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Clinical and Molecular Characteristics and Management of Adults with Rhabdomyosarcoma and Screening of Targeted Polytherapy in vitro

Sarah Baccouche Dumont

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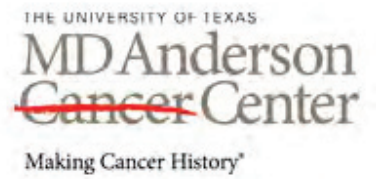
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Université Claude Bernard Lyon 1



Dissertation for the Degree of

DOCTOR OF PHILOSOPHY

**Clinical and Molecular Characteristics and Management of Adults with
Rhabdomyosarcoma and Screening of Targeted Polytherapy *in vitro***

Sarah DUMONT, MD



ABSTRACT

Introduction

Rhabdomyosarcoma is a rare entity in adult patient with unfavourable outcome. This work describes the clinical and molecular specificities of adolescent and adult type of rhabdomyosarcoma that lack literature and investigates the optimal integration of targeted therapy to doxorubicin, sarcoma standard of care through a screening of multiple therapies of targeted agents on small cell sarcoma cell lines *in vitro*.

Material and Methods

We retrospectively analyzed 239 patients, 10 years of age and greater, diagnosed with RMS at MD Anderson Cancer Center from 1957 through 2003 and their *PAX-FOXO1* fusion gene status by fluorescence *in situ* hybridization on tissue microarray. Three small cell sarcoma cell lines were exposed to increasing concentrations of targeted agents alone, in doublets, then with doxorubicin. Cell viability, cell cycle and apoptosis were assessed by MTS assay, Annexin V and caspase 3/7 activity.

Results

Patients with metastatic rhabdomyosarcoma were found to have a 18% survival rate at 5 years from diagnosis with an 12% survival past 15 years. This outcome was even poorer for patients over 50 of age, even with localized disease. Younger patients were more likely to receive multidisciplinary therapy than their older counterparts. The presence of *PAX-FOXO1* translocation was significantly associated with a higher frequency of metastatic disease. The four agents with the exception of abacavir synergized two by two with each other *in vitro* but the triple combinations did not perform better than the bitherapies. The dual therapies vorinostat (HDAC

inhibitor) plus 17-DMAG (Hsp90 inhibitor) added with doxorubicin achieved better results than dual or triple therapies.

Conclusion

Adolescent and adult patients with rhabdomyosarcoma present similar molecular and clinical characteristics compared to pediatric patients but outcome decreases with age partly due to a less multimodal management. Moreover, targeted combinations should be integrated to chemotherapy backbone.

Key words: rhabdomyosarcoma, *PAX3/7-FOXO1*, FISH, targeted therapy, cell line, combination

RESUME EN FRANCAIS

Introduction

Le rhabdomyosarcome de l'adulte est une tumeur rare dont le pronostic est plus défavorable que celui des patients pédiatriques. Cette différence peut être due à la nature même de la maladie, définissant ainsi deux entités distinctes, répondant à des caractéristiques cliniques et moléculaires propres. Cependant, des différences de prise en charge peuvent également être en cause et ouvrent des pistes d'amélioration des pratiques. Afin d'élucider ce point, le présent travail propose d'étudier les caractéristiques cliniques et moléculaires ainsi que les différences de prise en charge des adolescents et adultes atteints de rhabdomyosarcome. De plus, l'intégration de combinaison de thérapies ciblées à la doxorubicine, chimiothérapie de référence dans le sarcome a été étudiée sur lignées cellulaires *in vitro*.

Patients et méthode

Nous avons analysé rétrospectivement 239 patients âgés de 10 ans ou plus, ayant reçu un diagnostic de rhabdomyosarcome de l'adulte au MD Anderson Cancer Center entre 1957 et 2003 et leur statut fusionnel pour *PAX-FOXO1* par hybridation *in situ* en fluorescence. Trois lignées cellulaires de sarcome à petites cellules ont été soumises à des doses croissantes de différentes thérapies ciblées seules puis en doublets et enfin combinées à la doxorubicine. La viabilité cellulaire a été ensuite testée ainsi que le cycle cellulaire, la mesure de l'apoptose par test MTS, Annexin V et l'activité de caspase 3/7.

Résultats

Deux cent trente-neuf patients furent inclus sur cette période. Parmi ces patients, 163 avaient une maladie localisée et présentaient une médiane de survie globale de 3.8 années. Un âge supérieur à 50 ans et une infiltration tumorale étaient significativement associés à une survie plus courte pour ces patients non-métastatiques, tandis que l'emploi d'une stratégie intégrant chimiothérapie avec un contrôle locale par chirurgie ou radiothérapie était significativement associée à un meilleur pronostic. Les patients de plus de 50 ans avaient une survie globale à 5 ans de 13 % (médiane de survie à 1.7 ans) en dépit d'une maladie localisée. Soixante-seize patients étaient métastatiques au diagnostic. Leur médiane de survie était de 1.4 années. Approximativement 13 % de patients métastatiques de moins de 50 ans ont eu une survie prolongée de plus de 15 ans. L'utilisation d'une stratégie thérapeutique triple, intégrant chirurgie, chimiothérapie et radiothérapie était significativement associée à une survie prolongée. Par ailleurs, l'emploi de thérapie multimodale ainsi que l'utilisation d'ifosfamide étaient moindre chez les patients de plus de 50 ans comparé aux groupes de patients plus jeunes, parmi ces patients métastatiques. Au niveau moléculaire, 52% des patients présentaient une fusion de type *PAX3-FOXO1*, 15% la fusion *PAX7-FOXO1*, tandis que 33% des patients n'étaient porteurs d'aucune fusion. La présence du transcrit de fusion *PAX3/7-FOXO1* était significativement liée à un risque accru de maladie métastatique à l'image des données pédiatriques. Bien que la significativité statistique ne soit pas atteinte, une tendance à une survie plus longue était observée chez les patients négatifs pour la recherche de la fusion. L'étude *in vitro* de thérapies ciblées a permis d'identifier que les 4 agents à l'exception de l'abacavir étaient synergétiques 2 à 2 mais qu'une triple thérapie de ces agents ne permettait pas d'obtenir des résultats supérieurs aux bithérapies de thérapies ciblées. La combinaison du vorinostat plus le 17DMAG associée à la doxorubicine obtenait

une meilleure efficacité que les bi- ou trithérapies. En termes de séquence de traitement, une administration concomitante était supérieure en efficacité à un traitement séquentiel.

Conclusion

Les caractéristiques cliniques et moléculaires dont le statut *PAX-FOXO1* du rhabdomyosarcome de l'adolescent et de l'adulte sont proches de celles de l'enfant, notamment au niveau du caractère péjoratif de la translocation *PAX3/7-FOXO1*. La prise en charge du rhabdomyosarcome de l'adolescent et de l'adulte semble souffrir d'une approche moins agressive en matière de recours à un traitement local ou dans le choix d'agent de chimiothérapie, ce qui peut expliquer son pronostic péjoratif comparé au rhabdomyosarcome pédiatrique. De plus, des combinaisons de thérapies ciblées peuvent être intégrées aux protocoles de chimiothérapies standards.

Un résumé substantiel en français figure dans les annexes.

Mots clés : rhabdomyosarcome, *PAX3/7-FOXO1*, FISH, thérapie ciblée, lignée cellulaire, combinaison

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DEDICATION

This work is dedicated to Ethan and Eden, whose joy and love give me strength and joy.

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I- FOREWORD

Rhabdomyosarcoma (RMS) is a rare entity in children and even rarer in adult patients. It is a model of collaborative studies and is at the stage of targeted therapy development to improve its so far unfavourable outcome.

When this work has started, we sought to describe and better understand the specificities of adolescent and adult type of RMS that lack literature, through a large retrospective study of RMS patients treated in the none pediatric sarcoma department at University of Texas-MD Anderson Cancer Center (UT-MDACC). Aside from the clinical knowledge of this tumor type, the biological approach can help better define targetable pathways. As specific translocations are linked to outcome in pediatric RMS patients, the second part of this work was to investigate the *PAX-FOXO1* fusion status in the same population of adolescent and adult RMS patients and to assess its prognostic value. Finally, the question of how to integrate targeted therapy to the therapeutic arsenal was addressed through an empiric approach of a screening of polytherapies among the promising ones in RMS and sarcoma. The idea was to test bi- and polytherapies of targeted agents on small cell sarcoma cell lines *in vitro* and to optimize targeted agent use in conjunction with standard chemotherapy doxorubicin in matter of sequence of administration. The underlying question was to determine whether or not we could completely remove standard chemotherapy from cell treatment and still obtain acceptable efficacy on our model.

Our work led us to the conclusion that the adolescent and adult patients with RMS present similar clinical characteristics compared to pediatric patients such as subtypes, tumor site but suffer from a less multimodal management, withholding of chemotherapy due to side effect avoidance; those differences increasing with age. Likewise, the presence of *PAX-FOXO1*

fusion was found to be associated with increased metastases at diagnosis and tend to be linked with poor outcome but significance was not reached.

Targeted agents were tested: the multi tyrosine kinase inhibitor (TKI) sorafenib, the histone deacetylase (HDAC) inhibitor vorinostat, the heat shock protein 90 (HSP90) inhibitor 17DMAG and the anti-telomerase abacavir were tested on a panel of small cell sarcoma cell lines (two RMS cell lines and one Ewing's sarcoma cell line). Three of the four agents (all but abacavir) synergized two by two with each other but the triple combination did not perform better than the bitherapies. The bi-therapies such as vorinostat plus 17DMAG inhibitor added with doxorubicin achieved better results than bi or tritherapies and a concomitant administration was superior to a sequential one. Finally, this model suggests that chemotherapy still has its place in a future marked by targeted therapy era.

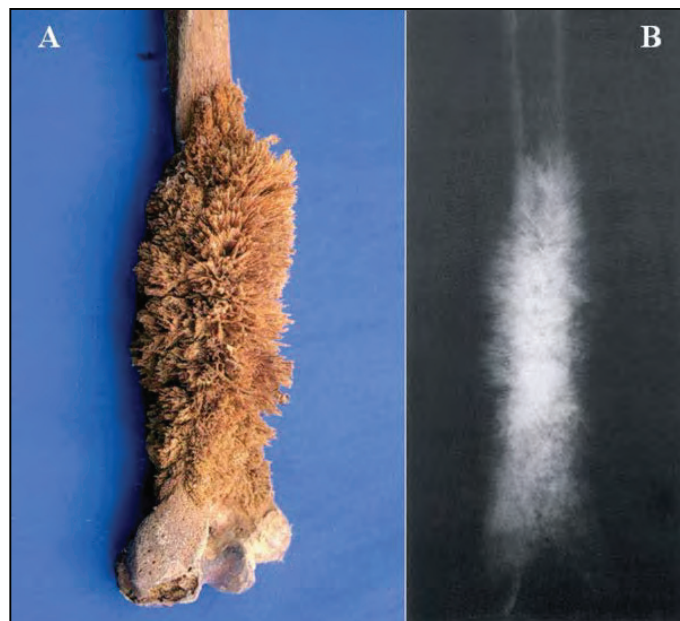
In the present document, we will first describe in an introduction the current state of knowledge of RMS including clinical aspects, staging, management and ongoing clinical trials. Secondly, we will expose the questions that drove this work and its objectives. Next, the different publications relative to our research work, two accepted and published and one submitted will be consecutively exposed. Finally, we will discuss the significance but also the limitations of this work. As a conclusion, we will describe what will be the future questions to be addressed as a follow up of this work.

