure to damaging stimuli. Experimental chemical stimulus with aphidicolin or gamma-radiation exposure to HTori-3 immortalized human thyroid cells (Caudill *et al.* 2005, Gandhi *et al.* 2010) and X-rays exposure to 8505C cells (human undifferentiated thyroid carcinoma cells) (Ito *et al.* 1993) or human normal thyroid tissues transplanted in scid mice (Mizuno *et al.* 1997, Mizuno *et al.* 2000) also resulted in the formation of RET/PTCs rearrangements. Thus, it can be concluded that on ionization exposure, DNA double-strand breaks (DSBs) are formed and since fusion participating genes are localized at common fragile sites, so during the breaks repair process they lead to formation and expression of RET/PTCs fusion rearrangements in the thyroid cells.

1.4.3.3. Activation of chimeric protein

During this gene translocation and recombination event, RET gene is truncated at the level of juxta-membrane position thus lose extra- and trans-membrane domains that exert negative regulatory constraints. Left-over intracellular tyrosine kinase domain fuses with N-terminal fragment of a heterologous partner gene to give rise to various RET/PTC oncofusions (Figure 5). As the inhibitory domains are located in the RET N-terminal which are lost during gene breakage thus the expression level of the fusion product is controlled by the promoter of the partner gene (Medves and Demoulin 2012) which drives a constitutive ligand-independent activation and expression of the resulting chimeric gene product and thus an illegitimate expression of the fusion protein (Santoro *et al.* 2004a, Puxeddu *et al.* 2005, Medves and Demoulin 2012). The partner gene is predicted to provide one or more coiled-coil domains, essential for dimerization and ligand independent activation of the truncated RET gene (Bongarzone *et al.* 1993, Tong *et al.* 1997, Monaco *et al.* 2001). The relocalization of the chimeric protein to the cytosol also contributes in the activation of the fusion product (Castellone and Santoro 2008). The truncated gene is devoid of N-terminal inhibitory domains and also the juxta-membrane domain; an integral part of the auto-inhibited RET dimer interface (Griffith *et al.* 2004, Knowles *et al.* 2006, Medves and Demoulin 2012),

hence TK domain is favored to adopt a constitutively active conformation. Furthermore, the subcellular aberrant localization of the rearranged protein not only prevents RET/PTC interaction with negative regulators present at plasma membrane *i.e.* tyrosine phosphatase like Protein Tyrosine Phosphatase Receptor type-J; PTPRJ (Iervolino *et al.* 2006) but also protects it from receptor mediated endocytosis and lysosomal degradation (Richardson *et al.* 2009) thus leading to an increased protein stability. Additionally, it avoids normal endosomal trafficking and thus are poorly regulated by ubiquitin-mediated lysosomal targeting (Richardson *et al.* 2009).

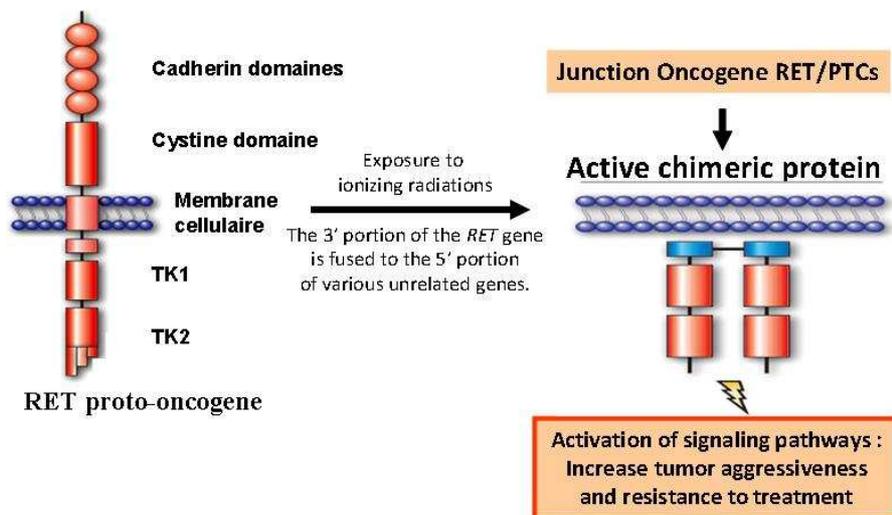


Figure 5: Mechanism of RET recombination and activation. Under the effect of ionizing radiations, RET gene fuses with another gene to give rise a junction oncogene. This fusion oncogene encodes a chimeric protein, which becomes cytoplasmic and constitutively active, to further activates the downstream signaling pathways leading to generation of a cascade of events for tumour progression.

1.4.3.4. Effect of RET recombination on tumour progression

RET/PTC expression causes TSH-independent proliferation of the thyroid follicular cells similar to cell growth in the presence of TSH. PC-PTC cells; PCCL3 (parental well differentiated rat thyroid cell line cells) with stable RET/PTC1 expression showed similar growth pattern in the absence of TSH as that of PCCL3 cells in the presence of TSH at 6 hrs post-treatment (Santoro *et al.* 1993). Over expression of either of RET/PTC1 or RET/PTC3 in transgenic mice leads to tumour development with papillary histological features (Jhiang *et al.* 1996; Santoro *et al.* 1996; Powell *et al.* 1998). The presence of RET fusions has also been correlated in a prospective multicentric study on 125 subjects where the growth rate of benign thyroid nodules was followed during 36 months which were found to grow at a higher rate than those having wild type RET (Sapio *et al.* 2011).

Numerous studies have contributed to highlight the transforming properties of RET as well as to identify the signal transduction pathways that participate in these neoplastic

transformations (Putzer and Drosten 2004, Wixted *et al.* 2011). All these pathways contribute to the cellular transformation after elevated and sustained RET activation while under normal conditions it is mediated in survival signalling pathways, of whom the details has yet to be illustrated. Thus, RET fusions lead to the up-regulation of genes involved in cell differentiation, growth and proliferation, cell signalling, neo-angiogenesis, tumour invasion and metastasis, adhesion (L-selectin) and extra-cellular matrix degradation (MMPs, uPA, uPA-R) etc (Hwang *et al.* 2003, Borrello *et al.* 2005, Wells and Santoro 2009, Bozec *et al.* 2010, Menicali *et al.* 2012). Once translocated and combined with RET oncogene, RET fusion partner leads to constitutive activation of effectors along the RAS-RAF-MEK-MAPK pathway (Kimura *et al.* 2003; Soares *et al.* 2003), either by the formation of RET/PTC fusion oncogenes (Santoro *et al.* 2002) or activating point mutations in RAS (Lemoine *et al.* 1988; Namba *et al.* 1990; Suarez *et al.* 1990) or most notably BRAF genes (Cohen *et al.* 2003; Fukushima *et al.* 2003; Kimura *et al.* 2003; Namba *et al.* 2003; Soares *et al.* 2003; Puxeddu *et al.* 2004).

RET/PTC oncofusion activates the RAS-RAF-MEK-MAPK, PI3K-AKT-mTOR (Phosphatidylinositol-3 Kinase-mammalian target of rapamycin) and calcium-calmodulin pathways (Miyagi *et al.* 2004, Castellone *et al.* 2009, Guerra *et al.* 2013) by recruiting signalling adaptors to phosphorylated Tyr1062 on the intracellular domain of the RET fusion protein (Hayashi *et al.* 2000). That's the reason that RET/PTC is detected in follicular thyroid tumours (FTA and FVPTC) where the PI3K-AKT pathway has a primary role in tumorigenesis (Xing 2010a) whose proliferative function is mediated by mTOR. It was also shown that additionally to MAPK and PI3K-AKT pathways; which are required for cellular transformation, RET/PTCs also activate additional TRAF pro-inflammatory pathways to further outline the tumour progression (Wixted *et al.* 2011). Thus generation of onco-fusions leads to start of a cascade of events that completely modify the conformation and properties of the transformed compared to original structure.

1.5. RET mutations, translocations and oncofusions in PTC

RET is a paradigm of a single TK gene that induces different types of human cancers depending on the mutation or transformation and thus represents one of the most frequently occurring disorders found in human malignancies (Futreal *et al.* 2004). Oncogenic conversion of TK receptor occurs *via* four main mechanisms: retroviral transduction, genomic rearrangements, point mutations or overexpression (Blume-Jensen and Hunter 2001). Since, it is an important regulator of intracellular signal transduction but if mutated or

structurally altered, it becomes a potent oncogene, causing cellular transformations by enhanced constitutive kinase activity with qualitatively and quantitatively altered downstream signaling (Blume-Jensen and Hunter 2001).

Different point mutations at specific sites in the extra-cellular cysteine rich domain or intra-cellular domains (Figure 4) in the RET protein lead to constitutive activation of RET-TK activity either by promoting dimerization or by altering conformation and favouring kinase activity and are thus responsible for the development of the various cancer syndromes (Jhiang 2000, Mulligan 2014). It is well established that gain- or loss-of-function germline mutations in PTKs (Grieco *et al.* 1990; Putzer and Drosten 2004; Lodish and Stratakis 2008) and/or by gene amplification (Pai *et al.* 2006) are responsible for the development of PTC and MTC and a subset of other rare malignancies like pheochromocytomas and paraganglioma (Burnichon *et al.* 2011). Thus germline point mutations in a single amino acid substitutions in the extracellular cysteine-rich domain of RET cause three related inherited cancer syndromes: multiple endocrine neoplasia type 2 (MEN2A and 2B) (Jhiang 2000) and familial MTC (Eng *et al.* 1996; Brandi *et al.* 2001; Santoro *et al.* 2004) in more than 95% of patients (Figure 6) (Mulligan 2014). These substitutions make unpaired cysteine residue capable to form intermolecular bonds and ligand-independent receptor dimerization and constitutive RET signaling (Asai *et al.* 1995).

Familial MTC arise by mutation in the intracellular domain (where Y791F or S891A mutations lead to ligand independent activation), is characterized by MTC without other features and is generally the most indolent form of disease with a disease onset at a later age. MEN2 is an autosomal dominant multi-tumour syndrome and is characterized by early onset of MTC. Its sub-type; MEN2A is the most common disease, found in more than 90% cases, is formed by constitutive activation of the kinase either as monomer or dimer, ligand activation further increase the kinase activity and is characterized by MTC and pheochromocytoma (neuroendocrine tumor of adrenal medulla, in about 50% of cases) and parathyroid hyperplasia or adenoma and/or the skin rash condition (lichen planus amyloidosis, in one-third of patients). MEN2B is the most severe disease sub-type, is characterized by MTC and pheochromocytoma with other developmental anomalies consistent with known roles of RET in embryonic development, including marfanoid habitus (elongated long bones and fingers, as well as hyperflexible joints), ganglioneuromas (neuronal tumors) of the mouth and gut and delayed puberty (Mulligan 2014). Other rare gain-of-function somatic mutations also include lung adenocarcinoma (Kohno *et al.* 2012,

Lipson *et al.* 2012) and chronic myelomonocytic leukaemia (Ballerini *et al.* 2012). Conversely, loss of function RET mutations lead to congenital aganglionosis of the gastrointestinal tract, leading to congenital aganglionic megacolon or Hirschprung's disease (Angrist *et al.* 1996, Manie *et al.* 2001, Airaksinen and Saarma 2002).

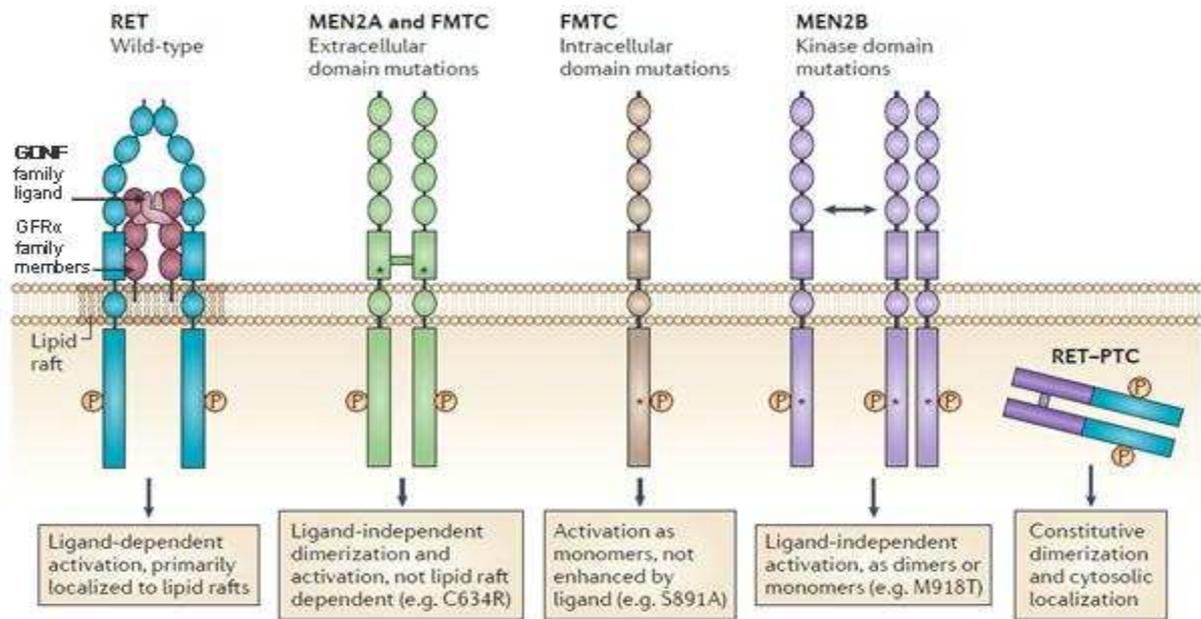


Figure 6: Molecular mechanisms of oncogenic RET mutations. The activation of wild-type RET requires the binding of a ligand (GDNF family member, GFL) and co-receptor (GFR α family member) complex. Each of these ligands interacts with RET via a cell surface co-receptor of the GDNF family receptor- α (GFR α) family members, which consists of two to three globular protein domains linked to the cell membrane by a glycosyl-phosphatidyl-inositol anchor. Each GFL interacts primarily with a GFR co-receptor to form a hetero-dimeric complex which then recruit RET into lipid raft membrane subdomains. Once bound to the ligand-co-receptor complex, conformational changes facilitate RET monomer association via the cadherin domains and lead to receptor dimerization and auto-phosphorylation (P). However, mutant forms of RET identified in human cancer can have one of several different activation mechanisms. Mutations (indicated by asterisks) of the extracellular domain which are found in multiple endocrine neoplasia type 2A (MEN2A) and familial MTC (FMTC), lead to constitutive dimerization of mutant RET which may occur anywhere in the membrane (not just within lipid rafts). FMTC RET mutations that affect the intracellular domain, such as Y791F or S891A, do not seem to require any ligand interaction for activation. These proteins are phosphorylated (P) and signal as monomers (again, not limited to lipid rafts) and this does not seem to be increased by the presence of ligands. The most severe group of RET mutations, which is found in MEN2B, leads to altered conformation, increased ATP-binding and constitutive activation of the kinase. The kinase activity can be further increased by the presence of the RET ligands which suggests activity either as monomeric forms or dimers. In addition, chimeric rearrangements of RET, as seen in PTC (RET/PTC), lung adenocarcinoma or chronic myelomonocytic leukaemia result in constitutively active cytosolic proteins. These proteins are able to dimerize via domains that are located in the amino-terminal regions of the chimeric protein which arise from the partner gene sequences that are fused upstream of the RET kinase domain. Ref. Mulligan 2014.

The RET/PTC fusion oncogenes are only present in the papillary carcinomas cells while normal thyroid follicular cells are devoid of these fusions and even have very low or no expression of wild type RET (Menicali *et al.* 2012). The low expression is due to presence of inhibitory domains in the extra-cellular N-terminal region (Medves and Demoulin 2012). BRAF^{V600E} is the most common genetic mutation found in PTC, usually present at advance

stages of cancer (Guerra *et al.* 2013). RET/PTC rearrangements were demonstrated by immuno-histochemical studies as a fraction of BRAF-mutated PTCs (Xu *et al.* 2003) but others concluded that BRAF mutation and RET/PTC rearrangements are alternative events in PTC (Kimura *et al.* 2003, Soares *et al.* 2003, Guerra *et al.* 2012b). Only rare PTCs harbor both oncogenes (Henderson *et al.* 2009) and the cases found to harbour dual alterations are usually associated with a higher rate of disease recurrence (Henderson *et al.* 2009). But recently simultaneous occurrence of BRAF mutation and RET/PTC rearrangement has been found by pyro-sequencing and Southern blot analysis of RT-PCR products in about 20% cases i.e. 14 PTCs out of 72 total samples. In other words, half of the tumours harbouring RET/PTC were found to have BRAF^{V600E} and thus was concluded that concomitant appearance of the two is a frequent event in PTC (Guerra *et al.* 2013). The role of RAS oncogene in thyroid carcinogenesis has been extensively studied (Unger *et al.* 2004, Guerra *et al.* 2011, Lee *et al.* 2011, Vitale 2012) and is more oftenly found in FTC and ATC while approximately 10% of PTCs harbour RAS (Omur and Baran 2014). The occurrence of all three RET/PTC fusion, BRAF and RAS mutations in a single PTC has been debated for long time and now has been accepted as a rare co-occurrence. Thus, there is a need to explore more crucial genetic events in relation with RET/PTC rearrangement in PTC progression (Vitale 2013).