II- Introduction

Sarcoma is a mesenchymal tumor that originates within non specialized or specialized connective tissue such as vascular, muscle or fat tissues. This explains the fact that it can arise from almost any anatomic site of the body. The greek prefix “*sarko*” means “*flesh*” as first described and different types of sarcomas are named according to the normal tissue they resemble rather than their tissue of origin.

Sarcoma has the peculiarity to be the first known cancer that appeared on Earth, discovered on a North American fossil fish *Phanerosteon mirabile* that lived about 300 million years BP. ¹

The first case of human osteosarcoma was discovered on the femur of a native Peruvian (A) dating to ca. 800 BP, with the typical radiographic “sunburst” pattern (B). ²



II-1 Epidemiology

RMS is a rare sarcoma that affects individuals of a young age.^{3,4} It is the most common soft-tissue sarcoma (STS) in the first two decades of life, but accounts for less than 1% of adult cancers and is reported to account for less than 4% of adult STS in the United-States.^{5,6} Incidence of sarcoma in France is about 4000 à 4500 (4,8/100 000).^{7,8} In Europe, incidence of STS from Eurocare database is 5,5 cases/100 000⁹ and 3,6/ 100 000. According to a French study, the incidence rate of STS was 3.7 per 100,000/year.⁸

However, pediatric cancer being a rare, about 4 in 10 patients reported to have RMS are adults.¹⁰ According to sarcoma nomenclature, RMS is named after the skeletal muscle differentiation it mimics (“rhabdo” for striated in Greek). RMSs are classified into three main histologic subtypes: pleomorphic RMS (PRMS), embryonal RMS (ERMS), and alveolar RMS (ARMS). Each subtype: PRMS, ERMS and ARMS, has specific clinical features, diagnostic criteria, and treatments. ERMS is predominantly represented in children (70%) than other subtypes.¹⁰ Classically, ERMS is correlated with a better prognosis than either ARMS or PRMS.¹¹⁻¹⁶

It affects slightly more frequently male than female in childhood but is essentially equivalent between adult patient genders and has two peak incidences at adulthood, during the third and seventh decades of life.¹⁰

World Health Organization (WHO) Classification of Tumors of Soft Tissue 2013

<ul style="list-style-type: none"> • Adipocytic tumors 	<ul style="list-style-type: none"> • Vascular tumors
Dedifferentiated liposarcoma	Epithelioid haemangioendothelioma
Myxoid liposarcoma	Angiosarcoma of soft tissue
Pleomorphic liposarcoma	<ul style="list-style-type: none"> • Gastrointestinal stromal tumors
<ul style="list-style-type: none"> • Myofibroblastic tumors 	<ul style="list-style-type: none"> • Nerve sheath tumors
Fibrosarcoma	Malignant peripheral nerve sheath tumour
Myxofibrosarcoma	Epithelioid malignant nerve sheath tumour
<ul style="list-style-type: none"> • Smooth-muscle tumors 	Malignant Triton tumour
Leiomyosarcoma	Malignant granular cell tumour
<ul style="list-style-type: none"> • Skeletal-muscle tumors 	Ectomesenchymoma
ERMS	<ul style="list-style-type: none"> • Uncertain differentiation
ARMS	Synovial sarcoma
PRMS	Epithelioid sarcoma
Spindle cell RMS	Alveolar soft-part sarcoma
<ul style="list-style-type: none"> • Undifferentiated / unclassified 	Clear cell sarcoma of soft tissue
Undifferentiated spindle cell sarcoma	Extraskeletal myxoid chondrosarcoma
Undifferentiated pleomorphic sarcoma	Extraskeletal Ewing sarcoma
Undifferentiated round cell sarcoma	Desmoplastic small round cell tumour
Undifferentiated epithelioid sarcoma	Neoplasms with perivascular epithelioid
Undifferentiated sarcoma NOS	cell differentiation (PEComa)

The new WHO classification classifies soft-tissue sarcomas (STS) according to benign, intermediate (locally aggressive and rarely metastasizing) and malignant behavior. Here we only present soft tissue sarcomas of malignant potential.

II-2 Risk factors

- **Genetic predisposition**

In most cases, RMS arises sporadically, but can occur in the context of genetic diseases. Particularly, the inactivating mutation of the tumor suppressor gene *P53* described in Li-Fraumeni syndrome predisposes to different type of cancers including RMS and other sarcoma¹⁷, leukemia, breast cancer and brain tumors.¹⁸

Other sarcoma predisposition syndromes have been reported to be linked to RMS arisal in the literature such as neurofibromatosis or Von Recklinghausen disease¹⁹, Beckwith-Wiedemann syndrome, Noonan syndrome, causing preferentially ERMSs or the cardio-facio-cutaneous syndrome or Costello syndrome.²⁰

Some genetic abnormalities have been described as risk factor for certain subtypes of RMS. Hedgehog signaling pathway, involved in embryogenesis plays an important role in the pathogenesis of RMS. Indeed, ERMS seems to be associated to mutation of Pached 1, the receptor of Hedgehog.²¹

In the context of RMS, a referral for genetic counseling should be considered for all patients.

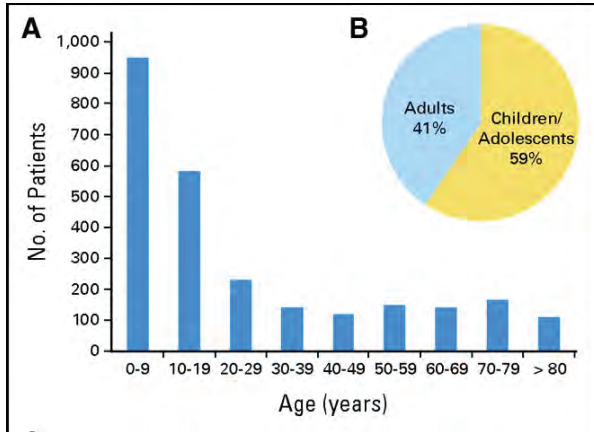
- **Radiation**

Radiation exposure is an important risk factor for small cell sarcomas, more frequently angiosarcoma. Although rare, a RMS can occur on a radiation therapy field, with a time lapse of about 5 years. When the initial treatment included chemotherapy, however, the median time frame is between 2 and 3 years after the initial treatment, most seen in secondary sarcoma after curative breast cancer treatment.²²

- **Other risk factors** were reported such as an association between marijuana consumption or cocaine up to one year before or during pregnancy and an increased risk for developing a RMS for the child,²³ suggesting a possible epigenetic mechanism.

II-3 Age spectrum and tumor site

RMS can affect almost any part of the body, even in those tissues lacking skeletal muscle. Using the National Cancer Institute source of cancer statistics in the United States called the Surveillance, Epidemiology and End Results (SEER) Program, a study compared clinical features of adults and pediatric RMS patients reported between 1973 and 2005 for a total of 2600 patients.¹⁰



Age distribution of the 2600 reported RMS patients

This study shows that while pediatric RMS occurs preferentially in the chest, abdomen, and pelvis area, it arises more often in the extremities for adult patients. The Intergroup Rhabdomyosarcoma Study Group (IRSG) defined a prognostic classification according to tumor location. For instance, the orbit or head and neck in general is considered a favorable site while a parameningeal site is of poor prognosis.

IRSG definition of favorable/unfavorable sites²⁴

Favorable site	Orbit; nonparameningeal head and neck; genitourinary tract other than kidney, bladder, and prostate; biliary tract.
Unfavorable site	Any site other than favorable.

However it appears that adult RMS are less likely to be favorably located than pediatric RMS which can partly explain their prognosis differences. ¹⁰

The most common primary site of adult RMS is the extremities (approximately 26% of all cases). ¹⁰

RMS incidence in adult according to tumor site from Sultan, *et al.*¹⁰

Primary site	Adult (%)	Children (%)
Extremities	26	14
Chest/abdomen/pelvis	23	21
Genitourinary (non bladder/non prostate)	17	14
Head and Neck	9	17
Parameningeal	9	10
Other/unknown	9	6
Retroperitoneum	4	3
Bladder/prostate	3	7
Orbital	1	8

II-4 Extension

The disease has tendency to grow locally and is rarely encapsulated, often infiltrating adjacent tissues and is one of the few sarcoma to invade lymph nodes in 55% of cases in metastatic childhood RMS according to the European Intergroup study.²⁵ Moreover, about 17 % of pediatric patients with clinically negative nodes are found to have microscopic disease on biopsy.²⁶ In adult series, about 35% of patients have nodal involvement.^{5, 11}

We distinguish locally advanced disease when the size of the primary tumor is superior to 5 cm versus regional spread when lymph nodes are involved versus distant metastases.

More than the location by itself, the presence of metastasis at diagnosis, as well as size > 5cm are important prognostic factors. About 28% to 44% of adult RMS patients present with metastases at diagnosis.^{5, 10, 27} Moreover, half of the patients have a primary tumor that measures more than 5 cm at diagnosis.^{5, 11, 27} Alveolar and ERMS, as small cell sarcomas, have a tendency to metastasize to lung, bone and bone marrow and should be considered micro metastatic thus requiring chemotherapy.²⁸ Conversely, the rules for the treatment of PRMS match these of conventional STS in adults, where chemotherapy is not recommended in all patients in adjuvant setting.²⁹

II-5 Diagnosis

RMS, as any STS should be addressed to reference centers for sarcoma at the time of the clinical diagnosis. ESMO guidelines recommend that patients should be referred to expert centers as soon as a sarcoma is suspected, meaning a deep mass of soft tissues or a superficial lesion of soft tissues having a diameter of >5 cm, or arising in pediatric age.²⁹ Indeed, survival of patients seems to correlate with adherence to guidelines.³⁰

II-5-1 Imaging

MRI is recommended as the main imaging modality for the primary tumor. CT scan performs equally than MRI in retroperitoneal RMS. Plain X-ray of the tumor can determine if the tumor originates from the bone.²⁹

PET scan is highly helpful to assess the spread (including bone) and early response to chemotherapy³¹.

II-5-2 Biopsy of the mass

Upon identification of a tumor nodule, a surgical biopsy should be done by or planned by an experienced surgeon (with sarcoma surgery experience if a RMS is suspected clinically or radiographically) who would be performing the excision of the tumor by an open biopsy, to prevent dissemination of tumor cells in the path of the biopsy needle. Interventional radiologist-guided needle biopsy can be chosen as an alternative to the open excisional

biopsy.³² ESMO guidelines recommend multiple core-needle biopsies with at least a ≥ 16 G needle. Indeed, the tumor biopsy is the critical first step to allow an accurate pathology classification of the tumor.^{29, 33}

II-5-3 Pathology

The biopsy tissue is fixed in formol, embedded in a paraffin block and read by a sarcoma trained pathologist. Because of the tumor rarity and its heterogeneity, even a well-trained pathologist can be challenged by a RMS specimen. In some studies a third of RMS samples remain non-specified, leading to a less accurate therapeutic strategy that may explain the relatively poorer prognosis of patients in this situation. Sometimes, a re-biopsy is necessary to have more material. Generally, a second opinion from an expert sarcoma pathologist may lead to a more accurate classification of the tumor.³⁴ Indeed, a high rate of discrepancy between initial diagnosis and central review has been reported in term of grading (18.5% of cases) and histology type (10.7%) in the Rhone-Alpes region.³³ In France, every sarcoma specimen benefit from a second lecture through the RRePS network (Réseau de Référence en Pathologie des Sarcomes des tissus mous et des viscères) composed of sarcoma trained pathologists.

RMS is composed of small round and spindle cells whose origin is still unknown. Based on the tumor architecture, RMS is first classified into embryonal, alveolar, and pleomorphic types but then confirmed by molecular testing.

ERMS and ARMS are frequently composed of small round blue cells and can be misdiagnosed with other entities resumed in the LEMONS acronym:

L for lymphoma,

E for Ewing's sarcoma and other small cell sarcomas such as RMS, desmoplastic small round cell tumor

M for medulloblastoma

O for olfactory/ other (esthesioneuroblastoma, Merkel cell carcinoma),

N for neuroblastoma

S for small cell carcinoma³⁵

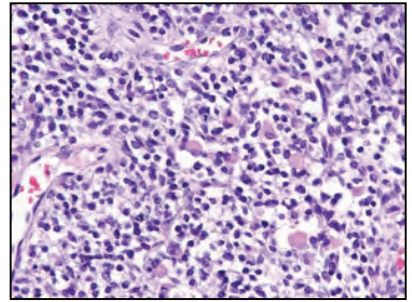
PRMS is usually composed of large spindle cells and can be confused with a variety of other types of spindle cell sarcomas. Because the treatment differs for each entity, the pathology identification is a critical step.

1) Tumor Architecture

The pattern of arrangement of the tumor cells in the tissue defines the architecture. The round cells can be arranged in solid or discohesive groups, and the spindle cells can be oriented in fascicles. The tumor architecture is another factor in classifying the RMS subtype.

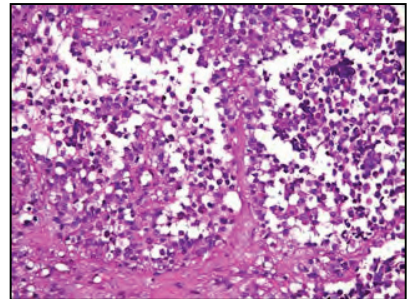
ERMS

Malignant small round cells growing in sheets in ERMS. Larger cells show evidence of skeletal muscle differentiation.



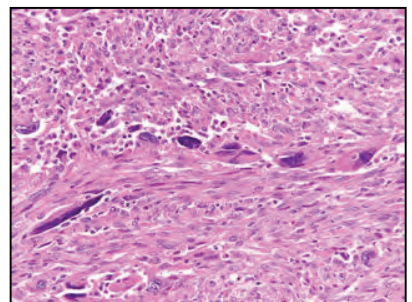
ARMS

Malignant round cells arranged in loose aggregates typical of alveolar RMS.



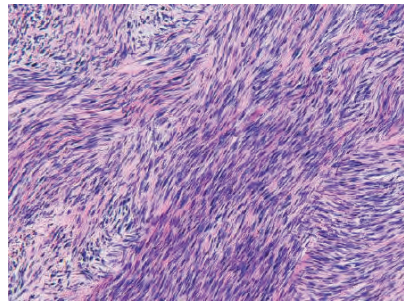
PRMS

Malignant large spindle cells oriented in fascicles in PRMS. It has transiently been regarded as a pleomorphic variant of malignant fibrous histiocytoma that no longer exist according to the 2013 WHO classification and other pleomorphic myogenic soft-tissue sarcoma. This subject is no longer of controversy due to recent ultrastructural studies that allow myofibroblastic phenotype identification that distinguished pleomorphic RMS from the other entities.³⁶⁻³⁹



Spindle cell RMS

Spindle cell RMS is a rare variant now included to the new WHO classification 2013. It affects more frequently young male in the paratesticular or head and neck region and is associated with a favorable outcome in children compared to other subtypes. It is composed of long spindle cells with fusiform or cigar-shaped nuclei and an eosinophilic cytoplasm arranged in fascicles or whorls. This tumor can be confused with multiple entities, such as leiomyosarcoma, spindle cell carcinoma, desmoplastic melanoma, or fibrosarcoma. Immunohistochemical is in favor of a sarcomeric differentiation with reactivity for desmin, myogenin, and MyoD1 markers.⁴⁰



Most tumors with mixed embryonal and alveolar pattern lack *PAX3-FOXO1* fusion. These are therefore considered as ERMS according to the 2013 WHO classification.

2) Immunohistochemistry

Myogenic differentiation markers define tumor histological type: myogenin, MyoD1, desmin, vimentin, actin can be positive in RMS.⁴¹ None of these immunohistochemical markers are subtype-specific. However, a strong positivity of Myogenin is associated with alveolar subtype.^{42,43}

3) Molecular biology

Morphology is sometimes not sufficient to discriminate one subtype from another. For instance, because of overlapping morphologic features, particularly with ERMS and solid pattern ARMS, molecular analysis is often used as a complementary diagnostic tool.⁴⁴

Some chromosome rearrangements are specific of a subtype and allow more precise classification of the tumor, thus prognostic implication.

In ARMS, a translocation involves either chromosomes 2 and 13 $t(2;13)(q35;q14)$, resulting in the *PAX3-FOXO1* fusion protein or chromosomes 1 and 13, $t(1;13)(p36;q14)$ resulting in the *PAX7-FOXO1* fusion⁴⁵. Moreover, pediatric patients with fusion-positive involving either *PAX3* or *PAX7* ARMS have a shorter survival than those with fusion-negative ARMS. In pediatric patients with ARMS, a translocation involving *PAX3* is associated with shorter survival than a translocation involving *PAX7* (4-year overall survival: 75% for *PAX7-FOXO1* vs. 8% for *PAX3-FOXO1*; $P = 0.0015$).⁴⁵ Interestingly, according to the European Pediatric Soft Tissue Sarcoma Study Group (EpSSG), the patient outcomes of fusion-negative ARMSs

are very similar to those of ERMSs⁴⁶. A recent study on adult RMS showed a similar relationship with *PAX3/7-FOXO1* fusion. Patients with fusion-negative RMS trended towards better outcome than those with fusion-positive RMS. However, the type of translocation *PAX3/FOXO1* or *PAX7/FOXO1* failed to show any prognostic significance.⁴⁷

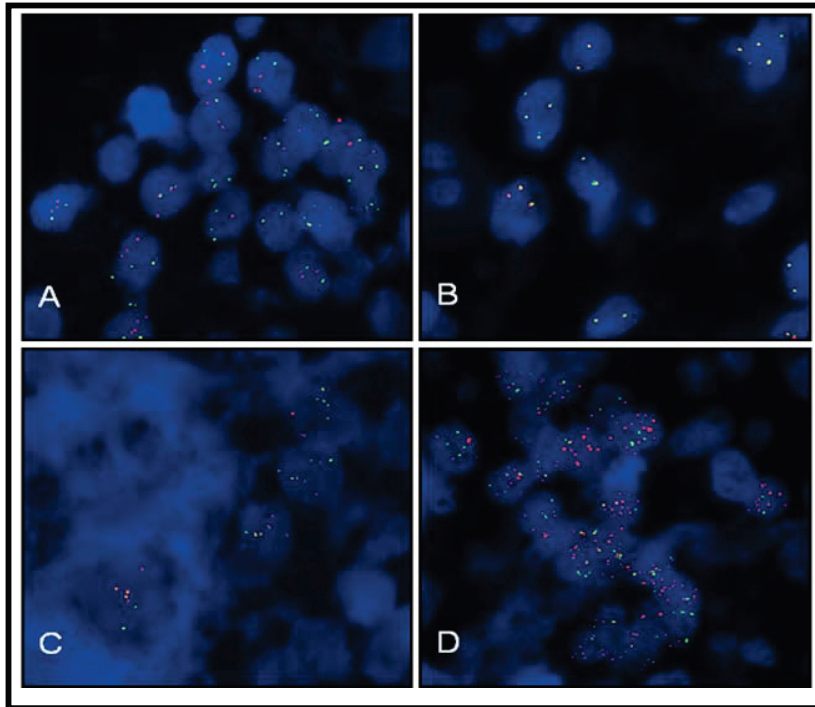
PRMS belongs to the so-called group of sarcomas with complex genomic rearrangements..

Among other complex karyotype abnormalities such as hyperdiploid with extra copies of chromosomes 2, 8, 9, 11, 12, 13, 17, 18, 19, and 20, ERMS can present a loss of a part of chromosome 11 on its p15.5 locus.^{48, 49} Other rearrangements have been described: *t(2;20)(q35;p12)*⁵⁰ or *der(16)t(1;16)*.⁵¹

Because of its prognostic implication^{44, 45, 48, 49, 52-56} and the aggressiveness of the treatments²⁴, the fusion status is systematically evaluated in pediatric patients and is likely to be relevant for adult patients as well.⁴⁷

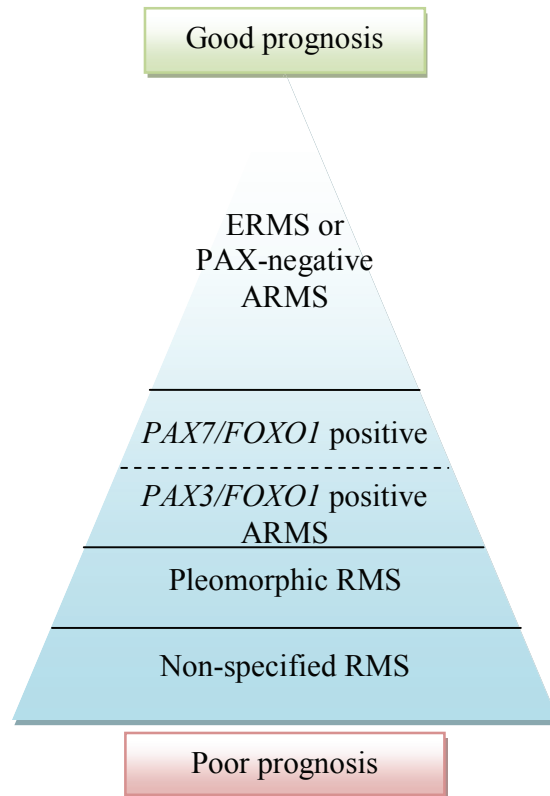
4) Fluorescent in situ hybridization (FISH)

One of the techniques used to identify the fusion is the FISH and uses fluorescent probes to track the broken ends of chromosomes and the way they abnormally fused together.