Although, RET/PTC rearrangements are usually found in radiation-induced tumours but have also been detected in variable proportions of sporadic or non-radiation associated papillary carcinomas. RET/PTC fusions are more commonly found in classical PTCs but can also occur in benign FTA (follicular thyroid adenoma) and follicular variant of PTC (FTC) (Marotta *et al.* 2011). RET rearrangements were found in 84% (16/19) of papillary carcinomas and 45% (9/20) of follicular adenomas in the patients received external radiations for benign or malignant conditions (Bounacer *et al.* 1997). Similarly, somatic chromosomal rearrangements of RET (RET/PTC) are found in both sporadic (5-30%) and radiation-induced (60-70%) PTCs (Jhiang 2000). As an estimate up to approximately half of the PTC cases in the United States and Canada harbour RET/PTC rearrangements.

So far about 13 RET/PTCs fusions have been identified wherein RET fuses with 12 different other genes (Menicali *et al.* 2012, Romei and Elisei 2012) but the most commonly found rearrangements are: RET/PTC1; an inversion and fusion of RET with H4/CCDC6 [coiled-coil domain-containing gene 6, position:10q21, inv(10)(q11.2q21] Grieco *et al.* 1990), followed by RET/PTC3; occurs between RET and ELE1/NcoA4 (nuclear receptor co-

activator 4, position:10q11.2, Bongarzone *et al.* 1994, Santoro *et al.* 1994) and occasionally RET/PTC2; RET+RI α encoding the regulatory subunit of protein kinase A [a translocation of 3' RET-TK with 5' RI α : t(10;17)(q11.2;q23)] (Sozzi *et al.* 1994, Tallini and Asa 2001). Both RET/PTC1 and RET/PTC3 are intra-chromosomal paracentric inversions because RET and the fusion partner genes (H4/CCDC6 and ELE1/NcoA4) are located on the same chromosome 10q (Grieco *et al.* 1990, Pierotti *et al.* 1992, Minoletti *et al.* 1994, Santoro *et al.* 1994). While RET/PTC2; a chromosomal translocation between RET (at chromosome 10) to RIA gene (on chromosome 17) and other rare fusions are inter-chromosomal translocations (Sozzi *et al.* 1994, Castellone and Santoro 2008, Menicali *et al.* 2012). These RET rearrangements were mostly reported in young people especially in children of 5-18 years, although the pattern of sites of translocation was different (Nikiforov *et al.* 1997).

1.5.1. CCDC6 / H4 / D10S170 (Coiled-coil domain-containing gene 6)

RET/PTC1 is the most commonly found rearranged form of RET in sporadic papillary carcinomas. Combined cytogenetic and molecular analysis demonstrated a paracentric inversion of the long arm of chromosome 10; inv(10) (q11.2q21), at the origin of the fusion of RET with H4/D10S170 (Pierotti *et al.* 1992) where RET fuses with first 101 amino acids of H4/CCDC6 [D10S170; DNA segment on chromosome 10 (unique) 170] (Puxeddu *et al.* 2005; Merolla *et al.* 2007). Virtually, the resulting translocated RET fusion protein is folded into coiled coils hence named (Santoro *et al.* 2006).

The gene encoding H4 is present on chromosome 10q11.2 in humans, on 10B5.3 in mouse and on 9q36 in rat (Gandhi and Nikiforov 2011). H4 is a ubiquitously expressed nuclear and cytosolic protein of 55 kDa molecular weight which is phosphorylated by extracellular signal-regulated protein kinase following serum stimulation and is demonstrated to have pro-apoptotic activity (Celetti *et al.* 2004). It not only provides a recombination domain of 60 amino acid fragment (Merolla *et al.* 2007) for ligand independent activation of the RET tyrosine kinase in PTC but also recombines with PDGFR β gene located on chromosome 5, in a typical chronic myelogenous leukemia characterized by a t(5;10)(q33;q22) translocation (Kulkarni *et al.* 2000; Schwaller *et al.* 2001). H4/PTEN fusion was also reported in cell lines derived from spontaneous or after ionizing radiation exposure benign and malignant human thyroid tumors and in human PTC specimens (Puxeddu *et al.* 2005). This suggests that H4 gene has high susceptibility for recombination with various partners (Merolla *et al.* 2007).

CCDC6 (or H4) was the first RET-associated oncogene discovered in 1990 by transfection assay (Grieco *et al.* 1990) and cloned and sequenced in 1994 (Grieco *et al.* 1994). H4 nucleotide sequence of 3 kb transcript has an open reading frame (ORF) of 585 amino acids and the respective protein has no transmembrane domain. The extensive regions in the alpha helical conformation have a 30% homology to the same domains of several other proteins like tropomyosin, vimentin, keratin and the tail region of myosin heavy chain. A putative SH3 binding site present at the carboxy terminus suggests its role as a cytoskeletal protein (Grieco *et al.* 1994). Primer extension assay revealed the transcriptional start sites of H4 to be localized in a region of 100-190 bp upstream of the translation initiation site (ATG) while the luciferase assay H4 promoter was found in a region at 259 bp upstream of the ATG site (Tong *et al.* 1995). Interestingly, protein sequence analysis indicated a potential coiled-coil domain in the N-terminal region of H4 (Tong *et al.* 1995).

1.5.2. NcoA4 / ELE1 / RFG (Nuclear receptor coactivator 4)

RET/PTC3 (RET/ELE1) fusion was firstly detected in 6 out of 18 PTC cases positive for RET fusions (Bongarzone *et al.* 1994). Northern blot and PCR analysis with ELE1 specific oligonucleotides showed that it is ubiquitously expressed in different human tissues including normal thyroid gland and using a panel of human-rodent somatic cells hybrid DNAs it was determined to be localized on chromosome 10 (Bongarzone *et al.* 1994). The hybrid fusion RET/PTC3 gene was found to be associated with synthesis of 2 constitutively phosphorylated isoforms (p75 and p80) of the oncoprotein in Western blot (Bongarzone *et al.* 1994; Santoro *et al.* 1994) which were generated by alternative splicing of the C-terminal portion of RET gene (Bongarzone *et al.* 1994). Concurrently, in another laboratory, this oncogenic version of RET proto-oncogene was concomitantly identified in a case of PTC where constitutively active tyrosine kinase domain of RET was reported to fuse with a new 790 bp long sequence; was named accordingly as Ret-Fused Gene (RFG) (Santoro *et al.* 1994). Despite to have the homology in the sequence, the gene was named differently by different laboratories accordingly as it was detected.

Later, the members of multiple nuclear receptor co-regulatory proteins were firstly introduced on discovery of proto-typical nuclear receptor coactivator; Steroid Receptor Coactivator 1 (SRC1) in 1996 (Katzenellenbogen *et al.* 1996). This family of co-regulatory proteins includes both coactivators and corepressors and plays an important role in modulating physiological and pathological functions of steroid hormone and of other nuclear receptors. Nuclear receptor coactivator-4 (NcoA4) is one of the family members and plays a

role in development, carcinogenesis, inflammation, erythropoiesis and cell cycle progression (Kollara and Brown 2012). It is found to ubiquitously express in adult mouse tissues including heart, adrenal gland, lung, intestine, spleen, skeletal muscle and brain (Yeh and Chang 1996; Siriatt *et al.* 2006; Kollara and Brown 2010) excluding cerebral cortex (Yeh and Chang 1996) while there is controversy about presence (Siriatt *et al.* 2006) and absence (Yeh and Chang 1996) of hepatic expression. Recent studies suggest that NcoA4 may also play role in cancer development and progression that may be independent of its role as a receptor coactivator (Kollara and Brown 2012).

1.5.2.1. Structural properties of NcoA4

The gene encoding NcoA4 is found on chromosome 10q11.2 in humans, 14B in mouse and on 16p16 in rat (Gandhi and Nikiforov 2011). The gene consists of ten exons in both species, resulting in mature transcripts encoding proteins of 614 and 625 amino acids in human and mouse respectively (<http://www.ensembl.org/>). Two NcoA4 transcript variants have been identified to date in humans: a full-length NcoA4 cDNA that encodes a 614 amino acid protein (NcoA4 α) with an estimated molecular weight of 70 kDa (Yeh and Chang 1996) and a shorter cDNA possessing an internal 985 base pair deletion encoding a 296 amino acid protein (NcoA4 β) with an estimated molecular weight of 35 kDa (Alen *et al.* 1999). Amino acids 37-167 and 198-332 of the human NcoA4 sequence correspond to evolutionarily conserved regions referred to as ARA70 family domains I and II respectively. Human NcoA4 α and ARA70N contain two nuclear receptor interaction motifs: an LXXLL motif (LYSLL, where L = leucine, Y = tyrosine and S = serine), initially identified in p160 family members (Heery *et al.* 1997), located in the N-terminal region (92-96 amino acids) and a FXXLF motif (FKLLF, where F = phenylalanine, K = lysine and L = leucine) at 328-332 amino acids (Heinlein *et al.* 1999; Zhou *et al.* 2002). LXXLL motif is involved in the interaction of NcoA4 with PPAR γ , VDR and TR (Heinlein *et al.* 1999; Moore *et al.* 2004; Ting *et al.* 2005) while FXXLF motif interacts with ligand binding domain (LBD) of AR and PR (Zhou *et al.* 2002; Hsu *et al.* 2003; Hu *et al.* 2004; Dubbink *et al.* 2006). Site-directed mutagenesis studies provided the evidence that FXXLF motif is essential for NcoA4–AR and NcoA4–PR interactions (Hsu *et al.* 2003; Dubbink *et al.* 2006; van de Wijngaart *et al.* 2006). Although exogenous androgens like dihydrotestosterone (DHT) and testosterone promote NcoA4–AR interaction (Yeh and Chang 1996; Hsiao and Chang 1999) but this interaction could also occur in the absence of exogenous androgen (Alen *et al.* 1999). The mouse full length NcoA4 sequence is notably different from human sequence and devoid of LXXLL

motif and thus only contains FXXLF motif at amino acids 334–338 (Kollara and Brown 2012) (Figure 7).

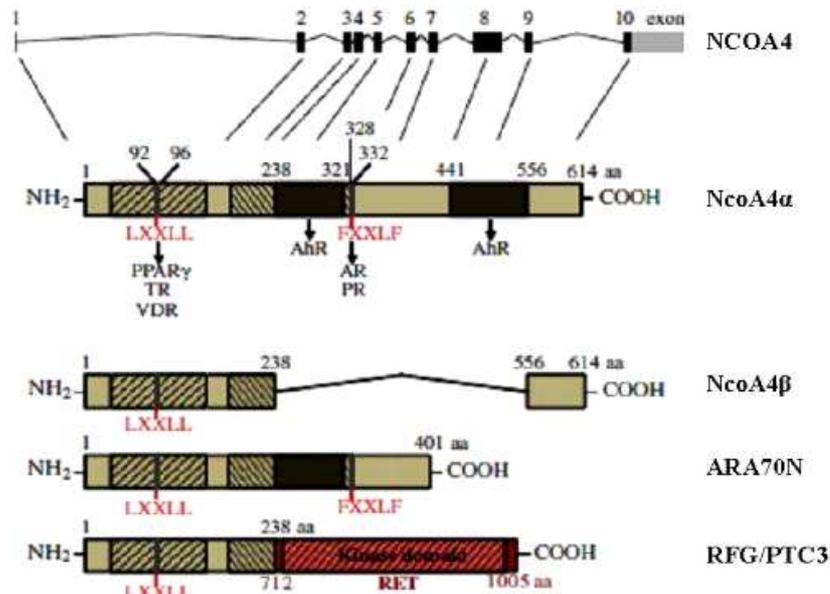


Figure 7: Schematic representation of human NCOA4 variants, ARA70N and RFG/PTC3. Ensembl Human Genome Browser software indicates the NCOA4 gene consists of 10 exons. The full length NcoA4 cDNA encodes a protein of 614 amino acids (NcoA4 α). Conserved ARA70 domain I (amino acids 37–167) and domain II (amino acid 138–332) are shown as hatched areas and include a FXXLF and LXXLL motif. The FXXLF motif located at amino acids 328–332 is involved in interaction with AR and PR. The LXXLL motif located at amino acids 92–96 is involved in interaction with VDR, TR and PPARc. Amino acids 231–321 and 441–556 are necessary for optimal AhR co-activation. The shorter NcoA4 variant (NcoA4 β) lacks internal amino acids 238–556 of NcoA4 α and lacks the FXXLF motif and most of ARA70 domain II. ARA70N consists of the first 401 amino acids of NcoA4 α and thus contains both the LXXLL and the FXXLF motifs. RET Fused Gene (RFG/PTC3) is a fusion protein consisting of the first 238 amino acids of NcoA4 joined to amino acid 712 of the C-terminal region of RET. This fusion protein thus contains the RET tyrosine kinase domain at amino acids 724–1005. Ref: Kollara and Brown 2010.

1.5.2.1. Functional properties of NcoA4

Yeh and Chang firstly described the function of intact NcoA4 gene product in yeast two-hybrid screen as an androgen receptor (AR) interacting protein and was shown to potentiate AR transcriptional activity (Yeh and Chang 1996). Thus was referred to as Androgen Receptor (AR)-associated protein 70 (ARA70) since it was initially thought to be AR-specific. However, subsequent studies indicated that NcoA4 interacts with and regulates the function of various other nuclear receptors including estrogen (ER) (Lanzino *et al.* 2005), progesterone (PR) (Gao *et al.* 1999), glucocorticoid (GR) (Gao *et al.* 1999), vitamin D (VDR) (Ting *et al.* 2005), thyroid hormone (THR) (Moore *et al.* 2004), peroxisome proliferator-activated α and γ (PPAR α γ) (Heinlein *et al.* 1999; Heinlein and Chang 2003) and aryl hydrocarbon (AhR) receptors. It has also been detected in both female (uterus and ovary) and male (prostate, testis, seminal vesicles, and epididymis) murine reproductive tissues (Thin *et al.* 2002; Kollara and Brown 2010) and found highly expressed in mouse ovarian surface

epithelial (OSE) cells compared to other ovarian cells. Gene expression profiling of these murine OSE cells at different reproductive stages indicated the highest NcoA4 expression at pro-estrus compared to estrus, mid-pregnancy and juvenile stages (Gava *et al.* 2008). Similarly, in human uterine myometrium showed higher NcoA4 levels in pregnant compared to non-pregnant tissues (Rehman *et al.* 2003); suggesting that reproductive hormones regulate its expression. Real time RT-PCR also detected NcoA4 mRNA expression in human luteinized granulosa cells in oocytes from *in vitro* fertilized patients (Chang *et al.* 2005) and was positively correlated with ER α and aromatase expressions. Thus, NcoA4 have significant contribution in development of follicles and hormonal regulation during various reproductive stages.

1.5.2.3. Role of NcoA4 as an androgen receptor co-regulator

NcoA4 is mostly involved in increasing the transcriptional activity of AR (Kollara and Brown 2012) to at least 10 folds by ligand-dependent interaction (Yeh and Chang 1996; Yeh *et al.* 1999). However, most of the studies were performed with ARA70N rather than full length NcoA4 and thus it was established that N-terminal portion of the protein provides optimal AR interaction and transactivation enhancement (Yeh *et al.* 1999; Yeh *et al.* 1999; Thin *et al.* 2002; Rahman *et al.* 2003; Chen and Chang 2007). The studies with full length NcoA4 generally report lower levels of AR potentiation in the range of 2-6 folds (Alen *et al.* 1999; Gao *et al.* 1999; Zhou *et al.* 2002; Bonagura *et al.* 2007; Kollara and Brown 2010; Wu *et al.* 2011) which suggests that C-terminal region might harbors inhibitory sequences. Similarly, though N-terminal region (amino acids 1-501) of mouse NcoA4 interacts directly with the LBD of AR but full length sequence is not able to interact with AR (Siriett *et al.* 2006), which further supports the existence of C-terminal inhibitory effects. Interestingly, despite lacking the FXXLF motif and flanking residues, NcoA4 β is also an effective AR coactivator as NcoA4 α (Alen *et al.* 1999; Peng *et al.* 2008), likely due to its interaction with the N-terminal AR domain.

A unique aspect of NcoA4 activity at the AR is its reported ability to diminish ligand specificity (Kollara and Brown 2012). Over-expression of NcoA4 has been shown to enhance AR transactivation induced by ligands other than active androgens like 17 β -estradiol (E2) (Yeh *et al.* 1998; Han *et al.* 2001; Chen and Chang 2007), androstene-3 β ,17 β -diol (Adiol) (Miyamoto *et al.* 1998) and the phytoestrogen daidzein (Chen and Chang 2007) which may have negative implications for the success of hormonal therapies used in the treatment of prostate cancer. Other ER ligands such as diethylstilbestrol (DES), estrone, 17 α -estradiol and

tamoxifen fail to induce AR transactivation in the presence of NcoA4 overexpression, indicating that this effect is ligand-specific (Yeh *et al.* 1998).

1.5.2.4. Potential involvement of NcoA4 in PTC progression

The involvement of NcoA4 in RET/PTC3 fusion oncogenesis has been extensively studied (Santoro *et al.* 1994, Powell *et al.* 1998, Monaco *et al.* 2001, Richardson *et al.* 2009, Gandhi *et al.* 2010, Kollara and Brown 2012). The ligand independent dimerization of RET/PTC protein leads to chronic stimulation of MAPK and PI3K-AKT signalling pathways and thus transformation and increased proliferation of thyroid cells (Richardson *et al.* 2009; Nikiforov and Nikiforova 2011). The oncogene also activates NF- κ B and pro-inflammatory mediators in thyroid follicular cells (Russell *et al.* 2004). Transgenic mice expressing human RET/PTC3 develop thyroid hyperplasia and metastatic cancer (Powell *et al.* 1998).