***PAX3/7-FOXO1* translocation.** **A**, FISH analysis with the *FOXO1* break-apart probe set demonstrates split red and green signals indicative of a *FOXO1* rearrangement. **B**, The fused red/green signals are consistent with no *FOXO1* rearrangement. **C**, FISH analysis using *PAX3-FOXO1* dual spanning probe set shows fused red/green signals indicative of a *PAX3-FOXO1* fusion. **D**, FISH analysis with a *PAX7* break-apart probe showing split red and green signals and amplification, common with *PAX7* rearrangements. ⁴⁷

Molecular biology prognostic significance at a sight



Validated in pediatric patients but not in adult RMS

II-6 Prognosis

In RMS patients, the 5-year overall survival (OS) prognosis decreases with age. According to the IRSG that initiated multiple major clinical trials on RMS, patients greater than 10 years of age have a poorer prognosis than younger patients.^{16, 24, 57} Five-year OS of adult patients with localized disease ranges from 20% to 40%¹¹, whereas it is between 60% and 80% for their pediatric counterparts.⁵⁸ Survival at 5 years of diagnosis for adult patients with metastatic RMS is, according to the same study group, inferior to 5%.^{5, 27, 59}

According to a recent study based on SEER data, survival decreases with age following a quasi-linear slope. Indeed, young children have a better prognosis with 67% 5-year OS than teenagers with 47%, while teenagers have a better prognosis than adults. Among the adult population, every stage pooled together leads to a 5-year overall survival of 36% for a young adult, while it is from 29% for middle age patients to 11% for geriatric patients.¹⁰

II-6-1 Staging

In adult RMS, the recently updated American Joint Committee on Cancer (AJCC 7) staging system is used for PRMS while ARMS and ERMS are staged according to the Children's Oncology Group (COG, IRSG merged with major cooperative paediatric cancer treatment groups).²⁸ This staging guides treatment algorithm.⁶⁰

Staging of A and ERMS follows the RMS staging system by IRSG while the TNM classification of AJCC version 7 applies to PRMS and undifferentiated RMS.

1) Childhood RMS staging system

The staging process of the Soft Tissue Sarcoma Committee of the COG follows 3 steps:

- 1. Pretreatment Staging System** TNM-based pretreatment staging system uses a TNM that differs from the regular AJCC TNM classification for STS.

T1	Tumor confined to anatomic site of origin (noninvasive).
T2	Tumor extension and/or fixation to surrounding tissue (invasive).
A	Tumor ≤ 5 cm in maximum diameter.
B	Tumor > 5 cm in maximum diameter.
N0	No clinical regional lymph node involvement.
N1	Clinical regional lymph node involvement.
NX	Regional lymph nodes not examined; no information.
M0	No metastatic disease.
M1	Metastatic disease.

Stage	Sites of Primary Tumor	T Stage	Tumor Size	Regional Lymph Nodes	Distant Metastasis
1	Favorable sites	T1 or T2	Any size	N0 or N1 or NX	M0
2	Unfavorable sites	T1 or T2	a, ≤ 5 cm	N0 or NX	M0
3	Unfavorable sites	T1 or T2	a, ≤ 5 cm	N1	M0
			b, > 5 cm	N0 or N1 or NX	
4	Any site	T1 or T2	Any size	N0 or N1 or NX	M1

2. Surgical-pathologic Group System

Group	Incidence	Definition
I	13%	Localized tumor, completely removed with microscopically clear margins and no regional lymph node involvement. Lymph node biopsy or sampling is encouraged if lymph nodes are clinically or radiographically suspicious.
II	20%	Localized tumor, completely removed with: (a) microscopic disease at

Group	Incidence	Definition
		the margin, (b) regional disease with involved, grossly removed regional lymph nodes without microresidual disease, or (c) regional disease with involved nodes, grossly removed but with microscopic residual and/or histologic involvement of the most distal node from the primary tumor.
III	48%	Localized tumor, incompletely removed with gross, residual disease after: (a) biopsy only, or (b) gross major resection of the primary tumor (>50%).
IV	18%	Distant metastases are present at diagnosis. This category includes: (a) radiographically identified evidence of tumor spread, and (b) positive tumor cells in cerebral spinal fluid, pleural, or peritoneal fluids, or implants in these regions.

3. Risk Group Classification

Risk Group	Histology	Stage	Group
Low risk	Embryonal	1	I, II, III
	Embryonal	2, 3	I, II
Intermediate risk	Embryonal	2, 3	III
	Alveolar	1, 2, 3	I, II, III
High risk	Embryonal or Alveolar	4	IV

To date, the PAX3/7-FOXO1 fusion status is not incorporated in the risk classification but recent data suggest that intermediate risk patients may benefit from a treatment algorithm based on fusion status.⁶¹

2) Adult STS staging system follows staging of STS according to AJCC 7th edition

TX	Primary tumor cannot be assessed.
T0	No evidence of primary tumor
T1	Tumor \leq 5 cm in greatest dimension
T1a	Superficial tumor
T1b	Deep tumor
T2	Tumor $>$ 5 cm in greatest dimension
T2a	Superficial tumor
T2b	Deep tumor
NX	Regional lymph nodes cannot be assessed.
N0	No regional lymph node metastasis.
N1	Regional lymph node metastasis.
M0	No distant metastasis.
M1	Distant metastasis.

Anatomic Stage/Prognostic Groups

Stage IA	T1a	N0	M0	G1, GX
	T1b	N0	M0	G1, GX
Stage IB	T2a	N0	M0	G1, GX
	T2b	N0	M0	G1, GX
Stage IIA	T1a	N0	M0	G2, G3
	T1b	N0	M0	G2, G3
Stage IIB	T2a	N0	M0	G2
	T2b	N0	M0	G2
Stage III	T2a, T2b	N0	M0	G3
	Any T	N1	M0	Any G
Stage IV	Any T	Any N	M1	Any G

II-6-2 Grading of malignancy

The WHO classification uses the grading system of the Fédération Nationale des Centres de Lutte contre le Cancer (FNCLCC), taking account of histology subtype, tumor necrosis, mitotic index.

FNCLCC histological grading criteria

Score	Tumor differentiation	Necrosis	Mitotic count (n/10 high-power fields)
0		Absent	
1	Well	< 50%	n<10
2	Moderate	> 50%	n 10-19
3	Poor		n≥20

Grade WHO corresponds to the sum of the scores of the three criteria²⁹

Grade 1: 2, 3

Grade 2: 4, 5

Grade 3: 6, 7, 8

II-7 Management

II-7-1 Management of PRMS and undifferentiated RMS

PRMS treatment strategy follows STS guidelines

1) Surgery

ESMO guidelines recommend a wide excision for all patients followed by radiation therapy for high-grade (G2–3), deep >5 cm lesions. R0 is the goal of surgery with margin depending on the adjacent structures. Re-excision is mandatory in case of R1 resection. In case of R2 resection, a re-operation is necessary after pre-operative chemotherapy or radiation to increase the chance to get a R0 resection and avoid mutilating surgery. Amputation is rarely necessary in RMS of the extremities. Resection of metastatic localizations of the tumor is always considered after chemotherapy response.²⁹

2) Radiation Therapy

RT improves local control, but has not demonstrated benefit on overall survival (OS). It is recommended for low- or high-grade, superficial, >5 cm and low-grade, deep, <5cm STS.

The timing of RT is debated. The standard of care is postoperative RT given at a dose of 50 to 60 Gy, with fractions of 1.8–2 Gy, possibly with boosts up to 66 Gy in case of residual disease.

The preoperative RT is an alternative to post-operative RT at a recommended lower dose of 50 G to reduce wound healing complications after surgery. According to a multicenter randomized trial comparing the two preoperative vs. postoperative RT, local control rates

(93% vs. 92%) and OS (73% vs. 67%, $P = .48$) were similar but wound healing complications were increased in the preoperative RT arm.⁶²

RT is currently investigated in retroperitoneal sarcoma through the STRASS trial (NCT01344018).

3) Chemotherapy

RMS is thought to be a systemic disease and has a good chemosensitivity. While adjuvant chemotherapy is not a standard in STS⁶³ is discussed in high-risk patients based on high-grade, depth, size >5 cm.²⁹ Nomograms to predict OS and DFS in sarcomas are decision tool to define the postoperative management. Among them, a recently reported nomogram for primary resected retroperitoneal STS predicts outcome more accurately than the current AJCC staging algorithm.^{64, 65} It consists of 4 cycles of adriamycin and ifosfamide.⁶⁶ Neoadjuvant chemotherapy is an option if the tumor is not resectable or to limit the morbidity of surgery. Due to its chemosensitivity, chemotherapy is given in most cases in PRMS. In metastatic STS, first line adriamycin is the most commonly used agent, either as a single agent or in combination therapy with ifosfamide.

As subsequent lines of therapy, for PRMS, gemcitabine plus docetaxel⁶⁷, trabectedin^{68, 69}, pazopanib^{70, 71}, eribulin^{72, 73} are valuable options.

II-7-1 Alveolar and ERMS management: the pediatric approach

Alveolar and ERMS in adult patients are treated according to pediatric RMS guidelines, thus follow the rule that chemotherapy will be used in all patients in conjunction with either radiation therapy or surgery or both.⁷⁴⁻⁷⁶

In non-metastatic disease, two different approaches are debated. The International Society of Pediatric Oncology Malignant Mesenchymal Tumor ([MMT) Group supports the use of front-line chemotherapy followed by surgery, while radiation therapy is used for residual disease or lymph node involvement.⁷⁶ Conversely, the COG-STS group's strategy consists of initial surgery followed by RT for patients with residual disease.⁷⁵

Comparison of outcomes based on treatment algorithms for RMS

Trial	5 year-OS(%)	5 year-EFS(%)
MMT89 ⁷⁶	71%	57%
IRS-IV ⁷⁴	84%	78%

Based on these trials, the use of early local therapy, including RT seems to be beneficial in term of survival. However, the MMT approach is preferred reduces morbidity.

1) Sugery

Initial surgery is performed in small tumors or no risk of morbidity such as disfigurement, substantial functional compromise, or organ dysfunction is expected.⁷⁷ The feasibility of this initial surgery conducts to cluster patients into Surgical-pathologic Groups. Group III

corresponds to the cases where surgery has not been complete with gross residual disease or no surgery has been attempted. About half of the patients fall in this category.

Correlation between initial surgery and outcome

Group	5 year OS (%) of the IRS- III study ⁵⁸
I	90
II	80
III	70

In case of microscopic disease, surgery is also used to remove the residual disease tend to improve prognosis.⁷⁸ In case of macroscopic residual disease, a debulking of greater or equal to 50% of the tumor volume seems to be beneficial for patients with retroperitoneal and pelvic RMS in term of failure-free survival.⁷⁹

Lymph node dissection

The COG-STS recommends systematic aggressive lymph node dissection for patients with extremity primary tumors, even with clinically and radiographically negative nodes.⁸⁰ Sentinel lymph node mapping is employed at the surgeon convenience but its benefit is no yet clearly established.^{81, 82}

In metastatic setting, resection of metastatic disease at diagnosis (Stage 4, M1, Group IV) not recommended.⁸³ Surgical resection of isolated lung metastatic disease can be discussed as

these patients present a slightly better prognosis than patients with other single-site metastasis.⁸⁴

2) Radiation Therapy

Every patient but completely resected, Group I patients will need Radiation therapy (85% of patients) for the local control of the disease.⁸⁵

Radiation Therapy (RT) Dose According to RMS Group, Histology, and Site of Disease⁸⁶

Group		Treatment
I	ERMS	no RT
	ARMS	36 Gy to involved (prechemotherapy) site
II	N0	36 Gy to involved (prechemotherapy) site
	N1	41.4 Gy to involved (prechemotherapy) site and nodes
III		50.4 Gy with volume reduction after 36 Gy if excellent response to chemotherapy and noninvasive pushing tumors; no volume reduction for invasive tumors.
IV		As for other groups and including all metastatic sites, if safe and possible. <i>Exception:</i> lung (pulmonary metastases) treated with 15 Gy if aged 6 years or older, 12 Gy if younger than 6 years

The dose of radiation correlates with the amount of residual disease and therefore, increases with surgical group classification. The treated volume is determined by the extent of tumor at diagnosis prior to surgical resection and prior to chemotherapy plus a 2 cm margin including clinically involved

regional lymph nodes. RT is given over a 5 to 6 week period, after 1 to 3 months of chemotherapy. Chemo regimen might be changed to avoid radiosensitizing agents such as dactinomycin and doxorubicin. RT should be used with caution since radiation of bone marrow may severely limit the ability to administer chemotherapy.⁸⁷⁻⁹⁰

3) Chemotherapy

All patients with ERMS and ARMS should receive chemotherapy. However, the duration and intensity of the chemoregimen depend on the risk group classification.

Low risk patients

The COG-D9602 study defined the guidelines for low-risk patient group and further distinguished between two prognostic subsets:

Subset A: stage 1, group I/IIA; stage 2, group I; and stage 1, group III (orbit only) ERMS
This patient population present high survival rate >90% with a two-drug chemotherapy regimen that includes vincristine and dactinomycin (VA) and no RT unless residual disease or group II or III.⁹¹

Subset B: stage I, group IIB/IIC or group III (nonorbit), had a lower than expected outcome.

Other subgroups of low-risk patients achieved 90% survival rates of with three-drug chemotherapy with VAC (total cyclophosphamide dose of 28.6 g/m²) plus RT for residual tumor.⁹¹

These finding have been confirmed by the preliminary results of the most recent COG-ARST0331 trial. In this study, the subset A of patients had an excellent outcome (2-year EFS,

88%; overall survival (OS), 98%) with short therapy duration (22 weeks) while subset B had 3-year EFS of 66%.^{92, 93}

Intermediate-risk patients

The COG- D9803 stratified intermediate-risk patients between VAC and VAC plus topotecan. No difference in 4-year EFS was observed between the 2 arms (73% and 68%, respectively).⁹⁴ This study echoed with the prior IRS-IV trial⁹⁵, which failed to demonstrate a benefit of a rotation of VAC with ifosfamide- etoposide. Finally, an MMT study compared the European standard RMS therapy vincristine, actinomycin D and ifosfamide (VAI) to carboplatin, epirubicin, and etoposide with no observed difference in outcome.^{96, 97}

High-risk patients

The standard systemic therapy for high risk patients with RMS is the three-drug combination of VAC.⁹⁴ Many clinical trials attempted to substitute or add agents but these modifications of the standard VAC never led to an improved outcome.⁹⁸

To test new drugs and assess their objective response rate, window phase II studies have been designed. The agent of interest is given upfront, during a 4-to 8-week “window” period before the administration of standard therapy in previously untreated high risk patients. Window studies demonstrated activity of irinotecan and its potentiation with vincristine that is found in the rationale of current trials.⁹⁹⁻¹⁰² (table)

The COG-ARST0431 study included high-risk patients in a dose dense regimen with interval-compressed chemotherapy (VDC/IE) and vincristine/irinotecan (VI) [23]. This

intensive regimen was feasible and showed a modest improvement in 3-year EFS (38% and 29%, respectively) compared to prior studies in high-risk patients. This effect was greater in ERMS (60% and 37%, respectively).⁹³

As second line therapy, irinotecan ¹⁰³ seems to be a promising drug. Its activity being dose and schedule-dependent, trials exploring dose-escalation ^{104, 105} or protracted administration ¹⁰⁶ had been reported. Irinotecan-based combination such as irinotecan-temozolomide¹⁰⁷ or topotecan-endoxan¹⁰⁸ have been evaluated with encouraging results. Finally, the alkylating agent temozolomide showed synergy with irinotecan in preclinic and activity in early phase trials.^{107, 109}

II-7- 3 Phase II studies in RMS patients

Regimen	CR (%)	PR (%)	SD (%)	PD (%)	Response (%)	Nb of patients	Reference
Vincristine/irinotecan	2	68	22	8	70	50	¹¹⁰
Vincristine/melphalan	4	51	37	8	55	64	¹⁰²
Ifosfamide/doxorubicin	11	41	41	7	52	152	¹⁰¹
Topotecan/cyclofosfamide	4	46	31	19	50	61	¹⁰⁸
Topotecan	3	46	20	31	49	48	¹¹¹
Irinotecan	0	45	23	32	45	19	¹¹²
Ifosfamide/etoposide	5	36	52	7	41	64	¹⁰²
Rebeccamycin Analogue	5	10	N/A	N/A	15	126	¹¹³
Temsirolimus	0	6	25	69	6	52	¹¹⁴
Trabectedin	0	5	5	90	5	50	⁶⁹
Pemetrexed	0	0	7	93	0	68	¹¹⁵
Oxaliplatin	0	0	0	100	0	113	¹¹⁶
Ixabepilone	0	0	0	100	0	59	¹¹⁷
Vinorelbine/cyclofosfamide	8	28	24	42	36	50	¹¹⁸
Imatinib	0	0	50	50	0	2	¹¹⁹

Cixutumumab (IMC-A12) has been tested in a phase I study showing no activity in RMS patients but the effective was of only 2 patients.¹²⁰ Another phase II study including 10 RMS patients showed activity of the combination of temsirolimus plus cixutumumab in STS but specific RMS response rates were not reported.¹²¹

Rebeccamycin Analogue showed a 15% response rate but its development stopped due to frequent myelosuppression.¹¹³

Imatinib was tested on STS in a phase II study that included only 2 RMS patients.¹¹⁹

II-7-4 Current clinical trials

As maintenance therapy, the combination of vinorelbine with oral cyclophosphamide is being evaluated in a randomized trial for high-risk RMS patients by the EpSS group.¹²²

The rôle of doxorubicin is still unclear. It is withheld in pediatric and low risk patients due to its cardiotoxicity. However, but this issue will be addressed through the EpSSG RMS 2005 trial who randomly assigns patients with high-risk RMS to IVA versus IVA with doxorubicin.

Targeted therapies are investigated for the treatment of RMS. One of the most exciting target is the Insulin Growth Factor Receptor 1 (IGF1R) which is upregulated in PAX3-FOXO1 positive ARMS.¹²³

The COG-ARST08P1 that opened on January 2010 for accrual, uses the same backbone of the ARST0431 with the addition of cixutumumab (IMC-A12), an antibody against insulin-like growth factor 1 receptor (IGF1R) and/or temozolomide in a series of three sequential pilot studies.⁹⁸ In refractory patients, cixutumumab was well tolerated and showed activity in preclinical models.^{120, 124, 125}

As maintenance therapy, the combination of vinorelbine with oral cyclophosphamide is being evaluated in a randomized trial for high-risk RMS patients by the European Paediatric Soft Tissue Sarcoma Study Group (EpSSG).¹²²

The COG-ARST0921 study is a ongoing randomized phase II trial that incorporates two targeted therapies: bevacizumab or temsirolimus in combination with vinorelbine/cyclophosphamide for relapse or progressive RMS. Bevacizumab is a

monoclonal antibody that targets the five isoforms of human vascular endothelial growth factor (VEGF)¹²⁶. It has shown activity in RMS xenograft models.¹²⁷ Temsirolimus is an inhibitor of the mammalian target of rapamycin (mTOR)¹²⁸. In RMS, the mTOR pathway is activated and mTOR inhibitors such as rapamycin and temsirolimus are active on RMS *in vitro* and *in vivo* models.^{114, 129, 130}

The multikinase inhibitor sorafenib is under investigation through a phase II clinical trial that is currently recruiting in refractory RMS patients (NCT01502410). Panobinostat is an histone deacetylase (HDAC) inhibitor. It failed to show efficacy in a STS trial but it included only one PRMS patient.¹³¹

Sorafenib and HDAC inhibitor mechanism of action will be developed later in the rationale.

II-7-5 Promising targets for future clinical trials

- Pazopanib is the first targeted agent FDA-approved for adult metastatic STS. It lead to a 3-month improvement in PFS compared to placebo in the PALETTE trial.⁷⁰ A phase II trial of pazopanib is about to open for accrual in pediatric patients with solid tumors from the COG (NCT01956669).
- Crizotinib is a dual ALK and c-met inhibitor. ALK amplification is common in RMS, particularly in ARMS and metastatic ERMS.¹³² C-met expression is also common in RMS and associated with inferior outcome.¹³³

- It is FDA approved for ALK-positive locally advanced or metastatic non-small cell lung cancer.¹³⁴
- Alisertib is an Aurora kinase A inhibitor showed in vitro activity¹³⁵ and a phase I COG study COG in phase I trial.¹³⁶
- The Hedgehog pathway, composed of Sonic hedgehog (Hh) and its receptor Patched 1 (Ptch) is also a promising target in RMS.¹³⁷⁻¹⁴⁰ Indeed, high expression of Ptch1 in tumor samples correlated with reduced survival in patients with embryonal and fusion-negative RMS.¹⁴¹ Vismodegib (GDC-0449) is a Hh antagonist that is being examined in phase I/II trials for advanced or metastatic sarcomas (NCT01154452).¹⁴²
- Erlotinib has been tested as phase I in RMS patients with a good tolerance profile¹⁴³ but no Phase II trial is ongoing so far.
- CXCR4 is overexpressed and can be a targetable biomarker in RMS.¹³³ Finally PARP inhibitors are under investigation in preclinical models.¹⁴⁴

Small cell sarcoma such as RMS and Ewing's sarcoma are chemosensitive tumors but the relapse rate is fairly high with a poor median survival in metastatic setting.¹⁴⁵ Despite its heterogeneity, doxorubicin and ifosfamid are the standard of care in most sarcoma, but this regimen confers only less than 10 % survival at 5 years in metastatic sarcoma.¹⁴⁶⁻¹⁵⁰ RMS, after a twenty year phase of improving outcome, is experiencing a plateau with no better regimen than VAI using standard chemotherapy despite innovative methodology such as "window" trials.

The development of targeted therapies has changed the landscape of sarcoma research.^{151, 152} Combination therapy is assumed to be the optimal strategy to potentiate targeted therapy use and achieve clinical significance. To combine targeted agents two by two or in polytherapy might overcome resistance and the inevitable relapse seen with single agent therapy.¹⁵³ Targeted agents may also potentiate standard chemotherapy, allowing it to reduce doses, or prolong its use, which would be relevant regarding doxorubicin dose limiting cardiotoxicity. The eventual question would be: could we spare standard chemotherapy with the use of a targeted polytherapy, translating the HIV model?

For the last years, its management has been at the challenging phase to incorporate targeted therapies to its therapeutic landscape. As single therapy, no targeted agents have demonstrated its efficacy in clinic. The common approach is to test targeted agents one by one in addition to standard VAI regimen.

After many failed early phase clinical trials, to find strong preclinical rationale before pushing further investigation in patients seems necessary.