1.6. RNA interference (RNAi)

RNAi was first discovered in 1998 when dsRNA was found to cause sequence-specific gene silencing in nematode worm *Caenorhabditis elegans* (Fire *et al.* 1998). Three years later, siRNA was successfully demonstrated to be used in mammalian cells for specific gene knockdown (Caplen *et al.* 2001; Elbashir *et al.* 2001). The first successful use of siRNA for gene silencing in mice was started from hepatitis C shortly thereafter (Spankuch and Strebhardt 2005). Since then RNAi has been extensively used for the treatment of various diseases including cancer (Davis *et al.* 2010, Gilbert-Sirieix *et al.* 2010, Hobel *et al.* 2010, Koga *et al.* 2010, Gunther *et al.* 2011, Feng and Guang 2013, Mollaie *et al.* 2013).

RNA interference (RNAi) is a powerful gene-silencing process that holds great promise in the field of cancer therapy. RNAi is a post-transcriptional mechanism of gene silencing through chromatin remodelling, direct mRNA degradation or inhibition of protein translation (Caplen 2004, Dorsett and Tuschl 2004, Shankar *et al.* 2005, Ramachandran and Ignacimuthu 2013). It can be induced by introduction of single stranded DNA or RNA sequences of 13–25 bases called as antisense oligonucleotides (AS-ONs), by plasmid DNA (pDNA) that expresses short hairpin RNAs (shRNAs) which subsequently processed to siRNAs by cellular machinery (McManus and Sharp 2002; Scherr *et al.* 2003; Kim and Rossi 2007) or by synthetic small interfering RNAs (siRNAs) consisting of 19-27 base pairs in length. The silencing activity by these major approaches is highly sequence specific and although is due to their base-pairing capacity with complementary cellular nucleic acid sequences but their method of gene inhibition is relatively different.

1.6.1. Antisense Oligonucleotides

AS-ONs selectively inhibit the expression of target genes by hybridizing with their specific 'sense' sequences in messenger RNA (mRNA) or DNA (Akhtar and Agrawal 1997) molecules. Ribonuclease H (RNase H) takes up these molecules and help in the degradation of complementary target mRNA in a catalytic manner (Walder and Walder 1988, Eder and Walder 1991, Schultz and Champoux 2008, Tadokoro and Kanaya 2009). This enzyme specifically cleaves the target RNA strand of RNA-DNA heteroduplexes, leading to release of AS-ONs and to bind with another target mRNA strand (Walder and Walder 1988; Sitikov 2012).

1.6.2. Small interfering RNAs¹

Synthetic siRNAs are small double stranded RNAs (dsRNAs) and are substrate of RNA induced silencing complex or RISC (Figure 8) (McManus and Sharp 2002). The introduction of foreign dsRNA initiates a potent cascade of sequence-specific degradation of endogenous mRNA after binding a complementary mRNA sequence in the host genome (Fire *et al.* 1998; Wilson and Doudna 2013). A dsRNA, as far as, introduced into the cytoplasm, at first initiation phase, is processed by the RNase-III enzyme Dicer to cleave the long strand into small pieces of 21-23 nucleotide duplexes (Lee *et al.* 2004; Lim do *et al.* 2008) having 5'phosphate and 3'hydroxyl groups with 2'overhangs (Hamilton and Baulcombe 1999; Tuschl *et al.* 1999) and are called as small interfering RNAs. The two strands of this duplex are then separated, the sense strand (passenger strand) is degraded while anti-sense strand (guide or active strand) is used for gene silencing and serves as a template for recognition of homologous mRNA containing a complementary short sequence, usually in the 3'untranslated region. During the second effector phase, the siRNA active product assembles into a multi-protein RISC *via* an ordered pathway (Lim do *et al.* 2008). Then RISC associated siRNA strand is guided to a homologous sequence in a target mRNA and triggering its endonucleolytic cleavage and degradation by a slicer protein; Argonaute-2 (Ago2, hAGO2 in humans; an enzyme of the RISC complex) (Stevenson 2003, Lee *et al.* 2004c, Sontheimer 2005). The target mRNA is thus cleaved at a site in the center of the duplex region between the guide siRNA and the target mRNA, at about 10 nucleotides from the 5'end of the siRNA, resulting in gene silencing (Figure 8) (Elbashir *et al.* 2001a, Elbashir *et al.* 2001b). The anti-sense strand of the siRNA is protected within the RISC complex and therefore is preserved as a catalyst to degrade additional copies of the target mRNA once released from RISC complex

¹ It should be noticed that a review was wrote by me on this field (Ali *et al* 2012).

of first cycle. Although it was initially believed that effective RNAi require almost complete sequence homology throughout the length of the mRNA but it now appears that as few as seven contiguous complementary base pairs can direct an effective RNAi-mediated gene silencing (Jackson and Linsley 2004; Sitikov 2012).

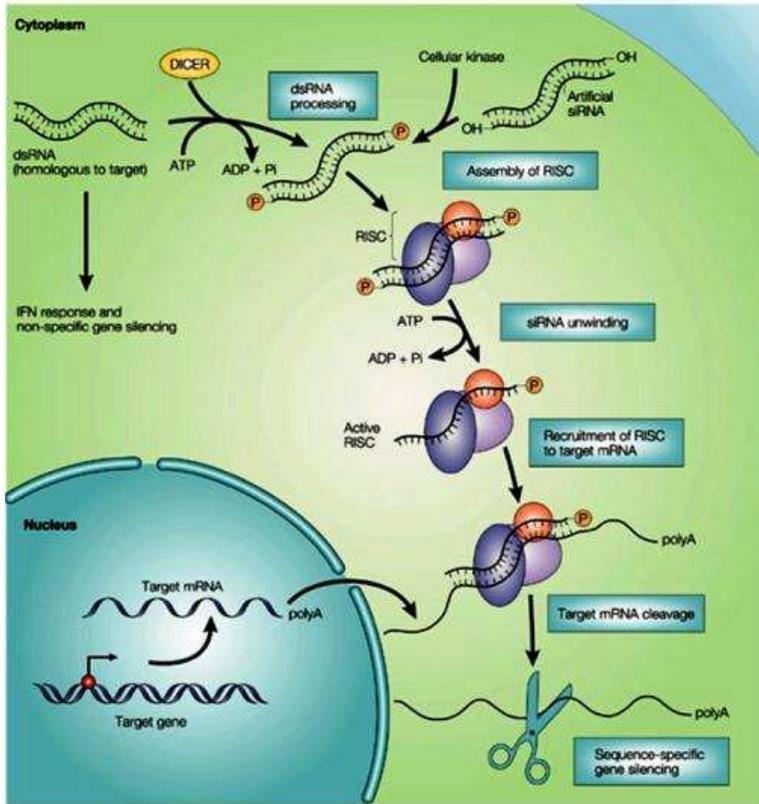


Figure 8: Molecular mechanism of siRNA mediated gene-silencing pathway.

Upon entry into the cytoplasm, an enzyme Dicer processes and trims the double stranded RNAs into short RNA pieces called as small interfering or siRNAs. These processed siRNAs are incorporated into the RNA-induced silencing complex (RISC). The sense strand of the duplex is separated and degraded. While anti-sense strand along with RISC complex attaches with the target messenger RNA, in a sequence specific manner, to trigger its cleavage and degradation by the slicer protein; Argonaute-2, resulting in target gene silencing. Thus siRNA serves as a template for recognition of homologous target mRNA containing a complementary sequence.

These siRNAs sometimes lead to unintended degradation of non-target mRNAs due to partial complementarity in sequence. If siRNAs are recognised as foreign, can lead to activation of immune system by release of type-I interferon (IFN), interleukin-8 and TNF α . These effects are called as off-target effects.

Ref: Stevenson 2003.

1.6.3. Application and uses of RNAi technology

Deregulation of gene expression is a common feature in cancer. The possibility to inhibit the expression of an oncogene at the mRNA level, instead of blocking the function of the gene product, elicited a great interest in this tool as a potential therapeutic application since long time. Therefore, RNAi offers promising new opportunities to very specifically target the genes deregulated in various pathologies. RNAi technology has been used to silence the expression of dominant mutant oncogenes, to inhibit gene amplifications and translocations and to restrain the function of viral oncogenes and their interaction with other genes in a number of critical cellular pathways (Ameyar-Zazoua *et al.* 2005, Leung and Whittaker 2005, Pai *et al.* 2006).

RNAi tool can be used to target a large number of genes involved in a number of distinct and critical cellular pathways especially in cancer development and progression. Most of the RNAi candidate genes are involved in the pathways that contribute to tumor growth; either *via* increased tumour cell proliferation or reduced tumour cell death or both, mutated

cancer oncogenes, negative regulators of endogenous tumor suppressors and also proteins involved in cellular senescence or protein stability and degradation. In this context, major cellular pathways altered include receptor tyrosine kinase (RTK), adenomatous polyposis coli (APC), glioma-associated oncogene (GLI), phosphoinositide 3-kinase (PIK3), hypoxia-inducible transcription factor (HIF), retinoblastoma (Rb), p53 and apoptosis (APOP) pathway (Vogelstein and Kinzler 2004). RNAi was also applied against the genes involved in cell cycle regulation and progression, tumour growth and metastasis, anti-apoptotic proteins, repair molecules against the damage caused by chemo- and radiotherapies. RNAi inhibiting pro-angiogenic genes also serve as a potential cancer therapy by halting blood and nutrient supply to the proliferating tumours tissues. siRNA against VEGF (Filleur *et al.* 2003; Schiffelers *et al.* 2004; Takei *et al.* 2004) and carcino-embryonic antigen-related cell adhesion molecule-1 (CEACAM1) was found to limit VEGF-induced morphogenesis (Kilic *et al.* 2005) and extra-cellular matrix degradation enzymes (cysteine and serine proteases and matrix metalloprotease) to retard tumour angiogenesis, metastasis and growth progression (Edovitsky *et al.* 2004, Gondi *et al.* 2004a, Gondi *et al.* 2004b, Lakka *et al.* 2005) and then leads to decreased tumor angiogenesis, metastasis and tumour progression.

Nevertheless the presence of tumor-specific antigens and the established cell and antibody mediated immune surveillance system; majority of tumors manage to escape from body immune response and immune-mediated destruction (Khong and Restifo 2002; Dunn *et al.* 2004). Thus an effective RNAi leads to efficient immune response against tumor cells *in vitro* and *in vivo* (Gorelik and Flavell 2001; Yin *et al.* 2003; McCarthy *et al.* 2004; Rubinstein *et al.* 2004). RNAi therapy also targeted multidrug resistance genes to counteract cancer resistance against chemotherapy and the efflux of chemotherapeutic agents in various cell models (Wu *et al.* 2003; Duan *et al.* 2004; Peng *et al.* 2004; Yague *et al.* 2004; Zhang and Li 2013).

1.6.4. Merits of siRNA

It is now well known that siRNAs and AS-ONs can inhibit their specific target genes and therefore could be used for the treatment of many disease conditions (Sitikov 2012; Ramachandran and Ignacimuthu 2013). Their high specificity, covering a wide range of biomedical applications allows the inhibition of target proteins that are not easily accessed and modulated by conventional small molecular weight or protein drugs (de Fougerolles *et al.* 2007). The siRNAs can be designed against any member of the entire genome including any anti-apoptotic gene, tumour survival or growth promoting factor, the participant of a

signalling pathway or a gene essential for cell survival or proliferation. Small interfering oligonucleotides could be used to target oncogenic transcription factors which are not accessible by conventional drug therapies and could equally be employed to eliminate the cancer stem cells within a tumour that are relatively resistant to chemotherapy and radiations. The drug efflux systems that are a source of drug resistance are also not able to eliminate these small RNA drugs. Once a target is identified, it takes much less to design and assess a potent siRNA candidate compared to relatively long period to identify an active chemical compound. The amount required for an efficient cancer inhibition is extremely low (picomolar) for siRNA compared to therapeutic dose of a drug (Petrocca and Lieberman 2010). RNAi-based therapeutics are able to block differentiation or can cause death of a highly malignant (sub)population of tumor-initiating cells that may be responsible for tumor resistance, relapse, possible metastasis, might resistant to conventional cytotoxic therapies or which are disseminated in the body in the metastatic cancers and not possible to control by surgical intervention (Petrocca and Lieberman 2010).

1.6.5. Drawbacks of siRNA

Along with desired effects of target gene silencing, siRNAs also lead to induce unintended consequences including immune stimulation, toxicity and off-target silencing in addition to transitory interference due to miscellaneous reasons (Kanasty *et al.* 2012).

1.6.5.1. Transitory gene silencing effects

The efficiency of siRNA is limited due to short-lived nature of siRNA itself and thus transient gene silencing effects due to relative instability in the biological fluids. Extracellular gradation of siRNA peaks around 36–48h after its entry and begins to decrease after 96h *in vivo*. According to the type of target tissue or cell, the RNAi efficiency varies as well, due to difference in ability of target to take-in siRNA. The level of gene silencing also not remains absolute because of high inter-individual variation (Ryther *et al.* 2005).

Linked with available level of siRNA is the duration of gene silencing activity. The efficiency decreases on dilution of siRNA concentration between dividing daughter cells with repeated cell divisions. This is especially important in physiological active and rapidly proliferating (tumour) cells, peaking at around 3 days and lasting for approximately 1 week. Whereas the gene silencing duration in differentiated or slowly dividing cells is relatively long which might be up to several weeks (Shankar *et al.* 2005). Efficiency is also affected by ongoing cellular enzymatic degradation and serum RNases which degrade these

oligonucleotides and thus subsequently are excreted *via* the reticuloendothelial system (RES) and rapid renal filtration (Juliano *et al.* 2009). Similarly, inhibition of cell differentiation or cell cycle arrest or senescence could be reverted with siRNA dilution. Thus a prolonged gene silencing is required for a stable therapeutic effect (Petrocca and Lieberman 2010).

1.6.5.2. Non-specific and off-target interference

Although RNAi is proficient in gene silencing but also shows the effects independent of specific gene targeting, the so-called off-target effects, due to similarities in nucleic acid sequences (Scherr and Eder 2004). If siRNA having partial complementarity to a non-target mRNA, it can repress translation of unrelated genes, can subject unintended degradation of mRNA or can enhance activity of other genes (Jackson *et al.* 2003, Holen *et al.* 2005, Kanasty *et al.* 2012) and thus can also lead to toxic outcomes (Fedorov *et al.* 2006). Similarly, if the passenger strand is incorrectly selected by RISC complex, this not lead to destruction of guide strand and only also lead to reduced inhibition of target gene but also silencing of off-target genes complementary to this selected passenger strand (Kanasty *et al.* 2012). Expression profiling of different siRNAs targeted against the same transcript revealed that as small as seven nucleotides complementarity between the 5' end of siRNA strand to a mRNA can cause an adequate reduction in transcript levels (Saxena *et al.* 2003, Jackson and Linsley 2004, Sitikov 2012). Similarly, sequence-independent gene suppression is also observed (Kariko *et al.* 2004; Kleinman *et al.* 2008; Cho *et al.* 2009) which might be due to large size (21bp or more) (Sledz *et al.* 2003) or high concentrations (Bridge *et al.* 2003; Sledz *et al.* 2003) of siRNAs.

1.6.5.3. Activation of immune response

Recognition of siRNA as foreign by immune sensors of invading infection can lead to activation of immune system and the release of type-I interferon, interleukin-8, TNF α (Kariko *et al.* 2004, Charlaftis *et al.* 2012, Kanasty *et al.* 2013) and/or pro-inflammatory cytokines response (Petrocca and Lieberman 2010) which can lead to systemic inflammation *in vivo* (Samuel-Abraham and Leonard 2010). Even sequence of siRNA can cause off-target effects due to imperfect pairing of guide strand with sequence motifs primarily in 3' UTR regions of cellular mRNAs and are called as microRNA-like off-target effects (Jackson and Linsley 2010). Both siRNAs and shRNAs activate macrophages and primary dendritic immune cells by specific endosomal toll-like receptors (TLR3, 7 & 8) that recognize these RNAs as foreign and trigger a proinflammatory response (Sledz *et al.* 2003; Kariko *et al.* 2004). Upon siRNA binding, there is also activation of cytosolic RNA-binding proteins like protein kinase R

(PKR) which then triggers the phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) and nuclear factor- κ B (I κ B- α) signalling, thus leading to a global inhibition of mRNA translation through production of type-I interferons - α & - β (Stark *et al.* 1998, Sledz *et al.* 2003, Garcia *et al.* 2006, Marques *et al.* 2006, McAllister *et al.* 2012).