III-Questions addressed and objectives of the work

STS is rare and a heterogeneous group of tumor. These two fundamental characteristics are major issues when trying to improve the standard of care.¹⁵⁴ Indeed, clinical trials suffer from inhomogeneous disease natural history or lack of power due to small number of patients in over specific subtypes.¹⁵⁵ Therefore, a better understanding of the clinical and biological characteristics of each sarcoma subtype is critical. As a result, sarcoma research has been an example for global collaboration for research, database and clinical trials¹⁵⁶ but also for the need to have a strong preclinical rationale before pushing further drugs or combinations into clinic.

Question 1: Are the clinical characteristics of adolescent and adult patients with RMS and their management differ across age groups?

RMS in adults is a good model to explore the sarcoma paradigm. RMS is a heterogeneous disease across all age groups. However, few studies focused on this particular RMS patient population and showed that adult RMS may have a distinct biological and clinical pattern than pediatric RMS. Because of its rarity, knowledge specific to adult RMS lacks, leading to emulate diagnostic and treatment strategy for adult patients from the childhood RMS guidelines or from cohort studies.¹⁵⁵

This leads to several concerns regarding the appropriate management of adult RMS patients. First, there are no data to support the use of the same diagnostic, prognostic, and treatment approaches for pediatric and adult patient populations.

Because several studies suggested that patients greater than 10 years of age experienced an inferior prognosis compared to younger children, the first part of the present work focuses on 239 patients, 10 years of age and greater, diagnosed with RMS at MD Anderson Cancer Center (MDACC). Its goal is to provide insight into the difference in outcome between adult and pediatric RMS patients, whether it is due to differences in biology itself through clinicopathologic description or differences in patient management.

Question 2: Is PAX3/7- FOXO1 fusion status of importance in adolescent and adult patients with RMS?

PAX3/7-FOXO1 fusion status has not been shown to have prognostic value in adult patients; rather, there are limited data on translocation testing in this population and its clinical significance.

In synovial sarcoma, the adverse prognosis of adult patients compared to pediatric patients was linked to increased metastatic evented linked to genomic instability showed on CGH array data.¹⁵⁷

To determine whether *PAX3/7-FOXO1* fusion gene status is associated with outcomes in adult RMS, a large panel of specimens from this patient group was characterized by fluorescence *in situ* hybridization (FISH) and these findings were correlated with specific clinicopathologic parameters in the second part of this work.

Question 3: Is a combination of targeted therapy relevant in RMS and how to orchestrate its use with standard chemotherapy doxorubicin?

Doxorubicin and ifosfamide are the standard of care in most sarcoma, but these regimens confer only less than 10 % survival at 5 years in metastatic sarcoma including RMS.¹⁴⁶⁻¹⁵⁰ The development of targeted therapies has changed the landscape of sarcoma research, bringing hope to patients and clinicians to achieve better response rates and survival.^{151, 152} Yet, many early phase clinical trials have failed with targeted therapies as single agents.⁹⁶

Combination therapy is assumed to be the optimal strategy to potentiate targeted therapy use and achieve clinical significance. To combine targeted agents two by two or in polytherapy might overcome resistance and the inevitable relapse seen with single agent therapy. Targeted agents may also potentiate standard chemotherapy, allowing it to reduce doses, or prolong its use, which would be relevant regarding doxorubicin dose limiting cardiotoxicity. The eventual question would be: could we spare standard chemotherapy with the use of a targeted polytherapy, translating the HIV model?

Sorafenib

Sorafenib is a multikinase inhibitor targeting vascular endothelial growth factor (VEGF) receptors 1 and 2, Flt-3, c-kit, Raf-1, platelet-derived growth factor receptors (PDGFR) alpha and beta, and fibroblast growth factor (FGFR) receptor 1.^{158, 159} These are targetable pathways in RMS. Indeed, expression of PDGFR alpha and beta are associated with inferior outcome in RMS.^{160, 161} Moreover, RAF is activated by RAS and activating mutations of RAS are present in 35–50% of ERMS.^{162, 163} Finally, FGFR₁ expression has been demonstrated in RMS, associated with epigenetic modification.¹⁶⁴

The inhibition of these kinases results in anti-tumor activity through the inhibition of cancer cell proliferation and angiogenesis.¹⁶⁵ An action on sarcoma has been showed on synovial sarcoma in vitro.¹⁶⁶ It has been tested in a phase II study as a single agent on metastatic or recurrent sarcomas.¹⁶⁷ An activity was notable against angiosarcoma and was minimal against other sarcomas and concluded that further evaluation of sorafenib in these and possibly other sarcoma subtypes would appear warranted, presumably in combination with cytotoxic or kinase-specific agents.¹⁶⁸ Pazopanib as exemplified the use of TKI as single agents in STS.⁷⁰

Vorinostat

HDAC inhibitors represent a new class of anti-cancer drugs, currently in development. HDAC modifications have been identified in many tumors.¹⁶⁹ HDAC inhibitors induce differentiation, a cell cycle arrest, apoptosis of tumor cells and inhibit tumor growth in preclinical assays.^{170, 171} Among HDAC inhibitors most advanced in clinical studies, Suberoylanilide Hydroxamic Acid or vorinostat exert antitumor activity on tumors that express a multi-drug-resistant phenotype (MDR). A synergistic action has been shown with ionizing radiation and inhibitors of kinases. The vorinostat has shown promising results on in vivo models such as prostate and hematological malignancies. It is now approved in cutaneous T cell lymphoma.¹⁷² Several recent arguments suggest an activity of vorinostat on sarcoma cells. Most of the model tested were osteosarcoma and Ewing's sarcoma.^{170, 173, 174} However, in Ptch mutant mice with RMS, the combination of 5-aza-2'deoxyctidine, an hypomethylating agent and valproic acid, one of the first HDAC inhibitor discovered had an anti-tumor activity.¹³⁹ Several phase I trials have been performed with HDAC inhibitors including in children with refractory solid tumors.¹⁷⁵ They are also tested in combination with sorafenib in a phase I trial¹⁷⁶ and temsirolimus.¹⁷⁷

17-DMAG

HSP 90 is a member of heat shock protein family whose expression is increased when cells are exposed to stress such as elevated temperature.^{152, 178} Intracellular heat shock proteins are highly expressed in tumor cells and are essential to their survival within a tumor, because of the hypo oxygenation or post chemotherapy stress. Furthermore, HSP 90 is known to play a critical role in tumor cell survival and growth in several types of cancer. Inhibition of HSP 90 in RMS cell lines showed blockade of proliferation and migration and induced apoptosis in vitro, which was confirmed in vivo on mice.¹⁷⁹ The HSP 90 antagonist 17-DMAG is currently in clinical trials and may promise on sarcomas.^{174, 179-181}

Abacavir

Upregulation of telomerase in most cancer yields to protect tumor cells from aging and death of cancer cells.^{182, 183} An antiretroviral nucleoside analogue called abacavir (Ziagen) has potent antiretroviral activity and a telomerase inhibitory activity in various cellular systems and animal studies. It showed in solid tumors notably prostate cancer cell lines a reduction of proliferation and senescence.¹⁸⁴ It has not been tested on sarcoma cell lines so far.

Many crosstalks are being discovered between the different pattern of inhibition between this four drugs. For instance, on gastrointestinal stromal tumors, it has been observed that HSP 90 was a target for SAHA, which acetylates it then induces apoptosis.¹⁸⁵ Therefore, early phase clinical trials are underway to test targeted therapy combination such as sorafenib and vorinostat in patients with solid tumors.¹⁷⁶

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was a target for SAHA, which acetylates it then induces apoptosis.¹⁸⁵ Therefore, early phase clinical trials are underway to test targeted therapy combination such as sorafenib and vorinostat in patients with solid tumors.¹⁷⁶

The last part of this work aims to study targeted therapy combinations to offer a preclinical background to clinical trials. We sought to identify most active bi and tritherapy combinations of targeted agents and how to timely orchestrate them together and with standard chemotherapy doxorubicin in small cell sarcoma cell lines.

IV RESEARCH WORK

IV-1 Management and Outcome of 239 Adolescent and Adult Rhabdomyosarcoma Patients

The present work aims to study the age-related differences in management of adolescents and adults with RMS. Under an institutional review board-approved protocol, we retrospectively analyzed 239 patients, 10 years of age and greater, diagnosed with RMS at MD Anderson Cancer Center from 1957 through 2003.

Of the 239 patients, 163 patients with localized RMS had a median OS of 3.8 years (95% CI 2.8-7.6). In the multivariate analysis, age >50 and invasion were significantly associated with shorter OS and RFS for patients with localized RMS. Meanwhile, chemotherapy given along with surgery or radiation was significantly associated with longer OS. Non-metastatic patients over 50 years of age had a poor 5-year OS of 13% (median OS 1.7 years) compared to the younger patients. Metastases were present in 76 patients, the median OS was 1.4 years in this population. Approximately 13% of metastatic patients less than 50 years-old were found to have a long-term survival exceeding 15 years. Triple modality therapy, including surgery, radiotherapy and chemotherapy was significantly associated with longer OS in metastatic patients. The use of bi- and triple modality treatment decreased in metastatic patients over 50 years of age compared to younger patients.

This is the largest single institution series combining adolescent and adult patients with RMS. While contributing to a more defined description of the disease, that appears to be

more similar to pediatric RMS than previously suspected, this work demonstrates age-related differences in management of patients that may partly explain their poor prognosis and suggests that increased use of multidisciplinary therapy may improve older patient clinical outcome.

ORIGINAL RESEARCH

Management and outcome of 239 adolescent and adult rhabdomyosarcoma patients

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Introduction

Rhabdomyosarcoma (RMS) is a rare mesenchymal tumor [1, 2]. It is the most common soft-tissue sarcoma in the first two decades of life, but accounts for <1% of adult cancers and is reported to account for <4% of adult soft-

Abstract

Adult rhabdomyosarcoma (RMS) is a rare tumor that has inferior outcome compared to younger patient population. The present work aims to study the age-related differences in management of adolescents and adults with RMS. Under an institutional review board-approved protocol, we retrospectively analyzed 239 patients, 10 years of age and greater, diagnosed with RMS at MD Anderson Cancer Center from 1957 through 2003. Of the 239 patients, 163 patients were nonmetastatic with a median overall survival (OS) of 3.8 years (95% CI 2.8–7.6). In the multivariate analysis, age >50 was significantly associated with shorter OS and recurrence-free survival (RFS) for primary patients. Metastases were present in 76 patients, the median OS was 1.4 years. Approximately 13% of metastatic patients <50 years old had a long-term survival exceeding 15 years. Multimodality therapy, including surgery, radiotherapy, and chemotherapy was significantly associated with longer OS in primary and metastatic patients. Use of bi- and triple modality treatment decreased in metastatic patients over 50 years of age compared to younger patients. RMS in adolescents and adults has a poor outcome compared with younger individuals. Increased use of multidisciplinary therapy may improve older patient clinical outcome.

tissue sarcomas in the United States [3, 4]. Because cancer is fortunately a rare event during childhood, about four in 10 patients reported to have RMS are adults [5].

RMSs are classified into three main histologic subtypes: pleomorphic RMS (PRMS), embryonal RMS (ERMS), and alveolar RMS (ARMS). Classically, ERMS

is correlated with a better prognosis than either ARMS or PRMS [6–11].