1.6.5.4. Miscellaneous flaws of siRNA therapy

Among the miscellaneous side effects of siRNA, there include the development of resistance against siRNA therapy through mutation in target sequence which make the previously efficient siRNA ineffective (Westerhout *et al.* 2005; Wu *et al.* 2005), competition with RNAi machinery modulate normal gene regulation by endogenous microRNAs (miRNAs) (Khan *et al.* 2009), target sequence inaccessibility due to some physical hindrance caused by RNA-binding proteins or due to complex secondary structures (Westerhout *et al.* 2005; Pai *et al.* 2006). Mutations in RNAi machinery like RISC or Dicer could also be a cause of inefficiency of RNA silencing (Lee *et al.* 2004; Lim do *et al.* 2008). Interferon-mediated activation of certain interferon stimulated genes (ISGs) like Stat1, 2'-5'-oligoadenylate synthetase 2 & 3 and interferon inducible transmembrane proteins 1 & 2 were also observed at all (10nM, 25nM, 50nM and 100nM) concentrations of siRNA (Sledz *et al.* 2003). On activation, oligoadenylate synthetase causes a sequence independent degradation of cellular single stranded RNAs by activating RNaseL (Sledz *et al.* 2003). Since transcriptional gene knock down does not affect pre-existing proteins, thus post-translational products with a long half life cannot be affected, despite an efficient siRNA gene silencing (Pai *et al.* 2006). Briefly, multiple sequences should be targeted or multiple targets should be addressed with siRNA therapy in a single model to achieve a therapeutic gene silencing (Pai *et al.* 2006). Thus, siRNA have numerous non-specific and complicating effects beyond the selective silencing of target genes.

1.7. Hurdles of siRNA based cancer therapies

The most challenging hurdle for translating RNAi technology from an effective research tool into a feasible therapeutic strategy is the efficient delivery of these molecules to the target cells *in vivo*. Delivery of siRNA to the target cell/tissue/organ is a key challenge because the biological efficiency of siRNAs is hampered by their short plasmatic half-life in the potentially dividing cells *i.e.* between 36-48h in the rapidly dividing and around several weeks in slowly growing cells (Ryther *et al.* 2005). They are unable to transverse intact cell membranes to significant degree due to their highly hydrophilic and anionic character (De

Paula *et al.* 2007; Fuchs and Borkhardt 2007; Aigner 2008; Guo and Huang 2011; Ali *et al.* 2012). There is no natural mechanism for the highly hydrophilic polyanionic macromolecules to traverse the cellular membrane and due to the reason; the bioavailability of these agents in the intracellular compartment is minuscule. They are also relatively unstable in serum containing medium (*in vitro*) or in blood (*in vivo*) in their native form as they are degraded by nucleases and are also rapidly cleared from the body by renal excretion or might form aggregates with serum proteins (Juliano *et al.* 2009; Guo and Huang 2011).

Another key barrier for siRNAs to reach the target area is the phagocytes of the RES. These cells monitor the blood and remove foreign materials such as bacteria and viruses and also tend to treat administered macromolecules like siRNAs as foreign (Seth *et al.* 2012), thus accumulating these materials in hepatic Kupffer cells, splenic macrophages and other phagocytes rich sites (Underhill and Ozinsky 2002). In parallel, the systemic exposure of siRNAs can also induce some non-specific innate and acquired immune responses against oligonucleotides CpG motifs (Krieg 2002). Passing through the vasculature, ONs must disseminate through the extracellular matrix (ECM) which is a dense network of polysaccharides and fibrous proteins that also hinder ONs diffusion towards the target cells (Zamecnik *et al.* 2004).

Similarly, siRNA have also to face several other hurdles like susceptibility to nucleases, transient or poor biodistribution in the tumour tissue, non-target cellular or tissue up- or intake, inability to escape from endosomes/lysosomes into the cytosolic compartment etc (Seth *et al.* 2012). Thus, the revolutionary potency and selectivity of siRNA can enable improved targeted cancer therapeutics, if the appropriate means of systemic administration and targeted distribution to disseminated metastatic lesions is employed (Schiffelers *et al.* 2004).

1.8. Strategies to improve siRNA design and therapeutic effects

Although, siRNAs have medicinal application as drugs in human gene therapy with high specificity for molecular targeting; however, the target delivery of synthetic siRNA is still an unresolved issue. The *in vivo* delivery of siRNA depends on its structure, chemical modifications and substitutions and delivery formulations, affecting its stability in circulation, tendency to extravasate blood vessels, bio-distribution, ability of intra-cellular penetration and clearance from circulation without being delivered (Kanasty *et al.* 2012). Thus, instability and

degradation issues need to be resolved before to exploit the full efficiency of siRNA in gene silencing.

Various structural chemical modifications like substitution in the nucleotide linkages, resistant to exonuclease cleavage or cholesterol conjugation *via* binding with low-density lipoprotein (LDL) and/or attaching a lipidic, peptidic or polymer moiety can improve the half-life of small RNAs in serum and extra cellular fluids (Soutschek *et al.* 2004, Wolfrum *et al.* 2007, Whitehead *et al.* 2009, Kanasty *et al.* 2013). Chemical modification of guide strand seed region by 2'-OMe specifically at position 2, diminished the off-target effects to about 80% in a microarray study (Jackson *et al.* 2006). In addition, by complexing with larger particles, naked small RNAs could be avoided to be excreted by renal clearance *via* glomerular filtration (Whitehead *et al.* 2009). Similarly, length of siRNAs has role in triggering the interferon response, thus dsRNAs sequences shorter than 19 nucleotides are more likely to escape the interferon response (Pai *et al.* 2006). Substitutional use of complete dsRNAs instead of synthetic siRNAs will help to avoid off-target effects.

The siRNAs derived endogenously by cleavage of dsRNAs are generated by Dicer and selected by RISC complex will have a branded sequence and thus protect against the recognition and silencing of other unrelated endogenous genes. In case of inevitable design and synthesis of siRNAs against specific target and to achieve relatively sophisticated sequence, computational softwares can be used to minimize potential off-target effects (Boese *et al.* 2005).

1.9. Strategies to deliver the siRNA to the target tissue

A wide variety of approaches including viral and non-viral based delivery systems have been employed so far, to enhance *in vivo* target cells uptake and silencing potential (Stone 2010, Huang *et al.* 2011, Nayerossadat *et al.* 2012, Resnier *et al.* 2013). Various strategies to deliver the RNAi technology and their pros and cons are already discussed in detail in the review (Ali *et al.* 2012). Just to recall, these approaches are summarized in few sentences.

1.9.1. Use of viral vectors for siRNA delivery

Viral vectors are the most powerful tool for gene transfection which can stably integrate into the genome, deliver ONs to the target and mediate a long term knockdown of endogenous transcript *in vitro* and *in vivo*. The viral vector delivery systems include retroviral

(Anson 2004; Bushman 2007), lentiviral (Azzouz *et al.* 2002; Balaggan and Ali 2012), adenoviral (Vorburger and Hunt 2002; Mowa *et al.* 2010), adeno-associated viral (Wang *et al.* 2011), helper-dependent adenoviral (Morral *et al.* 1999; Croyle *et al.* 2005) vectors. Despite of the benefits, the safety of viral vectors is questionable due to immunogenicity and possible recombination with host genome (Lv *et al.* 2006), a general lack of specificity for gene delivery to the target cells, potential toxicity by the viral vectors themselves (Raper *et al.* 2003; Mowa *et al.* 2010) and possible infection of non-dividing cells (Reid *et al.* 2002; Wang *et al.* 2011).

1.9.2. Use of non-viral siRNA carrier systems

Although less efficient than viruses for gene delivery, non-viral carriers offer several advantages; particularly simple to use, offer trouble-free quality control, biocompatibility, generate little or no specific immune response (Zaldumbide and Hoeben 2008; Mintzer and Simanek 2009). Among the non-viral vectors are various types of nanoparticles (de Fougerolles *et al.* 2007; de Fougerolles 2008; Whitehead *et al.* 2009), see review Ali *et al.* 2012), carbon tubes (Güven *et al.* 2012; Lim *et al.* 2013), nano-diamonds (Lam and Ho 2009; Man *et al.* 2013; Xiao *et al.* 2013), nano-spheres (Maksimenko *et al.* 2005), nano-capsules (Lambert *et al.* 2000), liposomes (Halder *et al.* 2006), micelles (Kim *et al.* 2008), polyplexes (Verbaan *et al.* 2004), lipophilic conjugates, polymers and cell penetrating peptides (Soutschek *et al.* 2004; Halder *et al.* 2006) are used to deliver oligonucleotides to the target tissue. For specific cell targeting, nanoparticles could be attached to antibodies, fusogenic peptides, polymers, glycans, folates or aptamers that could bind with protein carriers for enhanced uptake by the targeted population of cells (Zhang *et al.* 2004; Derfus *et al.* 2007; Stoltenburg *et al.* 2007; Bartlett and Davis 2008; de Martimprey *et al.* 2008; Davis 2009; Kievit *et al.* 2009; Chou *et al.* 2011). This episode is not yet finished as neither of the current solutions is ideal and always has its drawbacks.

1.9.3. Outcomes of cationic systems for siRNA delivery

Although cationic complexes have advantage of being non-immunogenic but the toxicity of cationic transfectants either lipidic or polymeric; which are mostly used to prepare the non-viral vectors, raise major issues for clinical utilization (Lee *et al.* 2004; Lv *et al.* 2006; Kuo *et al.* 2009). These delivery systems also cause toxicity, can induce various off-target effects and can also stimulate immune system. Toxicity occurs through membrane destabilization and alterations in cellular signaling or gene expression of un-intended targets (Kanasty *et al.* 2012). These effects and the type and degree of immune response depends on

the size, charge, chemical structure and form of the material used to construct the delivery vehicle and biodistribution of the respective nanoparticles (Jackson and Linsley 2010). Thus, in the design of delivery services, pharmacokinetics and pharmacodynamics of the system must be considered that contribute in the desired therapeutic and other un-predictable effects that affect the overall potency and acceptability of a formulation. Therefore, it is always a need to introduce new concepts, based on the use of more efficient and safe biological materials which improve the way of siRNA delivery.

1.9.4. Use of neutral lipids for siRNA delivery

Alternatively, non-ionic or neutral lipids provide another methodology for the targeted delivery of ONs (Templeton and Senzer 2012; Desigaux *et al.* 2005; Trang *et al.* 2011). Conjugation of nucleic acids with neutral lipids provides greater metabolic stability, improved bioavailability and thus offers an interesting substitution. Many of the non-ionic lipids are safe, non-toxic, bio-compatible and additionally some of these are of natural origin (Raouane *et al.* 2011). Carriers based on neutral lipids are less likely to form aggregates with charged components in biological fluids, not filtered by hepatic screening system, not adhere to the endothelium or be taken-up by scavenging macrophages of reticulo-endothelial system (Landen *et al.* 2005; Halder *et al.* 2006). In this context, examination of neutral lipid emulsion carrier delivery of miR-124 revealed their equal distribution in whole blood, liver, kidneys and lungs, just 10 minutes post-intravenous injection (Trang *et al.* 2011) and unlike cationic lipid particles, were not to be preferentially accumulated in liver (Landen *et al.* 2005). Neutral lipids are found to be less toxic; as such formulation with miR-34a revealed neither elevated hepato-renal enzymes in serum nor induced a non-specific immune response in mice (Wiggins *et al.* 2010).

1.9.4.1. Squalene: a neutral lipid

Squalene is an isoprenoid compound and a neutral lipid, widely distributed in nature and is found in olive, palm, wheat-germ, amaranth, rice bran oils (Kelly 1999; Desmaele *et al.* 2012) and also in the liver of deep sea shark (Desmaele *et al.* 2012; Tjan 2013). It is naturally present in the body as a major component (about 13%) of skin surface lipids in the sebaceous glands (Gylling and Miettinen 1994) and protects human skin surface from lipid peroxidation on exposure of ultraviolet radiations and other sources of oxidative damage (Kohno *et al.* 1995). It is widely involved in the maintenance of enormous homeostatic and metabolic processes in the body (Tjan 2013) and is an intermediate compound in the biosynthesis of cholesterol (Eidinoff *et al.* 1958; Reddy *et al.* 2007). On dietary intake, squalene is absorbed

and converted into cholesterol in humans, without increasing serum cholesterol levels which might be due to concomitant elimination from the body (Strandberg *et al.* 1990; Miettinen and Vanhanen 1994) and thus maintains cholesterol level in the body. Higher liver and spleen accumulation of squalene did not demonstrate any appreciable side effects and hepato-toxic signs *in vivo* (Reddy *et al.* 2007).

1.9.4.2. Applications and uses of squalene

Squalene acts as a weak antioxidant and is not only used as prevention for skin (Murakoshi *et al.* 1992, Desai *et al.* 1996, Huang *et al.* 2009) and eye diseases but also therapeutically applied in the treatment of variety of ailments including malignant diseases (Trichopoulou *et al.* 1995; Rao *et al.* 1998; Smith 2000). Additionally, it provides protection against bacterial and fungal infections (Nowicki and Baranska-Rybak 2007) and coronary heart disease and aging (Sabeena Farvin *et al.* 2004; Buddhan *et al.* 2007). It is used as immunological adjuvant for vaccine delivery (Reddy *et al.* 2009) and an excipient for various drugs (Allison 1999; Wang *et al.* 2006), as emulsion for various medicinal substances and enhances the therapeutic efficiency of certain cholesterol lowering agents like pravastatin (Chan *et al.* 1996).

It is believed to have protective and preventive functions as been responsible for the low incidence of human cancer in the Mediterranean region where people's diets have high olive contents (Trichopoulou *et al.* 1993; Willett 1994; Owen *et al.* 2000; Owen *et al.* 2004) while experimentally also decrease lung tumor growth by 58% compared to control diet in mice (Smith *et al.* 1998). Similarly, squalene intake significantly decreased ($p < 0.05$) lung hyperplasia by 70% and also effectively inhibits mammary carcinogenesis (Reddy *et al.* 2009). It is used to prevent or arrest tumor growth alone or in combination with anticancer therapies in various experimental tumor models (Cohen *et al.* 1986, Murakoshi *et al.* 1992, Reddy 1992, Desai *et al.* 1996, Newmark 1997, Singh *et al.* 1997, Rao *et al.* 1998, Smith 2000). It is also employed as a non-viral gene carrier (Chung *et al.* 2001; Kim *et al.* 2003; Kwon *et al.* 2008) and as biomaterials drug carrier for several anti-cancer molecules (Couvreur *et al.* 2006a, Couvreur *et al.* 2006b, Reddy *et al.* 2007, Arias *et al.* 2008, Bekkara-Aounallah *et al.* 2008, Couvreur *et al.* 2008, Reddy *et al.* 2009).

It has also been used to prepare the nanoparticles by a method introduced by Prof. Patrick Couvreur and co-workers and was named 'squalenoylation' (Couvreur *et al.* 2006) (Figure 6). The nanoassemblies of SQgem (squalene + gemcitabine) were prepared and

found to be more cytotoxic *in vitro* (Reddy *et al.* 2007) and a more efficient anti-cancer therapy *in vivo* (Reddy *et al.* 2008a) in resistant leukemia model. Taking this concept, nanoparticles of squalene with siRNA RET/PTCs were prepared and tested in papillary thyroid carcinoma model *in vitro* and *in vivo* in the current study.

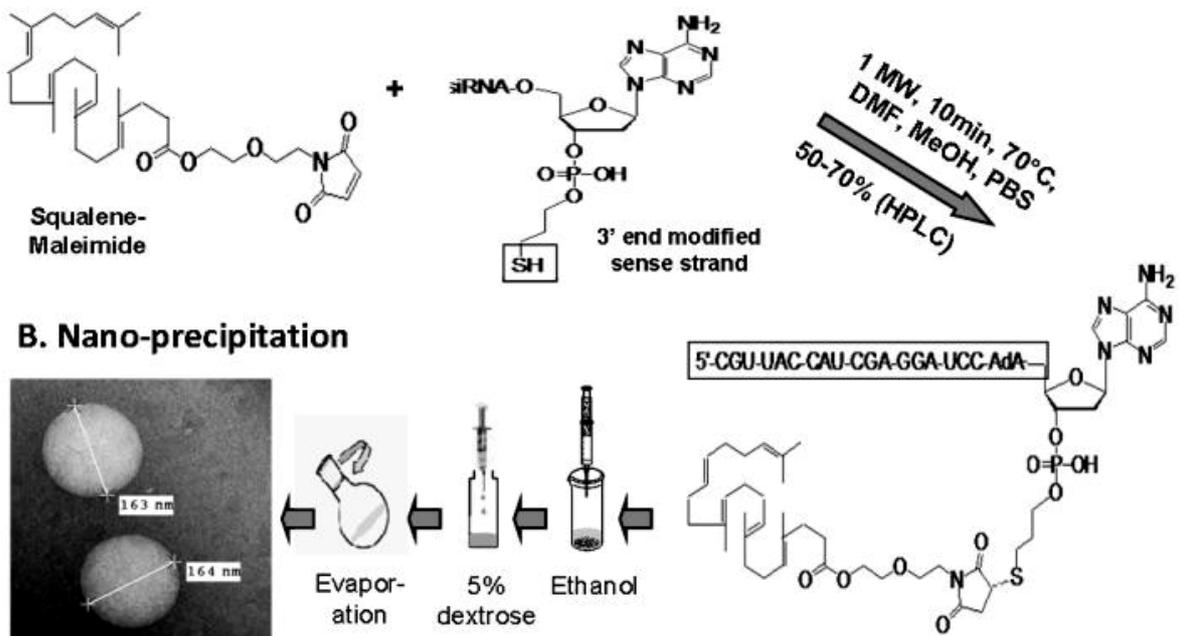
To summarize, squalene offers unique properties as dietary lipid constituents, used for cosmetic purposes, for vaccine, drug and gene delivery and also have several pharmacological functions. The diverse applications demonstrate that this natural triterpene has a very bright future in disease control and management and will be an active ingredient in the developing diverse squalene-based formulations for cancer therapy.

1.9.4.3. Squalenoylation

This method was used to covalently link the squalene with siRNAs to prepare the nanoassemblies (Couvreur *et al.* 2006b, Couvreur *et al.* 2008). The nanoparticles of siRNAs-SQ were prepared with (siRNA RET/PTC1-SQ GALA-Chol NPs) and with out GALA-Chol (siRNA RET/PTC1-SQ NPs) and tested *in vitro* and *in vivo* for their gene and protein silencing efficiency.

Briefly, a bioconjugate was prepared by linking maleimide attached squalene to 3' end of thiol-modified siRNA sense strand and then hybridized with antisense strand to obtain siRNA duplex. The bioconjugate was firstly lyophilized and then dissolved in 0.5ml of ethanol and later added drop by drop into 1ml of 5% aqueous dextrose solution under magnetic stirring (500 rpm). Then ethanol was completely evaporated using a Rotavapor to obtain an aqueous suspension of pure siRNA RET/PTCs-SQ nanoassemblies (Figure 9). The nanoparticles were characterized for their hydrodynamic diameter and ζ potential by Nanosizer 4 (Malvern Instrument Ltd, France) and also for size by transmission electron microscopy (TEM). All the nanoparticles were prepared using the same protocol as described previously (Raouane *et al.* 2011).

A. Bio-conjugation



B. Nano-precipitation

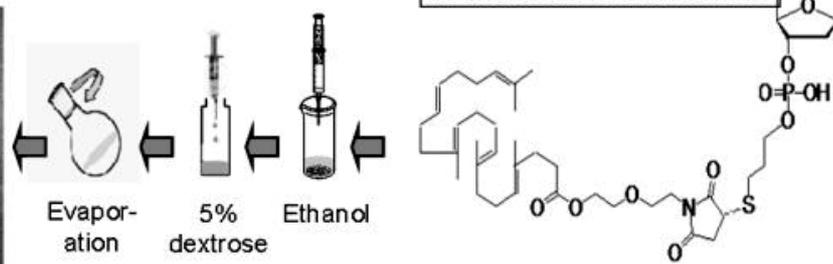
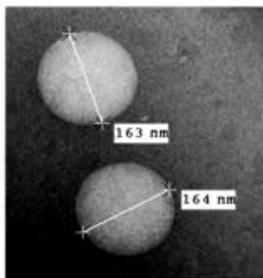


Figure 9: Preparation of siRNA RET/PTCs-Squalene nanoparticles by 'Squalenoylation'. A. Bioconjugate siRNA RET/PTCs-SQ was prepared by adding squalene-maleimide with pre-hybridized double stranded siRNA RET/PTC(s). B. Nanoparticles were then prepared by nano-precipitation. The bio-conjugate was dissolved in ethanol and added drop-by-drop in to aqueous 5% dextrose solution under magnetic agitation. The ethanol was completely evaporated using a Rotavapor and the resulting nanoassemblies were characterised by TEM. The work was done in collaboration with Pr. P. Couvreur and Dr. D. Desmaële : UMR 8612 CNRS (Brevet international, 2012). Ref: Raouane *et al.*, 2011.

1.10. Article – 1 :

Significance and applications of nanoparticles in siRNA delivery for cancer therapy

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and Liliane Massaad-Massade

Expert Review of Clinical Pharmacology 2012, 5(4):403-412.

Small interference RNA (siRNA) is an effective and powerful strategy for gene silencing in cancer therapy. The use of nanoparticles for siRNA delivery is an upcoming theme in the current medicine field for cancer therapy. The aim of this review is to give an overview of various approaches delivering siRNA to the target and to illustrate advantages and demerits of these delivery systems. This review describes nanoparticles (NPs) mediated siRNA delivery and includes various associated aspects like choice of an appropriate delivery system, salient shortfalls and delivery barriers of carrier services, various types of NPs employed in numerous studies, current status and awareness on nano-system-mediated siRNA delivery and finally the future insight. It also underlines the important parameters in terms of charge, size, dose and route of administration to obtain an efficient siRNA delivery system. In conclusion this review highlights that siRNA-based cancer therapy is promising, although challenges remain to be addressed and there are few issues that need to be solved to better exploit this technology.

1.11. Use of a fusogenic peptide GALA-Chol

GALA (glutamic acid - alanine - leucine - alanine) is an artificially designed, pH-sensitive water soluble α -helical amphipathic peptide composed of 30 amino acids (Subbarao *et al.* 1987; Nir and Nieva 2000; Yamauchi *et al.* 2010) and described to be a functional element for particle release from endosomal vesicle (Kakudo *et al.* 2004; Akita *et al.* 2011). It was the first peptide designed to interact with lipid bilayer at acidic pH to facilitate endosomal escape (Subbarao *et al.* 1987).

1.11.1. Properties and function of GALA

GALA was reported to induce membrane fusion (Parente *et al.* 1988; Parente *et al.* 1990), fragmentation (Subbarao *et al.* 1987) and permeabilization by pore formation for leakage of contents (Parente *et al.* 1990; Nir and Nieva 2000). The membrane-binding properties of GALA have been extensively investigated to explain its ability to perturb lipid bilayers (Subbarao *et al.* 1987; Parente *et al.* 1988; Parente *et al.* 1990; Parente *et al.* 1990; Strandberg *et al.* 1990; Goormaghtigh *et al.* 1991; Fattal *et al.* 1994; Haas and Murphy 2004; Haas and Murphy 2004). GALA has extremely low binding affinity to membranes at neutral pH but undergoes a structural transition from a random coil at pH 7.5 to an amphipathic α -helix at pH 5.0 due to neutralization of the negatively charged glutamic acid residues (Kakudo *et al.* 2004; Lochmann *et al.* 2004; Sasaki *et al.* 2008) and thus attains a high binding affinity to neutral and negatively charged membranes (Nicol *et al.* 1996; Simoes *et al.* 1998). At neutral pH, there is electrostatic repulsion between the carboxylic acid moieties of glutamic acid that destabilizes the helical structure. As soon as the pH is decreased, the neutralization of these residues promotes the helix formation, thereby increasing the hydrophobicity of glutamic acid side chains and consequently results an increased interaction with phospholipid membranes (Liu *et al.* 2013). Similar to GALA function, pH-sensitive lipids containing carboxylic groups have a net negative charge at neutral pH and thus provide a stable nanocarrier device. However, in acidic environment, carrier become protonated resulting its disintegration and release of attached substance (Liu *et al.* 2013). Likewise, inorganic substances like calcium phosphate and zinc oxide are relatively insoluble at neutral pH but are dissolved as non-toxic ions at acidic milieu as of endo/lysosome or solid tumours (Banerjee *et al.* 2011; Banerjee *et al.* 2012).

Taking the advantage of mildly acidic condition in the endosomes/lysosomes, the acid responsive delivery carriers cause facilitated cytosolic release of ONs. Thus has been an accepted approach to overcome the intracellular hurdle for ONs to be unable to escape from

the trap of lysosome (Cho *et al.* 2003). Hindering the acidification of the endosome environment by bafilomycin A₁, by inhibiting the vacuolar ATPase endosomal proton pump, significantly reduced the transfection efficiency. Thus highlighting the fact that the acidic pH of the endosomes is necessary for the fusogenic activity of GALA to be functional; to let escape the nanoparticles into cytosol (Wang *et al.* 2009). Hence in the acidic endosomal environment, GALA interacts with phospholipid membrane on fusion, aggregates and thus destabilizes the endosome by creating pores that cover a size of about 10 peptides (Parente *et al.* 1990; Nicol *et al.* 1996; Nicol *et al.* 2000; Liu *et al.* 2013) to release the endosomal trapped materials into cytosol (Figure 10).

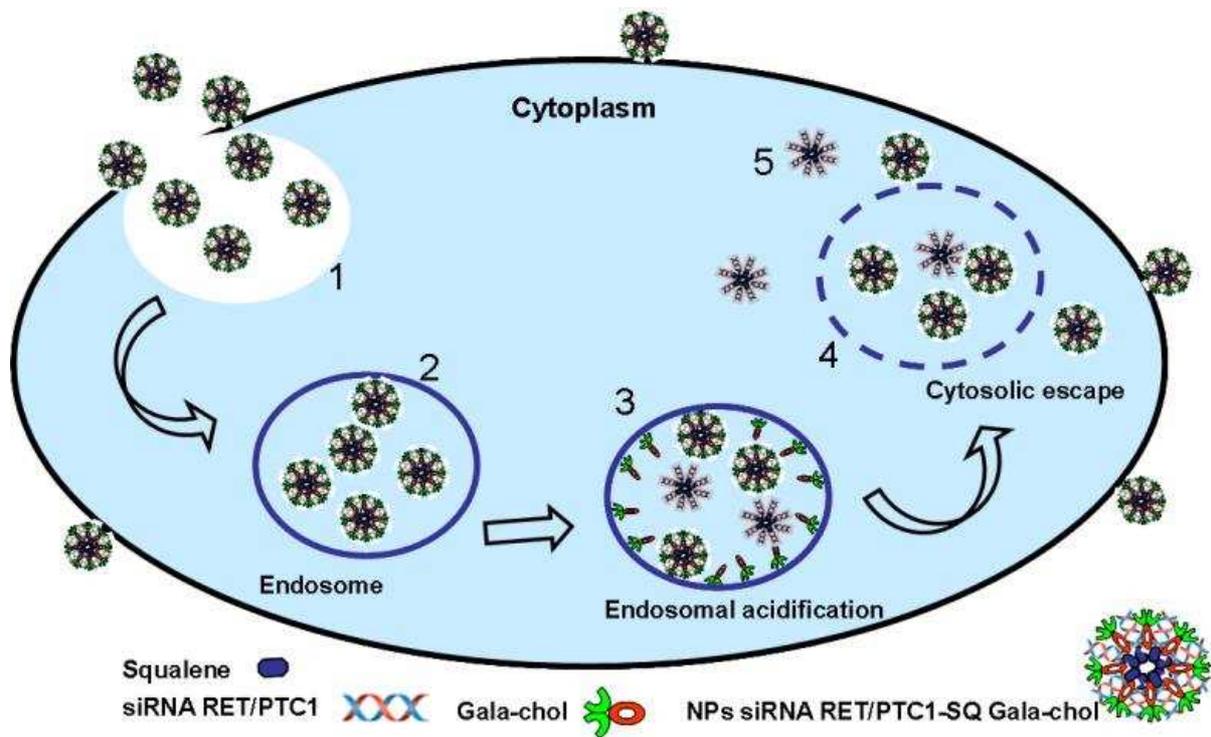


Figure 10: Trans-membrane and intra-cellular passage of Gala-Chol combined siRNA RET/PTC1-SQ nanoparticles. 1. Gala-Chol interacts with cellular membrane to promote the trans-membrane translocation of siRNA-SQ NPs. 2. While encapsulated within endosome / lysosome, 3. Gala undergoes a structural transformation in the acidic endosomal environment and thus attains an increased binding affinity to charged endo-/lysosomal membrane. It interacts with the phospholipid membrane, either released from or within NPs to cause fusion, aggregation and 4. thus destabilizes the envelop by creating pores, 5. to release out the entrapped contents into the cytoplasm

1.11.2. Why GALA as a choice

The properties of GALA render it an attractive candidate for investigation and has been used successfully to improve delivery of siRNAs (Futaki *et al.* 2005; Akita *et al.* 2010; Kusumoto *et al.* 2013; Liu *et al.* 2013; Toriyabe *et al.* 2013; Wang *et al.* 2013) and AS-ONs (Hughes *et al.* 1996). The synthetic GALA peptide was found to be more effective than other pH-sensitive 20 amino acid (Simoes *et al.* 1998) or 23 amino acid (Plank *et al.* 1994)

influenza HA-2 peptide in cytosolic release of endosomal bound material. GALA combined nanocarriers and having an additional targeting ligand, enter in the cell through coated pit-mediated endocytosis and deliver the the drug or gene in a pH dependant manner (Yamada *et al.* 2005). GALA effectively increased the delivery of pDNA by vector/pDNA complexes (designer biomimetic vectors; DBVs) into the cytosol *via* membrane destabilization of endocytotic vesicles in SK-OV-3 ovarian cancer cells (Wang *et al.* 2009). Transfection of cells by DBVs without GALA (DBV/GALA) showed a significant reduction in transfected cells from 30±4% to 16±3% (Wang *et al.* 2009). Addition of GALA in octa-arginine peptide-multifunctional envelope-type nano device (R8-MEND) pDNA/PEI condensed nano assemblies injected intravenously, significantly increased liver luciferase activity of both negatively (1640-fold) and positively (3-fold) charged particles compared to the particles without GALA which did not produce elevated levels of gene expression which might be due to inadequate intracellular trafficking. Similarly, relative luciferase expression per amount of pDNA delivered to the nucleus was also high in the presence of GALA in particles of both charges (Khalil *et al.* 2011). When GALA-associated cell surface blocking complexes were pre-incubated with the cells, an enhancement of transfection was observed independent of the composition and to some extent, the overall charge of the active complexes (Simoes *et al.* 1999).

1.11.3. Role of GALA location in the nanoparticles

The presence of GALA directly in the culture medium or within the aqueous compartment of transferrins-fluorescein isothiocyanate (Tf-FITC)-L (liposomes modified by Tf and labeled with FITC) was not found to promote the cytosolic release of sulfo-rhodamine from endosome of human chronic myelogenous leukemia K562 cells. Therefore, Tf-(FITC)-L GALA-Chol were synthesized, cholesteryl moiety was added to display GALA segment on the surface of liposomes. Thus, a highly efficient release of these GALA-Chol conjugated liposomes was observed by confocal laser scan microscopic (CLSM) analysis (Kakudo *et al.* 2004). While when GALA was encapsulated within the liposomes, no pH-dependent structural transition was observed and it might remain inactive due to rather disordered conformation even at low 5.0 pH (Kakudo *et al.* 2004). Similarly, considerable structural from random to helical conformational change was observed in the peptide at pH 5.0 which eventually cause disruption of lipid membranes by interaction with the phospholipid (Subbarao *et al.* 1987; Nir and Nieva 2000). This advocate that GALA should be at the surface of NPs to have a conformational change either to directly communicate with the

endosomal membrane or to disintegrate from the particles to make aggregates with the membrane phospholipids for pore formation when the endosomal pH is decreased.

1.11.4. Role of cholesterol in GALA function

GALA peptide without being attached to the cholesterol moiety was reported to interact only slightly with lipid membranes and to exhibit a rather random structure (Subbarao *et al.* 1987; Nir and Nieva 2000). While the cholesteryl moiety was attached, the peptide was probably forced to lie on the membrane to form a helical structure at low pH. Possible loss of repulsion between the negative charges of glutamic acid residues also changed the mode of interaction of the peptide with the membranes due to its structural modification and eventually facilitating membrane fusion (Kakudo *et al.* 2004). The capacity of GALA to form pores in cholesterol-containing bilayers for ANTS (1-aminonaphthalene-3, 6, 8-trisulfonic acid) leakage at pH 5.0 was studied in POPC/POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-phosphatidylglycerol) (5:1) liposomes at ascending cholesterol contents from 0 to 40 mol%. The cholesterol content up to 15% did not cause any effect but at 20% or further addition of cholesterol reduced the final leakage extents of ANTS from POPC/POPG/Chol liposomes. It was thus concluded that increasing cholesterol contents in the liposome formulation changes the lipid/peptide ratio which might cause modification in the pore formation process and subsequently decreases the membrane-binding affinity of the peptide and a decrease in leakage of contents (Nicol *et al.* 1996). Thus added cholesterol is essential for membrane binding properties of GALA but at a moderate concentration; to might not interact with serum cholesterol components and thus not to hinder GALA function.

1.12. Article – 2 :

Effects of silencing RET/PTC1 oncogene in papillary thyroid carcinoma by siRNA-squalene nanoparticles with and without fusogenic companion GALA-Cholesterol

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Thyroid, 2014 24(2): 327-338.

The incidence rate of RET/PTC1 is much higher compared to rest of all RET oncogenes found in papillary thyroid carcinoma. Previously, our team demonstrates the anti-tumour activity of siRNA RET/PTC1-SQ NPs *in vivo* but not *in vitro* (Raouane et al., 2012). Thus, in this study, to impede the expression of RET/PTC1 fusion oncogene, squalenoyl nanostructures of siRNA RET/PTC1 have been employed *in vitro* (two thyroid cancer cell lines; BHP 10-3 and TPC-1 carrying RET/PTC1 junction oncogene) and *in vivo* (nude mice). These nanoparticles were herein modified by the addition of a peptide GALA-Chol that favours the endosomal escape of the enclosed elements. The consequences of this modification were evaluated *in vitro* and *in vivo*.

Addition of GALA-Chol leads the siRNA RET/PTC1-SQ nanoparticles to enter into the cells which were previously unable to be internalized. The effects of siRNA RET/PTC1-SQ GALA-Chol nanoparticles were then tested *in vitro* on cell viability RET/PTC1 gene and protein expressions compared to siRNA RET/PTC1-SQ NPs without GALA-Chol in both cell lines. These modified nanoparticles were found to have considerable inhibitory effects *in vitro* which were further enhanced by addition of lipofectamine 2000 during transfection. After finding the suitable dose for parenteral administration, the efficiency of both nanoparticles, siRNA RET/PTC1-SQ NPs with and without GALA-Chol was compared *in vivo* via intra-tumoral and intra-venous injections in nude mice. Although siRNA RET/PTC1-SQ GALA-Chol NPs showed inhibitory effects *in vitro*, contrasting outcomes *in vivo* via both routes of administration compared to siRNA RET/PTC1-SQ NPs were observed. Therefore, siRNA RET/PTC1-SQ NPs (without GALA-Chol) were selected as potential therapeutic agent and were recommended for further studies.