Some chromosome rearrangements are specific to a subtype and allow a finer classification of the tumor, thus having a prognostic importance [12–20]. Therefore, the assessment of the translocation is becoming a tool for risk stratification in pediatric RMS [21, 22] and is likely to have relevance for adult RMS as well [23].

The Intergroup Rhabdomyosarcoma Study (IRS) made the major observation that patients older than 10 years of age, defined in the present work as adolescent and adult patients, had inferior outcomes compared to patients younger than 10 [11, 22, 24]. The 5-year overall survival (OS) for adult patients with localized disease is a dismal 20–40% [6], whereas in pediatric patients it is between 60% and 80% [25]. Five-year OS is <5% in adult patients with metastatic disease [3, 26, 27].

Because of its rarity, knowledge specific to patients over 10 with RMS comes mostly from cohort studies, explaining the fact that diagnosis and treatment strategy for adult patients is often emulated from the pediatric RMS guidelines [28].

Because several studies suggested that patients over 10 years of age experienced an inferior prognosis compared to younger children, the present work focuses on 239 patients, 10 years of age and greater, diagnosed with RMS at MD Anderson Cancer Center (MDACC).

We provide insight into the difference in outcome between adult and pediatric RMS patients, whether it is due to differences in biology itself through clinicopathologic description or differences in patient management.

Patients and Methods

Patients

We retrospectively analyzed 239 consecutive patients, 10 years of age and greater, diagnosed with RMS at MDACC from 1957 through 2003. Thirteen patients (5%) presented with relapse while the others received their primary treatment at our institution. We obtained a waiver of informed consent and the protocol was approved by the Institutional Review Board.

Patient demographics and tumor, treatment and survival data were reviewed. Tumors were classified as localized disease when they had not yet metastasized at the time of diagnosis. Locoregional lymph node involvement was included in the nonmetastatic group. Distant lymph node involvement was categorized as metastases.

The IRS staging system divides favorable from unfavorable sites and gives risk stratification management in pediatric RMS. The anatomic sites were at first clustered following this staging system but were broadened due to

lack of prognostic significance. Tumor size was based on the largest dimension of the localized tumor as reported on pretreatment imaging scans.

At least two cycles of chemotherapy were required to be included in the treatment analysis.

Pathology

Pathology review was performed at our institution by a sarcoma pathologist at the time of diagnosis for each patient and the specimens were rereviewed a second time at the inclusion to the study by a different sarcoma pathologist.

Statistical analysis

Patient outcome was assessed according to the following clinicopathological variables: age, gender, size and loca-

Table 1. Patient and tumor characteristics.

Description	No.	%
Gender		
Female	97	41
Male	142	59
Age (years)		
<20	122	51
20–50	88	37
>50	29	12
Subtype		
Alveolar	56	23
Embryonal	95	38
Pleomorphic	23	10
Unknown	65	27
Location		
Head and neck	87	36
GU	46	19
Trunk	23	10
Intraabdominal/pelvis	35	15
Extremities	48	20
IRS		
1	57	24
2	32	13
3	73	31
4	76	32
Unknown	1	0
IRS group		
I	52	22
II	13	5
III	88	37
IV	76	32
Unknown	10	4
Tumor size		
0–5 cm	88	37
5.01–10.00 cm	90	38
>10 cm	51	21
Unknown	16	7

GU, genitourinary; IRS, Intergroup Rhabdomyosarcoma Study.

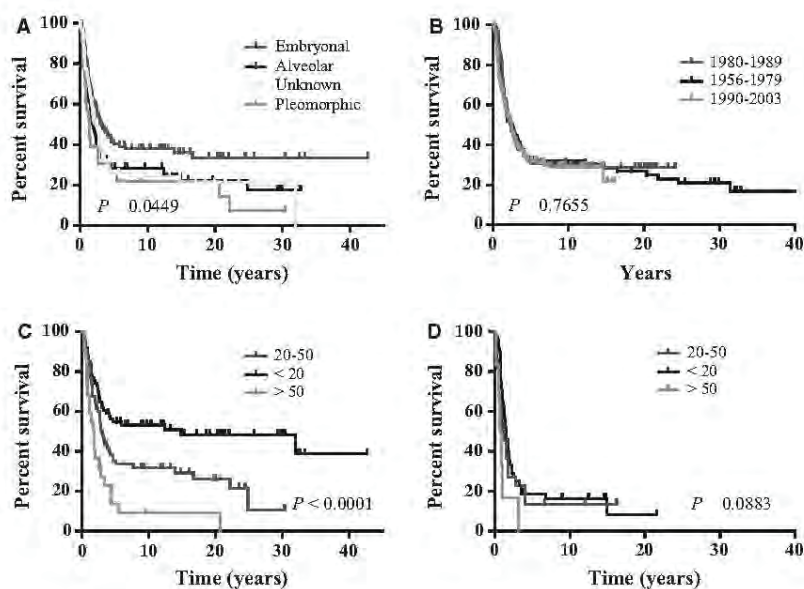


Figure 1. Kaplan Meier patient overall survival comparing (A) histology subtypes, (B) era of treatment, (C) age in localized, and (D) metastatic setting.

tion of localized tumor, nodal status, and IRS group classification. Treatment was analyzed according to the extent of multidisciplinary involvement as well as the type of chemotherapy agent.

The Kaplan and Meier product limit estimator estimated the median OS, recurrence-free survival (RFS) for nonmetastatic patients and progression-free survival (PFS) for metastatic patients from date of diagnosis to date of death or last follow-up. We used Cox proportional hazards regression to model OS, RFS, and PFS and to estimate the hazard ratios for several potential prognostic factors in a univariate fashion. We then included in a multivariate model all factors found to be significant at the 0.25 level and used backward elimination to remove factors until all remaining factors were significant at the 0.05 level. All statistical analyses were performed using SAS 9.1 for Windows (Copyright © 2002–2003 by SAS Institute Inc., Cary, NC).

Results

Patient demographics

The cohort included 239 patients, 10 years of age and older, followed and treated at our institution for a diagnosis of RMS. This represents only 1.2% of the 19,708 patients with sarcoma seen during the period of the study. The median age was 19 years with a range of 10–102, while 80% of patients were age 15 or older. There

were 97 (40.6%) women and 142 (59.4%) men (Table 1). ERMS was the most represented subtype and tended to have a longer survival compared with other subtypes (Fig. 1A). To reflect the evolution of RMS management over the 45-year study period, survival was analyzed according to three different time periods: 1957–1979, 1980–1989, and 1990–2003. No difference in 5-year OS was observed between the three time periods and was stable around 33% (Fig. 1B). Moreover, incidence of nodal involvement was used as a surrogate for imaging improvement. Nodal detection was stable overtime with a rate of 7%.

Localized disease

Patient demographics

163 patients (68%) had no evidence of metastases, including 63 (38.7%) women and 100 (61.3%) men. The median age was 22 with a range of 10–102. The mean age was 28.6 (standard deviation, 18.1).

Tumor characteristics

Sixty-two percent of the tumors were considered invasive at surgery or on imaging. The main tumor location was the head and neck (44%), followed by the genitourinary (GU) tract (20%) and the extremities (18%). The trunk and intraabdominal and pelvis locations each represented <10% of patients (Table 2).

Table 2. Factors associated with recurrence-free survival and overall survival for patients with localized disease.

	No. of Pts (%)	Recurrence-free survival				Overall survival			
		Median (years)	Uni variate P-value	Multivariate		Median (years)	Uni variate P-value	Multivariate	
				Hazard ratio	95% CI for HR			Hazard ratio	95% CI for HR
Gender									
Female	63 (39)	2.6	—	—	—	4.4	—	—	—
Male	100 (61)	1.4	0.142	—	—	3.3	0.396	—	—
Age									
<20	74 (45)	6.7	—	1.00	—	31.9	—	1.00	—
20–50	66 (40)	1.4	0.003	0.010	1.15–2.73	3.0	0.006	0.036	1.04–3.03
>50	23 (14)	0.9	<0.001	<0.001	1.98–6.12	1.7	<0.001	<0.001	2.92–12.42
Location									
Head and neck	72 (44)	1.5	—	—	—	3.0	—	—	—
GU	33 (20)	13.9	0.039	—	—	—	0.030	—	—
Trunk	14 (9)	1.0	0.630	—	—	2.4	0.708	—	—
Abdominal/pelvis	14 (9)	1.3	0.716	—	—	3.2	0.519	—	—
Extremities	30 (18)	2.2	0.617	—	—	3.4	0.611	—	—
IRS									
I	57 (35)	3.9	—	—	—	14.8	—	—	—
2	32 (20)	2.0	0.826	—	—	7.6	0.628	—	—
3	72 (44)	1.2	0.051	—	—	2.6	0.004	—	—
Unknown	1 (1)	—	—	—	—	—	—	—	—
IRS group									
I	52 (32)	1.9	—	—	—	13.9	—	—	—
II	13 (8)	2.4	0.969	—	—	2.9	0.657	—	—
III	87 (53)	1.7	0.652	—	—	3.3	0.166	—	—
Unknown	10 (6)	—	—	—	—	—	—	—	—
Chemotherapy									
Doxorubicin, no ifosfamide	63 (39)	1.7	—	—	—	2.8	—	—	—
Ifosfamide, no doxorubicin	5 (3)	1.3	0.376	—	—	4.2	0.954	—	—
Doxorubicin plus ifosfamide	19 (12)	1.2	0.095	—	—	2.1	0.192	—	—
Actinomycin D, no doxorubicin/ifosfamide	33 (20)	—	0.015	—	—	—	0.002	—	—
Any regimen, no actinomycin D/doxorubicin/ifosfamide	2 (1)	1.3	0.249	—	—	1.8	0.168	—	—
Sequential doxorubicin/ifosfamide	6 (4)	5.0	0.687	—	—	9.5	0.891	—	—
No chemotherapy	17 (10)	0.6	<0.001	—	—	2.8	0.323	—	—
Unknown	18 (11)	3.1	—	—	—	3.4	—	—	—
Treatment modality									
Chemotherapy	10 (6)	0.9	—	—	—	1.1	—	1.00	—
Surgery or surgery plus radiotherapy	15 (9)	0.5	0.113	—	—	2.9	0.417	0.300	0.21–1.61

Table 2. Continued.

	No. of Pts (%)	Recurrence-free survival				Overall survival			
		Median (years)	Uni variate P-value	Multivariate		Median (years)	Uni variate P-value	Multivariate	
				Hazard ratio	95% CI for HR			Hazard ratio	95% CI for HR
Surgery plus chemotherapy or chemotherapy plus radiotherapy	58 (36)	5.9	0.120						
Surgery plus chemotherapy plus radiotherapy	60 (37)	2.0	0.441		3.3	0.108	0.074	0.47	0.20–1.08
Unknown	20 (12)	–	–		–	–	–	–	–
Tumor Size									
0.00–5.00 cm	67 (41)	2.4	–		4.6	–	–	–	–
5.01–10.00 cm	61 (37)	1.6	0.998		4.4	0.645	–	–	–
>10.00 cm	28 (17)	1.5	0.627		3.1	0.285	–	–	–
Unknown	13 (8)	–	–		–	–	–	–	–

GU, genitourinary; IRS, Intergroup Rhabdomyosarcoma Study.