1.13. Article – 3 :

Effects of siRNA on RET/PTC3 junction oncogene in papillary thyroid carcinoma: from molecular and cellular studies to preclinical investigations

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Jean-Rémi Bertrand, Patrick Couvreur and Liliane Massaad-Massade
PlosONE, 2014 v.9 (xx)xxx accepted.

RET/PTC3 is the second most frequent fusion oncogene found in papillary thyroid carcinoma and shows more metastatic characteristics than RET/PTC1. It is mostly present in cases of thyroid cancer where patients were previously exposed to therapeutic or accidental radiations, particularly as in post-Chernobyl radiation fallout. It occurs mainly at young age with a short latency period and appears as an aggressive phenotype and has a poor prognosis. This research article reports the *in vitro* and *in vivo* effects of siRNA RET/PTC3-SQ NPs on targeting this onco-fusion. Due to the lack of cellular model expressing RET/PTC3, firstly a novel RP3 cell line was established that stably expresses RET/PTC3 junction oncogene. RP3 cells were found to be tumorigenic in nude mice contrasting to their parental cell line NIH/3T3 (mouse fibroblast). Although siRNA RET/PTC3 showed significant inhibitory effects on RP3 cell viability, invasion/migration, cell cycle, RET/PTC3 gene and protein inhibitions, the squalenoyl nanostructures of siRNA RET/PTC3 were found ineffective *in vitro*. However, when xenografted mice were treated with siRNA RET/PTC3-SQ NPs *via* intravenous injection, tumour growth was observed with significant gene and protein inhibitory effects along with induction of cell death in the tumour tissues. These findings concluded the effective use of siRNA RET/PTC3-SQ NPs as a new therapy against RET/PTC3 in young patients affected by PTC and thus support their application in further pre-clinical and clinical studies.

1.14. Discussion

The discoveries in cancer therapy are progressing everyday with advancement in the technology and discovery of innovative therapeutic applications. Despite of this progress and the current treatment plan, a considerable number of patients come across with recurrent disease with a resistance phenotype. Among the treatment strategies, RNAi has progressed from a powerful laboratory tool into a potential therapeutic modality in the diverse field of targeted therapy and therefore siRNA constitutes an area of important research in emerging therapies. In this context, a research project was launched in Massade's team to search an efficient siRNA based therapy against papillary thyroid carcinoma (PTC). The exploration of an efficient nano-medicine against RET/PTCs fusion oncogenes expressed in PTC was the main theme of my PhD project.

Previously in the laboratory, siRNA RET/PTC1 was designed and tested against RET/PTC1 junction oncogene (Gilbert-Sirieix *et al.* 2010). Later on, to overcome the limitations of siRNA in systemic application (reviewed in article-1, Ali *et al.*, 2012), 'squalenoylation' technology was employed by using squalene (SQ), to prepare the nanoparticles of siRNA RET/PTC1-SQ, to be used as *in vivo* therapy against RET/PTC1 onco-fusion. These nanoparticles, although showed significant inhibitory effects *in vivo* on tumour growth and RET/PTC1 gene and protein expressions, however they were found inefficient *in vitro* (Raouane *et al.* 2011). It was assumed that the inefficiency of the nanoparticles was due to their inability to cross the cell membranes, as they were not found in the intracellular compartment by fluorescence microscopy, except once transfected with lipofectamine 2000. To overcome this hurdle, a fusogenic peptide, GALA was selected which showed exciting results to improve the target delivery of antisense-ONs (Hughes *et al.* 1996) and siRNAs (Hatakeyama *et al.* 2009, Sakurai *et al.* 2009, Akita *et al.* 2010, Sato *et al.* 2010, Akita *et al.* 2011). GALA was thought to interact with the cell membranes in the target tissue, to achieve enhanced trans-membrane and intra-cellular penetration of GALA combined nanoparticles up to their destination. Thus, to improve the *in vitro* efficiency of siRNA RET/PTC1-SQ nanoparticles, GALA was added as GALA-Chol to enhance the translocation of respective nanoparticles up to the target.

In my PhD project, it was found that although the addition of GALA-Chol improved the *in vitro* gene and protein inhibitory efficiencies of siRNA RET/PTC1-SQ GALA-Chol NPs in both BHP 10-3 and TPC-1 cell lines but rendered them inefficient *in vivo* compared to siRNA RET/PTC1-SQ NPs (without GALA-Chol). The possible reason is the difference in

the molecular environment between *in vitro* culture medium and *in vivo* biological fluids which might lead to aggregations of NPs containing GALA with serum proteins *in vivo*, as discussed in article-2 (Ali *et al.* 2014a). It is also likely that the 10% molar concentration of GALA-Chol might be in excess that possibly provokes fusions/aggregations with serum proteins and thus showed unexpected results *in vivo* but this concentration was selected, as previously found effective in the literature (Khalil *et al.* 2011). The effects of siRNA RET/PTCs and their respective nano-formulations with and without Gala-Chol on *in vitro* and *in vivo* gene and protein silencing efficiency and tumour growth inhibition are summarized in Table-3 (results obtained in the laboratory). These controversial *in vitro* and *in vivo* results (Raouane *et al.* 2011, Ali *et al.* 2014a) also support the notion that only *in vitro* manipulations are not sufficient to make a decision to proceed towards clinical investigations rather *in vivo* studies are indispensable to make a reasonable conclusion. The inability of GALA-Chol containing nanoparticles to show proficient *in vivo* biological effects also halted the use of GALA for further studies. Therefore, siRNA RET/PTC3-SQ NPs were prepared without GALA-Chol to trial against RET/PTC3 oncogene. These nanoparticles showed comparable *in vitro* and *in vivo* results as that of siRNA RET/PTC1-SQ NPs, thus confirmed the *in vitro* inefficiency and *in vivo* effectiveness of these nanoparticles. Therefore, siRNA RET/PTCs-SQ NPs were confirmed to be an effective therapy against *in vivo* tumour models and in pre-clinical cancer cases.

Table 3: Gene and protein silencing efficiencies and tumour growth inhibitions by siRNA RET/PTCs-Squalene nanoparticles with and with out GALA-Chol.

References	Target fusion oncogene	Cell line	<i>In vitro</i>				<i>In vivo</i>				
			Without Lipo*		With Lipo*		Dose injected (mg/kg)	Route (i.t./i.v.)	Tumor growth inhibition	Gene	Protein
			Gene	Protein	Gene	Protein					
Martimprey <i>et al.</i> , 2008	si RET/PTC1	RP-1	---	---	89%	---	1.0	---	□ 70%	○ 85%	---
Gilbert-Sirieix <i>et al.</i> , 2010	si RET/PTC1	TCP-1	---	---	85%	---	---	---	---	---	---
	si RET	BT-474	---	---	50%	---	---	---	---	---	---
	si RET	MCF-7	---	---	80%	---	---	---	---	---	---
	si RET	RP-1	---	---	65%	---	---	---	---	---	---
Raouane <i>et al.</i> , 2011	si RET/PTC1-SQ NPs	BHP 10-3	---	---	80%	---	2.5	---	70%	80%	---
Ali <i>et al.</i> , 2014a ¹	si RET/PTC1-SQ NPs	BHP 10-3	00%	00%	80%	85%	0.5	i.t.	50%	80%	95%
	si R/PTC1-SQ GC NPs	BHP 10-3	30%	30%	85%	85%	0.5	i.t.	10%	00%	00%
	si RET/PTC1-SQ NPs	TCP-1	00%	00%	85%	85%	0.5	i.v.	60%	70%	98%
	si R/PTC1-SQ GC NPs	TCP-1	30%	60%	80%	90%	0.5	i.v.	07%	00%	00%
Ali <i>et al.</i> , 2014b ²	si RET/PTC3	RP3	---	---	60%	70%	---	---	---	---	---
	si RET/PTC3-SQ NPs	RP3	05%	10%	70%	80%	2.5	i.v.	60%	60%	95%

All the values represent relative (gene, protein and tumor growth) inhibitions compared to non-treated units (cells or tumors treated with 0.9% NaCl).

Lipo = Lipofectamine 2000, * = 24h post-transfection incubation, α = effects were studied by siRNA RET/PTC1-Chitosan NPs, si = siRNA RET/PTC, GC = GALA-Chol, *i.t.* = intra-tumoral, *i.v.* = intra-venous. Gene = gene silencing, Protein = protein inhibition.

Left alignment shows non-significant while right alignment shows significant results.

¹ = Ali, *et al.*, 2014a. Effects of silencing RET/PTC1 oncogene in papillary thyroid carcinoma by siRNA-squalene nanoparticles with and without fusogenic companion GALA-Cholesterol.

² = Ali, *et al.*, 2014b. Effects of siRNA on RET/PTC3 junction oncogene in papillary thyroid carcinoma: from molecular and cellular studies to preclinical investigation.

During the second part of the study, siRNA application was extended against the second most prevalent RET chromosomal rearrangement; RET/PTC3 and the results are presented in article-3 (Ali *et al.* 2014b). Four different siRNAs were designed against RET/PTC3 and tested against the fusion gene sequence in order to select the most appropriate and most efficient siRNA. After selection of a siRNA with most efficient RET/PTC3 gene silencing effects, an optimal dose was searched among the four tested concentrations (10nM, 25nM, 50nM and 100nM) *in vitro*, to obtain the maximum gene silencing efficiency and to employ this selected dose for *in vivo* experiments. The concentration of 50nM showed maximum gene and protein silencing efficiency while higher concentration (100nM) could be in excess and due to saturation of the RISC machinery possibly lead to ineffectiveness of the system which might be unable to preferentially discriminate and select antisense strand instead of sense strand. The *in vitro* efficiency of siRNAs in our experiments (Table-3) was found adequate enough to proceed towards further pre-clinical studies as a sufficient high rate of gene silencing *in vitro* is mandatory to obtain an efficient tumour growth inhibition *in vivo* (de Martimprey *et al.* 2008).

To have better experiment conditions, previously not only a scrambled sequence siRNA was used as a control but also a siRNA only against the wild-type RET gene (siRNA RET) was tested to scrutinize if a degradation of the RET gene might inhibits the junction oncogene (Gilbert-Sirieix *et al.* 2010). The siRNA RET was found to be efficient in RET/PTC1 silencing ($p < 0.001$) in TCP-1 and RP-1 cells (expressing RET/PTC1 fusion oncogene) as well as in MCF-7 and BT-474 human breast cancer cell lines expressing wild-type RET gene. Although siRNA RET could be sufficient to inhibit RET/PTC expression in cancerous cells, it will equally inhibit the RET expression in normal tissues including lungs, nervous, enteric, genitourinary and hematopoietic systems (Mulligan 2014). Remarkably, siRNA RET/PTC1 was highly efficient against RET/PTC1 fusion and was unable to inhibit RET expression in both MCF-7 and BT-474 cell lines which depicts its specificity for its own junction oncogene avoiding interaction with healthy cells (Gilbert-Sirieix *et al.*, 2010).

Thus we concluded that a siRNA specifically targeted against the junction sequence (including parts of both participating gene) is required to cause efficient inhibitory effects against the junction oncogene and also to avoid wild-type RET gene inhibition in other systems. In the same context, siRNA RET/PTC1 was used as another non-specific control in RP3 cells (expressing RET/PTC3 fusion oncogene). The non-inhibitory effects of siRNA RET/PTC1 in RP3 cell line further supports the specific targeting against the junction oncogene sequence. Vice versa, the siRNA RET/PTC3 was also unable to knockdown RET/PTC1 in BHP10-3 and TPC-1; PTC cell lines expressing RET/PTC1 junction oncogene.

In search of the biological consequences of RET/PTC3 gene silencing, we found that siRNA RET/PTC3 have dual effects; cell growth inhibition by blockage of cell cycle at G₀/G₁ phase and also induction of cell death by apoptosis and/or necrosis. Similarly, under the effect of siRNA RET/PTC1, apoptosis was detected by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay and a down regulation of E2F2 transcription factor was revealed by microarray and RT-qPCR analysis (Gilbert-Sirieix *et al.* 2010). E2F2 plays a crucial role in the cell cycle progression and is a key factor of regulation of cell proliferation (Lal *et al.* 2009). It was then concluded that siRNA not only induces an apoptotic response but also hold up the proliferation by blockage of cell cycle progression (Gilbert-Sirieix *et al.* 2010). In my study, cell death was found to be induced by siRNA RET/PTC3 along with cleavage of both caspase-3 and PARP-1. This demonstrates that siRNA treatment might causes DNA fragmentation that leads activation of the cell death machinery within tumour cells, initially by apoptotic pathway, and as siRNA effects prevail for a certain time, it later activates necrotic pathways. Several cell models including L929 fibro-sarcoma (Schulze-Osthoff *et al.* 1994), HT29 human colon (Meurette *et al.* 2007) and HT29 adeno-carcinoma (Wilson and Browning 2002) cancer cells demonstrated the execution of both apoptotic and necrotic pathways at the same time. Therefore, we concluded that siRNA provokes the cell death by a combination of both apoptosis and necrosis pathways. Since, the cell death is induced by the stimulation of death receptors by their specific ligands, it might be interesting to study, if siRNA treatment is able to modify the expression of apoptosis (CD95/FAS receptor) and necrosis (TNFR = tumour necrosis factor receptor) cell death receptors and/or their respective ligands (TRAIL = TNF-related apoptosis-inducing ligand, FASL or CD95L, TNF α = TNF ligand). Moreover, TRAIL (Frew *et al.* 2008, Bernardi *et al.* 2011, Bernardi *et al.* 2012, Dimberg *et al.* 2013) and CD95 (Martin-Villalba *et al.* 2013) are already used as anti-tumour therapy and potential

therapeutic tool for various malignancies and few TRAIL-based therapies are in phase I or II clinical trials (Bellail *et al.* 2009, de Wilt *et al.* 2013, Gasparini *et al.* 2013, Stuckey and Shah 2013), hence they could also be used in association with siRNA to test the effectiveness of this combined therapy.

Based on our previous experiments (Raouane *et al.* 2011) and promising *in vitro* effects of siRNA RET/PTC3, we decided to carry on *in vivo* experiments after vectorization of siRNA by squalene in the form of nanoparticles. This siRNA nanoparticles formulation was found to be efficient *in vivo* as caused significant inhibition (~60%) of tumour growth. As we observe in the Table-3 that compared to *in vitro* experiments, *in vivo* efficiency of nanoparticles was relatively decreased that might be due to their lose, disintegration or entrapment in the circulation and during transport through various organs of circulatory passage like liver, lungs, spleen and heart (Alexis *et al.* 2008, Tong *et al.* 2013). Depending on their size and surface charge and modifications, the nanoparticles are biologically distributed in different body organs (Alexis *et al.* 2008) and ultimately extravasate the wide fenestrations of tumoral endothelium to reside in the tumour tissue by EPR (enhanced permeability and retention) effect (Dubey *et al.* 2012). An improved circulatory half-life is obtained by a particle size of more than 100nm and a negative or neutral surface charge (Alexis *et al.* 2008). For the reason, the respective NPs were appropriately prepared in respect to size and negative zeta potential in our experiments, for stability of nano-system, to keep the repulsion among the charged particles and to prevent the possible aggregations.

To explain the disagreement between *in vitro* and *in vivo* results, the molecular moieties present in the micro-environment of physiological fluids should be considered. Nanoparticles are encountered with a diverse medium of biological components; mostly proteins including immuno-globulins, complement and coagulation factors, albumin, fibrinogen and tissue development factors (Saptarshi *et al.* 2013). Thus, due to their size and large surface-to-mass ratio, NPs form Bio/Nano complexes by interacting with these proteins and bio-molecules or with other nucleic acids, lipids and even biological metabolites (Saptarshi *et al.* 2013). Nanoparticles selectively adsorb various peptides and amino acids onto their surface to form NP-protein coronas by molecular interactions between chemical groups of nanoparticle surfaces and amino-acid residues. The coated material changes the NPs shape and surface properties, potentially enhancing or reducing the ability to deliver the carrier and thus the ultimate fate of the nano-agent (Xia *et al.* 2010). Protein interactions also affect the cellular uptake, accumulation, degradation and clearance of NPs from the

circulation (Saptarshi *et al.* 2013). Due to this reason, it is thus possible that nanoparticles containing GALA-Chol made aggregates with serum components and were not able to give as efficient *in vivo* results as *in vitro*.

As previously mentioned that nanoparticles interact with complex natural fluids components (Saptarshi *et al.* 2013) and this nano-bio interaction could hinder the absolute quantity of nanoparticles to be delivered to the target, can significantly reduce the kinetic release of the oligonucleotides from the squalenoylated nanoparticles and that's the reason the efficiency of vectorized siRNA is not parallel to that of non-vectorized oligonucleotides *in vitro* (Table-3). Thus, the fate of nanoparticles in the form of whole particle delivery must to be addressed, in order to increase the delivery and efficiency of nano-formulations and to prevent their metabolism by body immune system and subsequent rapid blood clearance by renal excretion. In this context, radio (radio-iodine¹²⁵) or fluorescent labelling by near-infrared fluorochromes (von Maltzahn *et al.* 2012) or quantum dots (Ballou *et al.* 2005) can be used to detect the destination of circulatory nanoparticles. However, appropriate structural modifications or binding of nanoparticles with a peptide (arginine residues), transferrin (Bartlett and Davis 2007) or antibodies exclusively targeting tumoral cells [Avastin (bevacizumab) or Herceptin (trastuzumab); monoclonal, specific antibodies against human VEGF (Lee *et al.* 2000) or HER-2 (Izumi *et al.* 2002)] can improve specific delivery.

Previously in the *in vivo* experiments, a significant gene inhibition was observed by siRNA RET/PTC1-SQ NPs *via intra venous* route at a cumulative dose of 2.5mg/kg/mouse (Raouane *et al.* 2011) but then a 5 times decreased dose (0.5mg/kg/mouse) was tested in the experiments (Ali *et al.*, 2013). The observed significant tumour growth inhibition even at this low concentration demonstrated the efficacy of the selective treatment. To start the experiments on RET/PTC3, at an initial step, previously selected higher cumulative dose of 2.5mg/kg/mouse was selected, as this fusion oncogene is more aggressive and highly metastatic. The assumption was that, in case of un-expected outcomes, it might not be controversial that whether the in-efficiency of the treatment is due to in-sufficiency of the administered dose or due to in-effectiveness of the treatment. The inhibitory trend in tumour growth even from the first NPs injection also showed the effectiveness of the therapy. The sustained effects of both siRNA RET/PTC1-SQ and siRNA RET/PTC3-SQ nanoparticles on tumour growth inhibition for several (7-9) days after the last injection, advocates the stability of the system and that, it could preserve the effects of siRNAs for a longer time which are usually transitory. Current observations also validated the efficacy of nano-precipitation of

siRNAs by squalenylation and also demonstrated that the siRNAs are still active after modification of passenger sense strand, use of ester hydrolysable linkage between the squalene moiety and siRNA and bio-conjugation with squalene. The comparable inhibitions of tumour growth by intra-venous route as that of intra tumoral injection suggest that the NPs are capable of protecting the activity of siRNA once injected by classical intra venous route of cancer treatment. Therefore, siRNA RET/PTCs-SQ NPs were confirmed to be effective therapy against *in vivo* tumour models, as summarized in Table-3.

The nanoparticles of siRNA RET/PTCs-SQ are a promising new therapy for the treatment of PTC but with a few limitations which lead to their lower *in vivo* efficiencies. For further improvement in the siRNA therapy, combinational therapies of RNAi in conjunction with chemo-, radio- and/or immuno-therapy could be potentially exercised. Hence, siRNAs RET/PTCs could also be combined with multiple key players of a single pathway or against multiple cancer-promoting pathways or along with other anti-cancer drugs or ¹³¹I radiotherapy, for more effective tumour therapies. Moreover, association of siRNA with ¹³¹I radio-therapy could open new perspectives since ¹³¹I is the most prevalent treatment used for thyroid carcinoma.

1.15. Conclusions

1. Both siRNA RET/PTC1 and siRNA RET/PTC3 are efficient and specific against their own junction oncogene and are not able to inhibit the expression of an alternate sequence.
2. RET/PTC3 junction oncogene is capable of tumorigenic transformation of NIH/3T3 embryonic mouse fibroblasts into RP3 cells which stably express this oncogene and have phenotypic differences from their mother cell line.
3. The GALA-Chol is efficient for intra-cellular penetration and trans-membrane delivery of siRNA RET/PTC1-SQ nanoparticles *in vitro*.
4. Addition of GALA-Chol renders the nanoparticles (siRNA RET/PTC1-SQ GALA-Chol NPs) efficient *in vitro* in gene and protein inhibitions and to decrease cell viability but make them in-effective in tumor growth inhibition *in vivo*.
5. The nanoparticles siRNA RET/PTCs-SQ are not able to cross the cell membrane and thus are in-efficient in gene and protein inhibitions *in vitro* except once transfected with lipofectamine.
6. Both nanoparticles (siRNA RET/PTC1-SQ and siRNA RET/PTC3-SQ) are efficient *in vivo* for tumor growth inhibition, to reduce RET/PTCs oncogene and oncoprotein expressions.
7. Both nanoparticles have similar non-efficient *in vitro* and efficient *in vivo* results in gene and protein inhibitions.
8. Nanoparticles of siRNA RET/PTC1-SQ inhibit the tumor growth even at five times less important cumulative dose (0.5mg/kg/mouse) than previously published results (2.5mg/kg/mouse).
9. Newly targeted RET/PTC3 junction oncogene with siRNA RET/PTC3-SQ NPs at 2.5mg/kg/mouse cumulative dose showed efficient *in vivo* tumor inhibitory results.
10. The nanoparticles of siRNA RET/PTC3-SQ induce cell death *in vitro* and *in vivo* by cleavage of both caspase-3 and PARP-1 and partially restore differentiation *in vivo* (decrease of Ki67 marker).
11. The "squalenylation" of siRNA offers a new pharmacological approach to target junction oncogenes, thus could be extended to other cancer therapy models.

Drug therapy based on siRNA is developing rapidly and new outcomes are appearing every day. It is the time of RNAi technology to be applied in clinics to exploit the potential of this powerful tool in cancer treatment. In conclusion, the significance of siRNA

RET/PTCs-SQ NPs have been demonstrated in preclinical studies for thyroid cancer therapy and further pharmacological and clinical investigations can be proceeded to set-in the remedy of thyroid carcinoma.

1.13. Perspectives

1. Lower concentrations of GALA-Chol will be tested to check their *in vitro* and later *in vivo* efficiency to resolve the dilemma of nanoparticles aggregation.
2. Lower cumulative dose of siRNA RET/PTC3-SQ NPs at 0.5mg/kg/mouse will be injected to observe the tumor inhibitory effects *in vivo*.
3. A longer treatment plan with more spaced injection schedule of siRNA RET/PTCs-SQ NPs will be applied to might enhance tumour regression *in vivo*.
4. The bio-distribution of nanoparticles will be monitored in the organs of drug elimination *i.e.* liver, spleen, kidney along with tumor tissue to well understand the fate and the availability of nanoparticles to the target organ.
5. The metabolites of nanoparticles should be screened out to observe the bio-degradation of NPs by the body immune and defense system.
6. The siRNA RET/PTCs-SQ NPs could be combined with ^{131}I radiotherapy and/or VEGF inhibitor to further improve the treatment plan of PTC.

Since the effectiveness of siRNA-squalene nano-precipitation is validated against papillary thyroid carcinoma, thus it will be extended to other cancer pathologies like prostate (TMPRSS2/ERG) and leukaemia (PDGFR β /D10S170) carrying junction oncogenes by using the generic platform of squalenoylation.

1.17. Synthèse du travail :

1.17.1. Introduction

Le cancer de la thyroïde est la tumeur endocrine la plus fréquente et représente 95% des cancers endocriniens (Keefe *et al.* 2010). Son taux d'incidence a augmenté au cours des dernières années (2005 à 2009) de 5,6% par an chez les hommes et de 7,0% par an chez les femmes dans la plupart des pays développés (Chen *et al.* 2009; Enewold *et al.* 2009; Kilfoy *et al.* 2009; Pellegriti *et al.* 2013, American Cancer Society, 2013). Aux États-Unis, 56 460 et 60 220 nouveaux cas de cancer de la thyroïde ont été rapportés en 2012 et 2013 (Siegel *et al.* 2012). L'incidence annuelle de cancer de la thyroïde varie considérablement entre les différents régions ; de 1,2 à 2,6 cas chez les hommes et de 2,0 à 3,8 cas chez les femmes par 100 000 personnes (Nagataki and Nystrom 2002; Venencia *et al.* 2002; Zaman *et al.* 2006). Le sexe ratio est plus élevé chez les femmes (trois patients sont des femmes sur quatre) ainsi que le taux de mortalité (1,3 fois plus élevé chez les femmes, *i.e.* 1 040 femmes *vs* 810 hommes).

Deux types de cellules endocriniennes sont responsables du développement du carcinome thyroïdien (TC) : les cellules épithéliales et des cellules parafolliculaires. Les formes TC dérivées des cellules épithéliales comprennent TC différenciés (DTC) [y compris papillaire (PTC; environ 70-80%) et folliculaires (FTC; 10-20%) TC] et le carcinome thyroïdien indifférencié ou anaplasique (ATC; 2-5%). La tumeur de la thyroïde dérivé de cellules parafolliculaires est nommé le carcinome médullaire de la thyroïde (MTC) et représente environ 3 à 10% de cancer de la thyroïde (Benvenga 2008; Zhu and Cheng 2009; Chougnnet *et al.* 2010; Keefe *et al.* 2010).

L'augmentation de l'incidence du cancer de la thyroïde est principalement due à celui du type papillaire (Leenhardt *et al.* 2004b, Pellegriti *et al.* 2013). L'exposition aux radiations ionisantes est un facteur de risque établi pour cancer de la thyroïde. Le PTC est fréquemment retrouvé chez les personnes ayant été exposées à de fortes radiations externes accidentelles, comme par exemple, à la suite de l'explosion du réacteur de la centrale nucléaire de Tchernobyl en 1986, ainsi que chez les personnes ayant déjà été traitées par radiothérapie. D'après plusieurs auteurs, la tomодensitométrie (TDM) utilisée en particulier en pédiatrie conduirait à une augmentation du risque de développer un PTC (Baker and Bhatti 2006, How and Tabah 2007).

Au niveau moléculaire, le carcinome papillaire de la thyroïde est principalement caractérisé par des réarrangements génomiques, entre le gène RET (REarranged during Transfection) et 12 autres gènes exprimés dans la thyroïde conduisant à 13 onco-fusions différentes type RET/PTC (Romei and Elisei 2012). Les variants les plus fréquemment observés chez les patients sont RET/PTC1 et RET/PTC3. Ces variants sont dus à une inversion para-centrique du chromosome 10 (10q11.2) entre la partie intracellulaire du gène RET et respectivement les gènes H4/CCDC6 ou ELE1/NCOA4. Chez l'homme, La proximité spatiale de RET entre le gène RET et les gènes partenaires, NCOA4 et H4 (situés dans la même région chromosomique 10q11.2) (Gandhi and Nikiforov 2011) serait à la base de la formation de réarrangements RET/PTC (Nikiforova *et al.* 2000, Gandhi *et al.* 2006). Ces altérations génétiques, présents dans environ 80% des cas de cancer de la thyroïde (Moses *et al.* 2010) sont à l'origine de l'activation de plusieurs voies de signalisation menant à une augmentation de la prolifération cellulaire dont celle des MAPK (mitogen-activated protein kinase) (Castellone *et al.* 2009).

Malgré des progrès dans le traitement du cancer suite à la découverte d'applications thérapeutiques innovantes, un nombre considérable de patients retrouvent une maladie récurrente avec un phénotype résistant. Parmi les stratégies de traitement, l'interférence à l'ARN (ARNi) a progressé d'un outil de laboratoire puissant vers des modalités thérapeutiques potentielles dans le domaine de la thérapie ciblée.

L'interférence ARN (ARNi) est un mécanisme post-transcriptionnel de l'inactivation de gènes par la dégradation de l'ARNm ou de l'inhibition directe de la traduction des protéines (Caplen 2004; Dorsett and Tuschl 2004; Shankar *et al.* 2005; Ramachandran and Ignacimuthu 2013). Elle peut être induite par l'introduction d'ARN ou d'ADN simple brin de 13 à 25 bases; les oligonucleotides anti sens (AS-ONs), par de l'ADN plasmidique (ADNp) qui exprime courts ARN (shRNA) qui par la suite sont traitées comme siRNA par la machinerie cellulaire (McManus and Sharp 2002; Scherr *et al.* 2003; Kim and Rossi 2007) ou par synthèse de petits ARN interférents (siRNA) consistant en 19-27 paires de bases en longueur. L'inhibition des gènes par ces approches est hautement spécifique et bien que leur capacité d'appariement de bases avec les séquences spécifiques d'acides nucléiques complémentaires est similaire, leur mode d'inhibition d'un gène est relativement différente. Les AS-ONs sont pris en charge par l'enzyme Ribonuclease H (RNase H) qui aide à la dégradation de l'ARNm complémentaire cible d'une manière catalytique, ensuite l'AS-ONs

est à nouveau libéré pour se lier avec un autre ARNm cible (Walder and Walder 1988, Eder and Walder 1991, Schultz and Champoux 2008, Tadokoro and Kanaya 2009, Sitikov 2012).

Les siRNA synthétiques sont des petits ARN double brin (dsRNA) et sont le substrat du complexe RISC ou RNA induced silencing complex (McManus and Sharp 2002). L'introduction de siRNA initie une cascade puissante de la dégradation spécifique de la séquence d'ARNm cible sur la liaison d'une séquence d'ARNm complémentaire du génome (Wilson and Doudna 2013). Les siRNAs sont des agents très prometteurs en thérapie anticancéreuse. Ils peuvent inhiber un grand nombre de cibles (récepteurs à activité tyrosine kinase, gène anti-apoptotiques et autres gènes de survie cellulaire). De plus, ils peuvent contrecarrer la résistance causée par l'efflux des drogues. En effet, il est peu probable que les siRNA soient expulsés des cellules cancéreuses et certains d'entre eux peuvent inhiber l'expression des protéines d'efflux. De plus, les siRNAs exercent leur activité à des concentrations extrêmement faibles (de l'ordre du pico molaire).

Sachant que la séquence d'un oncogène de jonction est unique, elle ne se trouve que dans les cellules tumorales et représente une cible spécifique pour des thérapies ciblées type siRNA, nous avons choisi d'appliquer cette technologie innovante au cancer papillaire de la thyroïde qui est la tumeur endocrine la plus fréquente.

Dans ce contexte, un projet de recherche a été lancé dans l'équipe de Dr Massade à la recherche d'un traitement efficace basé sur l'utilisation de siRNA contre le carcinome papillaire de la thyroïde (PTC). L'exploration d'une nano-médecine efficace contre les oncogènes de fusion RET/PTC exprimée dans les PTC a été le thème principal de mon projet de thèse.

Mais, la délivrance *in vivo* de siRNA est un défi majeur. De plus, l'efficacité biologique du siRNA est entravée par leur demi-vie plasmatique courte (mauvaise stabilité dans les liquides biologiques) et à une faible pénétration intracellulaire (caractère très hydrophile). La délivrance de siRNA dépend de: i) la structure, ii) des modifications et des substitutions chimiques qui affectent sa stabilité dans la circulation sanguine, iii) l'aptitude à s'extravaser dans les vaisseaux sanguins, iv) la bio-distribution, v) la capacité de pénétration intracellulaire et clairance de la circulation (Kanasty *et al.* 2012). Les problèmes d'instabilité et de dégradation doivent être résolus avant d'exploiter la pleine efficacité de siRNA pour l'inactivation des gènes. Les différentes approches utilisées pour délivrer les siRNA à la cible, leurs avantages et inconvénients sont résumés dans l'article de revue (Ali *et al.* 2012).

Afin de protéger les siRNA contre la dégradation et d'améliorer la pénétration intracellulaire, les siRNA RET/PTCs ont été vectorisés par méthode de "squalenoylation" (pour revue voir: Raouane *et al.* 2012, Ali *et al.* 2012). Le squalène (SQ) est un lipide neutre, précurseur du cholestérol, également utilisé dans diverses préparations pharmaceutiques comme excipient. Ce nouveau composé non-cationique a été découvert par l'équipe du Pr. COUVREUR pour préparer des nanoparticules (NPs) (Couvreur *et al.* 2006; Couvreur *et al.* 2008). Ainsi les nanoparticules de SQgem (squalène + gemcitabine) ont été préparées et ont été trouvées plus cytotoxique *in vitro* (Reddy *et al.* 2007) et possèdent une activité antinéoplasique plus efficace *in vivo* (Reddy *et al.* 2008a). Nous avons appliqué ce concept innovant pour vectoriser les siRNA thérapeutiques que nous avons mis au point au laboratoire.

1.17.2. Principaux résultats obtenus au cours de la thèse

Au Cours de sa thèse, Marie Gibert a conçu et testé l'efficacité et la spécificité des siRNA RET/PTC1 (Gilbert-Sirieix *et al.* 2010). Ensuite, Mouna Raouane a vectorisé les siRNA RET/PTC1 par la méthode de squalenoylation. Brièvement cette technique consiste à coupler d'une manière covalente le siRNA RET/PTC1 au squalène. Le bio-conjugué formé est capable de s'auto-assembler spontanément dans une solution aqueuse pour donner des nanoparticules. Les nanoparticules siRNA RET/PTC1-SQ ont montré des effets inhibiteurs significatifs (70% d'inhibition de la croissance tumorale). De plus, une inhibition de l'expression de l'oncogène et de l'onco-protéine sont observés (Raouane *et al.* 2011). En revanche, aucune efficacité des nanoparticules siRNA RET/PTC1-SQ ont été trouvés *in vitro*. **Nous avons supposé que l'inefficacité des nanoparticules est due à leur incapacité à traverser les membranes cellulaires, comme ils ne sont pas trouvés dans le compartiment intracellulaire par microscopie à fluorescence, sauf une fois transfectées par la lipofectamine 2000.**

Pour surmonter cet obstacle, un peptide fusogénique, le GALA a été choisi ayant montré des résultats intéressants pour améliorer l'internalisation des ONs anti sens (Hughes *et al.* 1996) et les siRNAs (Hatakeyama *et al.* 2009, Sakurai *et al.* 2009, Akita *et al.* 2010, Sato *et al.* 2010, Akita *et al.* 2011). Le GALA interagit avec les membranes cellulaires dans le tissu cible, pour obtenir un meilleur transport transmembranaire et pour améliorer la pénétration intracellulaire de nanoparticules. Ainsi, pour restaurer l'efficacité *in vitro* des nanoparticules siRNA RET/PTC1-SQ, le GALA a été ajouté en tant que GALA-Chol pour améliorer la translocation de nanoparticules jusqu'à la cible.

Afin d'améliorer la pénétration intracellulaire, nous avons combiné les siRNA RET/PTC1 SQ avec le GALA (répétition d'acide glutamique-alanine-leucine- alanine) (Subbarao *et al.* 1987). Le GALA s'incorpore dans la membrane endosomale et la déstabilise suite à un changement conformationnel induit par le faible pH endosomal, ce qui favorise l'échappement vers le cytoplasme. Le GALA sous une forme conjugué avec du cholestérol (GALA-Chol) a été synthétisé et fourni par le Pr Hideyoshi Harashima (Laboratory of Innovative Nanomedicine, Faculty of Pharmaceutical Sciences, Hokkaido University Kita 12, Japan). Ainsi, le GALA-Chol associé aux nanoparticules squalénées siRNA-SQ RET/PTC1 conduit à la formation de nanoparticules (NPs) siRNA-SQ-GALA-Chol. *In vitro*, les NPs siRNA-SQ GALA-Chol RET/PTC1, à la dose de 50nM sont efficaces et non toxique dans la lignée BHP 10-3 qui exprime RET/PTC1. En microscopie confocale et contrairement aux NPs siRNA-SQ RET/PTC1, les NPs siRNA-SQ GALA-Chol RET/PTC1 sont capables de franchir la membrane cellulaire et se situent dans le cytoplasme.

Ensuite, des expériences chez l'animal ont été effectuées. Les NPs siRNA RET/PTC1-SQ ainsi que les NPs siRNA-SQ GALA-Chol RET/PTC1 ont été injectés à des souris nues porteuses de tumeurs xéno greffées par BHP-10 soit par voie intraveineuse (*i.v.*) soit par voie intratumorale (*i.t.*). Après sacrifice des souris, l'expression de RET/PTC1 par RT-qPCR et par Western blot dans les tumeurs.

Les résultats montrent que seules les NPs siRNA RET/PTC1-SQ sont capables d'inhiber la croissance tumorale et l'expression de RET/PTC1. Donc le GALA améliore l'internalisation des NPs siRNA RET/PTC1-SQ *in vitro* mais n'a aucune activité antinéoplasique *in vivo* probablement dû à une agrégation des NPs siRNA RET/PTC1-SQ+GALA dans la circulation sanguine.

En conclusion, les nanoparticules siRNA-squalène sont définitivement accréditées pour délivrer les siRNA thérapeutiques et permettent de diminuer les doses de siRNA tout en maintenant leurs efficacités antinéoplasiques.

Dans la deuxième partie de l'étude, nous nous sommes intéressés à l'oncogène de jonction RET/PTC3 qui est à l'origine du cancer radio-induit agressif de l'enfant, observé avec une incidence élevée dans les zones fortement exposées aux radiations ionisantes après

l'accident de Tchernobyl. Il résulte de l'activation de RET par recombinaison au gène ELE1 suite à des irradiations précoces.

A notre connaissance, aucune lignée de cellules humaines exprimant RET/PTC3 n'a été établie. Dans un premier temps, nous avons établi une lignée cellulaire exprimant l'oncogène RET/PTC3 par transfection stable d'un plasmide d'expression exprimant RET/PTC3 dans la lignée NIH-3T3 issue de fibroblastes de souris (**baptisée RP3**).

La lignée RP3 a été caractérisée pour sa tumorigénicité chez la souris nude. Nous montrons que l'injection en sous-cutanée des cellules RP3 engendre le développement de tumeurs en 15 jours chez la souris nude qui confirme l'acquisition du phénotype tumoral.

Ensuite, quatre siRNA ciblant la jonction RET/PTC3 ont été dessinés et synthétisés, le plus efficace inhibe de 80% l'expression de l'ARNm de RET/PTC3 et diminue significativement l'expression de la protéine RET. Nous montrons que le siRNA RET/PTC3 est spécifique de la séquence RET/PTC3 car il est inefficace sur des lignées cellulaires exprimant l'oncogène RET/PTC1 (lignées de carcinome papillaire de la thyroïde TCP-1 et BHP10-3). Dans la lignée RP3, le siRNA RET/PTC3 inhibe la viabilité cellulaire (test MTT), la migration et l'invasion cellulaire (recolonisation de brèche sans et avec MatrigelTM respectivement, suivi d'une analyse en IncucyteTM), bloque les cellules en phase G0/G1 (cytométrie en flux après marquage des cellules à l'iodure de propidium) et induit l'apoptose (clivage de la caspase-3 et de PARP observés en Western blot). Ensuite, le siRNA RET/PTC3 a été vectorisé par squalénisation. Le siRNA RET/PTC3 couplé au squalène et administré sous forme de nanoparticules inhibe fortement la croissance tumorale et diminue l'expression de l'oncogène et de l'oncoprotéine (Ali *et al.* 2014b).

En conclusion, nous avons établi un modèle cellulaire (RP3) qui exprime l'oncogène de jonction RET/PTC3, qui est tumorigène chez la souris athymique. Nous avons défini des siRNA RET/PTC3 efficaces et spécifiques RET/PTC3 qui, administrés *in vivo* sous forme de nanoparticules, inhibent fortement la croissance tumorale et diminuent l'expression de l'oncogène et de l'oncoprotéine.

1.17.3. Discussion

Dans la première partie de ce travail de thèse, nous avons montré que les nanoparticules siRNA RET/PTC1-SQ combinées au GALA-Chol sont inefficaces *in vivo* mais augmentent l'internalisation du siRNA RET/PTC1 *in vitro*. Pour expliquer le désaccord entre résultats *in vitro* et *in vivo*, nous devons considérer les molécules présentes dans le microenvironnement des fluides physiologiques. *In vivo*, les nanoparticules se trouvent dans un milieu riche en protéines *e.g.* des immunoglobulines, des protéines du complément, des facteurs de coagulation, l'albumine, le fibrinogène et des facteurs de développement (Saptarshi *et al.* 2013). Ainsi, en raison de leur grande taille et du rapport surface-masse, les NPs forment des complexes Bio/Nano en interagissant avec des protéines et des biomolécules ou, avec d'autres acides nucléiques, des lipides et même avec des métabolites (Saptarshi *et al.* 2013). Les nanoparticules adsorbent sélectivement différents peptides et d'acides aminés sur leur surface pour former des coronas NPs-protéines par des interactions moléculaires entre des groupes chimiques des surfaces des nanoparticules et des résidus d'acides aminés. Ces composants absorbés sur les NPs modifient leurs propriétés de forme et de surface, ce qui pourrait accroître ou réduire la capacité de délivrance et donc le devenir ultime du nano-agent (Xia *et al.* 2010). Les interactions protéiques affectent également la pénétration cellulaire, l'accumulation, la dégradation et l'élimination des NPs de la circulation (Saptarshi *et al.* 2013). Pour cette raison, il est ainsi possible que les nanoparticules associées au GALA-Chol forment des agrégats avec des composantes sériques ce qui expliquerait la discordance entre les résultats *in vivo* et *in vitro*.

Comme mentionné précédemment, les nanoparticules interagissent avec les composants complexes de fluides naturels (Saptarshi *et al.* 2013). Cette interaction nano-bio pourraient entraver la quantité absolue de nanoparticules à être livré à la cible ce qui permet de réduire considérablement la cinétique de libération des oligonucléotides des nanoparticules.

Pour cela, des études de pharmacocinétique et de bio-distribution seront nécessaires pour comprendre le devenir des NPs siRNA-SQ. Nous supposons que ces nano-vecteurs sont capables de traverser le réseau vasculaire qui est désorganisé dans la tumeur et rendu par ce fait, plus perméable par rapport aux tissus sains. De plus, le système lymphatique tumoral est souvent compromis. Cet effet de perméabilité et de rétention tissulaire appelé EPR (Enhanced Permeability and Retention effect) (Maeda *et al.* 2013) permet une vectorisation passive c'est-à-dire, un effet spontané de translocation des nanoparticules de

taille inférieure à 200 nm à travers la paroi des vaisseaux néoformés de la tumeur. Dans le cas où les études de biodistribution montrent une accumulation importante dans les organes d'épuration, nous pouvons envisager de PEGyler les nanoparticules (Jokerst *et al.* 2011) pour empêcher les phénomènes d'opsonisation afin de réduire la capture massive des nanoparticules par le système réticulo-endothélial et par conséquent favoriser leur accumulation sélective au niveau de la tumeur (Brigger *et al.* 2001). Des modifications structurales peuvent être aussi envisagées en liant les nanoparticules soit à la transferrine (Bartlett and Davis 2007) soit à des anticorps ciblant exclusivement les cellules tumorales [Avastin (bevacizumab) ou Herceptin (trastuzumab), des anticorps monoclonaux spécifiques contre le VEGF (Lee *et al.* 2000) ou l'HER-2 (Izumi *et al.* 2002)].

Dans la seconde partie du travail, nous avons établi une nouvelle thérapie contre l'oncogène de jonction RET/PTC3 par siRNA et montré la spécificité du traitement en ciblant les deux gènes partenaires de la jonction RET et ELE1 et non seulement RET. Nous avons entrepris cette démarche car un siRNA dirigé uniquement contre RET pourra également inhiber l'expression de RET dans les tissus normaux, y compris les poumons, les systèmes nerveux, uro-génital et hématopoïétiques (Mulligan 2014). Le siRNA RET/PTC1 a été utilisé comme contrôle non spécifique des cellules RP3 (exprimant l'oncogène de fusion RET/PTC3). Les effets non inhibiteurs du siRNA RET/PTC1 dans la lignée cellulaire RP3 montre que le ciblage est spécifique contre la séquence d'oncogène de jonction. *Vice versa*, le siRNA RET/PTC3 est également incapable d'inhiber RET/PTC1 dans les lignées cellulaires BHP 10-3 et TPC-1 (exprimant oncogène de fusion RET/PTC1). Ainsi, nous avons conclu qu'un siRNA ciblé spécifiquement contre la séquence de jonction (y compris à deux parties de gène participant) est nécessaire pour provoquer des effets inhibiteurs efficaces contre l'oncogène de jonction et également d'éviter une inhibition de type sauvage du gène RET dans d'autres systèmes.

A la recherche des conséquences biologiques de l'inhibition de RET/PTC3, nous avons constaté que le siRNA RET/PTC3 à des effets doubles; inhibition de la croissance cellulaire par un blocage de cycle cellulaire en phase G0/G1 et également induction de la mort cellulaire par apoptose et/ou par nécrose. Dans notre étude, la mort cellulaire a été trouvée être induite par le siRNA RET/PTC3 par clivage de la caspase-3 et de PARP-1. Ceci démontre que le traitement par siRNA provoque la fragmentation de l'ADN qui conduit à l'activation de la machinerie de la mort cellulaire dans les cellules tumorales, d'abord par voie apoptotique et dès que les effets siRNA persistent pendant un certain temps, il active

ensuite les voies nécrotiques. Ces résultats ont été observé par d'autres auteurs dans plusieurs modèles cellulaires cancéreux, notamment de fibro-sarcome L929 (Schulze-Osthoff *et al.* 1994), et d'adénocarcinome colique HT29 (Wilson and Browning 2002, Meurette *et al.* 2007). Par conséquent, nous avons conclu que le siRNA provoque la mort cellulaire par une combinaison des deux voies; l'apoptose et la nécrose. Tant que, la mort cellulaire est induite par la stimulation des récepteurs de mort par leurs ligands spécifiques, il pourrait être intéressant d'étudier, si le traitement par siRNA est capable de modifier l'expression de récepteur de l'apoptose (CD95/Fas), de la nécrose (TNFR = tumour necrosis factor receptor) et/ou leurs ligands respectifs (TRAIL = TNF-related apoptosis-inducing ligand, FASL or CD95L, TNF α = TNF ligand). En plus, les TRAIL (Frew *et al.* 2008, Bernardi *et al.* 2011, Bernardi *et al.* 2012, Dimberg *et al.* 2013) ainsi que le CD95 (Martin-Villalba *et al.* 2013) sont déjà utilisés comme thérapie anti-tumorale et outil thérapeutique potentiel pour diverses tumeurs malignes et quelques thérapies à base de TRAIL sont en phase I ou II en essais cliniques (Bellail *et al.* 2009, de Wilt *et al.* 2013, Gasparini *et al.* 2013, Stuckey and Shah 2013). Par conséquent, ils pourraient également être utilisés en association avec le siRNA pour tester l'efficacité de cette thérapie combinée.

L'ensemble des résultats obtenus sur RET/PTC1 et RET/PTC3 montrent que nos siRNA mis au point auraient des applications thérapeutiques. Les expériences préalablement effectués au laboratoire montrent une inhibition significative (~70%) de gène RET/PTC1 par les siRNA RET/PTC1-SQ NPs lorsqu'ils sont injectés par voie intraveineuse à la dose cumulative de 2.5 mg/kg/souris (Raouane *et al.* 2011). Même à une dose 5 fois moins importante (0.5 mg/kg/souris) une inhibition significative de la croissance tumorale (Ali *et al.* 2014a) dans les tumeurs porteuses de RET/PTC1 a été montrée. Pour RET/PTC3, la dose de 2.5 mg/kg/souris a été choisie car l'oncogène de jonction RET/PTC3 est plus agressif et plus métastatique (Ali *et al.* 2014b). L'inhibition de la croissance tumorale, même à partir de la première injection de siRNA RET/PTC3 NPs montre l'efficacité de cette thérapie ciblée. Les effets persistants des nanoparticules siRNA RET/PTC1-SQ ou siRNA RET/PTC3-SQ sur l'inhibition de la croissance tumorale pendant plusieurs (7-9) jours après la dernière injection, montre la stabilité du système qui préserve les siRNA de la dégradation.

1.17.4. Conclusions :

1. Les deux siRNA RET/PTC1 et RET/PTC3 sont efficaces et spécifiques contre leur propre oncogène de jonction.
2. L'oncogène de jonction RET/PTC3 est capable de transformer les fibroblastes embryonnaires de souris NIH/3T3.
3. *In vitro*, les nanoparticules siRNA RET/PTC-SQ ne sont pas capables de traverser la membrane cellulaire et sont donc inefficace sur l'inhibition de l'oncogène et de son oncoprotéine, sauf si transfectées avec lipofectamine 2000.
4. *In vitro*, le GALA-Chol favorise l'internalisation des nanoparticules siRNA RET/PTC1-SQ et la libération du siRNA RET/PTC1 qui se traduit par une efficacité de l'inhibition de l'oncogène et de l'oncoprotéine RET/PTC1 mais il s'agrège *in vivo* aux protéines et rend les nanoparticules siRNA RET/PTC1 inefficaces.
5. Les deux nanoparticules (siRNA RET/PTCs-SQ NPs) sont efficaces *in vivo*, ils inhibent la croissance tumorale, réduisent l'expression de l'oncogène et l'oncoprotéine RET/PTCs.
6. Les nanoparticules de siRNA RET/PTC1-SQ inhibent la croissance tumorale, même à une dose cinq fois moins importante (de 0.5 mg/kg/souris) que les résultats publiés antérieurement (de 2.5 mg/kg/ souris).
7. Les nanoparticules de siRNA RET/PTC3-SQ induisent la mort cellulaire *in vitro* et *in vivo* par clivage de la caspase-3 et PARP-1 et restaurent partiellement la différenciation *in vivo* (diminution de marqueur Ki67).

1.17.5. Perspectives :

1. Des concentrations plus faibles de GALA-Chol seront testées pour vérifier leur efficacité *in vitro* et *in vivo* afin d'empêcher l'agrégation des nanoparticules aux protéines sériques.
2. Plusieurs protocoles de traitement par les nanoparticules siRNA RET/PTC-SQ seront testés afin de pouvoir améliorer la régression tumorale (*e.g.* des concentrations plus faibles en siRNA-SQ NPs, des injections plus espacés de siRNA-SQ NPs à des concentrations plus fortes).

3. Dans le but de comprendre la disponibilité des nanoparticules dans l'organe cible, la bio-distribution des nanoparticules sera étudiée dans les organes ainsi que le tissu tumoral.
4. Dans le but d'améliorer le traitement des carcinomes papillaires de la thyroïde, les siRNA RET/PTCs-SQ NPs devraient être combinés avec ^{131}I et/ou un inhibiteur de VEGF.
5. L'approche pharmacologique par siRNA-SQ NPs devrait être étendue à d'autres pathologies cancéreuses porteuses d'oncogène de jonction (*e.g.* TMPRSS2/ERG dans le cancer de la prostate).

1.17.6. Retombées cliniques :

Sachant que les oncogènes RET/PTC1 et RET/PTC3 représentent le *quasi* majorité des réarrangements RET observés dans les cancers papillaires de la thyroïde, leur inhibition par des siRNA permettrait d'introduire une nouvelle thérapie pour les patients réfractaires aux traitements actuels. L'avantage d'utiliser la méthode de squalénisation par rapport à celle des lipides cationiques pour délivrer les siRNA RET/PTCs est d'avoir un ratio en charge efficace (siRNA/squalène) de 1/1 (mole/mole) permettant ainsi de livrer plus de produit actif. Notre but ultime serait d'introduire dans les années qui suivent une nano-médecine à base de siRNA RET/PTC-squalène en phase clinique.

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