Tumors measured most often <5 cm (41%); whereas 37% were 5–10 cm and 17% were larger than 10 cm. Tumor size was not specified in 8% of cases.

Treatment

Very few patients underwent localized therapy alone (20 patients, 9%) while 6% (14 patients) had chemotherapy alone. Many patients had multimodality therapy integrating surgery, chemotherapy, and radiotherapy (37%) whereas 36% had chemotherapy-based therapy with either surgery or radiotherapy. Patients over 50 years of age were more likely to have multimodality therapy compared with younger patients but the rate of chemotherapy-based strategies was fairly similar over age in the localized patient population (Fig. 2A). A chemotherapy regimen that included actinomycin D was given to 23% of patients. The patients receiving actinomycin D were almost exclusively (91%) under 20 years old. Doxorubicin was administered to 54% of patients and ifosfamide to 18% of patients. Eighty percent of patients receiving doxorubicin plus ifosfamide were between 20 and 50 years of age. Patients over 50 were less likely to receive ifosfamide than patients between ages 20 and 50 (Table 3). Ninety-nine percent of patients with localized RMS received at least one of the three drugs.

Outcome

The analyses of RFS and OS are summarized in Table 2. One hundred twelve (69%) of the 163 patients had recurrent disease or died. Median follow-up for all 163 patients with localized disease was 3.3 years (range, 0.3–42.7 years). The 64 patients who remained alive had a median follow-up of 12.1 years (range, 0.6–42.7 years) while the 99 patients who died had a median follow-up of 1.8 years (range, 0.3–31.9 years).

The median RFS was 1.9 years (95% CI 1.3–2.8 years), the 1-year RFS rate was 0.67 (95% CI 0.59–0.73), the 2-year RFS rate was 0.469 (95% CI 0.390–0.544), and the 5-year RFS rate was 0.362 (95% CI 0.288–0.436). Thirteen patients had recurrent disease but remained alive at last follow-up. In the univariate analysis of RFS, increasing age, and “No Chemotherapy” were associated with shorter RFS, while GU location and “Any actinomycin D, No doxorubicin/ifosfamide” were significantly associated with longer RFS. Patients who did not receive chemotherapy had a complete resection with or without radiation therapy. However, in the multivariate analysis only an increasing age was associated with a shorter RFS (Table 2).

The median OS for all patients with localized disease was 3.8 years with 95% confidence interval (CI)

Table 3. Chemotherapy regimens according to age in localized and metastatic setting.

Disease stage	Localized						Metastatic					
	<20		20-50		>50		<20		20-50		>50	
Age	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Chemotherapy regimen	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Ifosfamide-free regimen												
Any doxorubicin, no ifosfamide	26	35	27	41	14	48	22	46	12	55	4	67
Any actinomycin D, no doxorubicin/ifosfamide	30	41	1	2	2	7	13	27	0	0	0	0
Any regimen, no actinomycin D/doxorubicin/ifosfamide	0	0	2	3	0	0	1	2	1	5	0	0
Ifosfamide-based regimen												
Any ifosfamide, no doxorubicin	3	4	2	3	0	0	4	8	1	5	0	0
Any ifosfamide plus doxorubicin	1	1	16	24	3	10	4	8	6	27	1	17
Sequential doxorubicin plus ifosfamide	2	3	4	6	0	0	3	6	1	5	0	0
No chemotherapy	4	5	9	14	4	14	0	0	0	0	0	0
Unknown	8	11	5	8	6	21	1	2	1	5	1	17
Total	74	100	66	100	29	100	48	100	22	100	6	100

2.8–7.6 years. Ninety-nine of the 163 patients died. The 1-year OS was 0.846 with 95% CI 0.781–0.893. The 2-year OS was 0.660 with 95% CI 0.582–0.727, and the 5-year OS was 0.441 with 95% CI 0.362–0.516. In the univariate analysis of OS, increasing age and IRS grade 3, were significantly associated with shorter OS, while GU location, “any actinomycin D, no doxorubicin/ifosfamide”, and “surgery plus chemotherapy or chemotherapy plus radiotherapy” were significantly associated with longer OS. In the multivariate analysis only increasing age and inclusion of chemotherapy (“surgery plus chemotherapy or chemotherapy plus radiotherapy”) were associated with longer OS (Table 2). The Kaplan–Meier survival curve presented a notable inflection point at 5 years followed by a plateau. Long-term survivors (>15-year) included 55% of patients younger than 20, 31% of patients between 20 and 50 years of age, but <10% of patients older than 50 (5-year OS 13%, median OS of 1.7 years, Fig. 1C).

Metastatic disease

Patient demographics

There were 76 patients with metastatic disease, including 34 (44.7%) women and 42 (55.3%) men. The median age was 18 (10–67 years). The mean age was 23.7 (standard deviation, 13.9).

Tumor characteristics

Localized tumors were found within the abdomen or pelvis in 28%, the extremity in 24% of the tumors were located in the extremities, the head and neck in 20%, in the GU region in 17%, and on the trunk in 12% of patients. Two thirds of the primary tumors measured >5 cm (68%) at

diagnosis (Table 4). The main metastatic sites were the lungs in 39% of cases, the bone marrow for 28%, distant lymph nodes in 20%, and the bone for 14% of patients.

Treatment

All the patients received chemotherapy. Thirty-nine percent had either surgery or radiotherapy associated with chemotherapy while 21% had all three modalities. Seventeen percent of patients received actinomycin D, 70% doxorubicin, and 26% ifosfamide. Only two patients received none of these three agents (3%). Patients with metastatic disease had a 14.4% 15-year survival treated with bimodality treatment (chemotherapy plus surgery or chemotherapy plus radiotherapy) and patients who were able to be treated with all three modalities (surgery plus chemotherapy plus radiotherapy) had a 37.5% 14-year survival. Use of surgery and/or radiation therapy in addition to chemotherapy decreased in metastatic patients over 50 years of age compared to younger patients (Fig. 2B), and their chemotherapy was less likely to include ifosfamide than patients between 20 and 50 years of age (Table 3).

Outcome

The analyses of PFS and OS are summarized in Table 4. Median follow-up for all 76 patients with metastatic disease was 1.4 years (range, 0.1–21.6 years). For the 14 patients who remain alive the median follow-up was 8.9 years (range, 1.1–21.6 years). For the 62 patients who died the median follow-up was 1.1 years (range, 0.1–14.9 years).

The median PFS was 0.9 years (95% CI 0.7–1.3 years). Sixty-seven (88%) of the 76 patients had progressive disease or died. The PFS rate at 1-year was 0.447 (95% CI

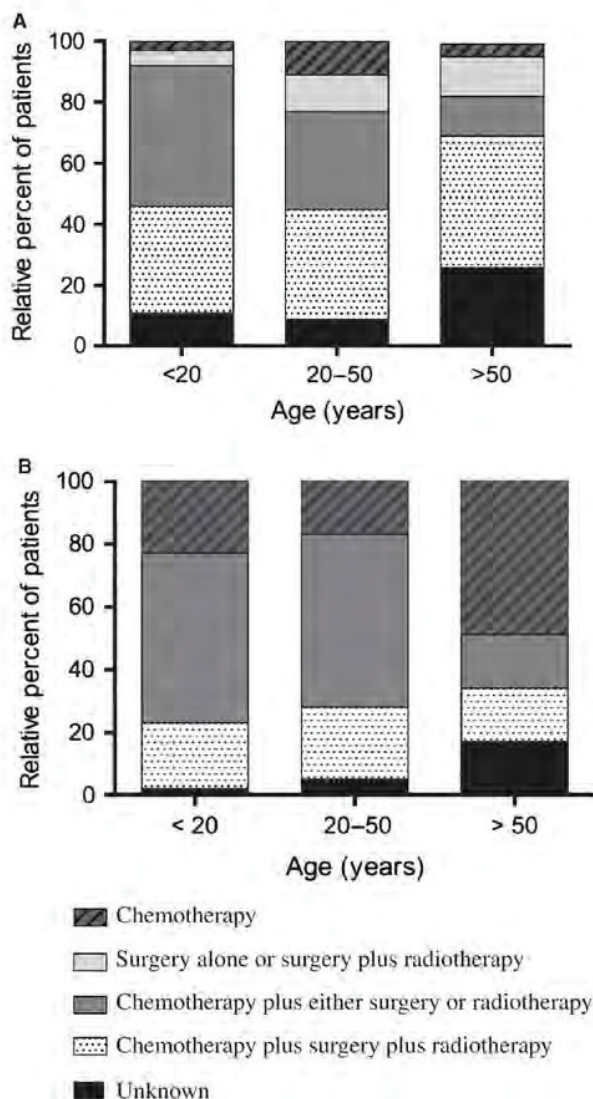


Figure 2. Treatment modality for (A) localized patients (B) metastatic patients.

0.334–0.555), at 2-years was 0.224 (95% CI 0.138–0.322), and at 5-years was 0.132 (95% CI 0.067–0.218). Five patients with progressive disease remained alive at last follow-up. In the univariate analysis age >50 and location in the trunk or abdomen/pelvis region were significantly associated with shorter PFS, while patients who were able to receive surgery, radiation, and chemotherapy were associated with longer PFS than those who did not. In the multivariate analysis these same factors were found to be associated with PFS, along with location in the extremities (Table 4).

The median OS for all patients with metastatic disease was 1.4 years (95% CI 1.0–1.8 years). The median OS at

1-year was 0.605 (95% CI 0.486–0.705), at 2-years was 0.323 (95% CI 0.221–0.429), and at 5-years was 0.180 (95% CI 0.102–0.277).

In the univariate analysis of OS, age >50 and location in the trunk or abdomen/pelvis were associated with shorter OS, while patients who were able to receive surgery, radiation, and chemotherapy were associated with longer OS compared with those who did not. These three factors remained significant in the multivariate analysis of OS in patients with metastatic disease (Table 4).

In the multivariate analysis of OS, location in the extremities was also found to be associated with a longer PFS. The Kaplan–Meier curve shows an inflection point around 3 years, followed by a plateau for patients younger than 50 suggesting a cure rate of ~17% for these metastatic patients (Fig. 1D).

Discussion

Adolescent and adult RMS is a rare entity that has inferior outcome compared to younger patient population. Our study shows not only that this patient population have a similar clinicopathologic pattern than pediatric RMS, but also that adolescent and adult patients before 50 years of age are more likely to receive multidisciplinary therapy and ifosfamide-based chemotherapy than their older counterparts.

The first major difference between pediatric and older patients is the referral pattern. Indeed, pediatric cancers are most likely to be referred to tertiary or quaternary cancer center, thus benefiting from a more accurate diagnosis, which is critical in this disease. According to the Surveillance, Epidemiology and End Results (SEER) study comparing adult and pediatric RMS patients between 1973 and 2005 for a total of 2600 patients, the histologic subtype was unknown for about 43% of adult patients versus 13.2% of pediatric patients [5]. SEER data reflecting the average population management, we could assume that in the early 70s, many malignant fibrous histiocytoma were mistaken for PRMS by nonsarcoma-trained pathologists. MDACC being a referral center for sarcoma treatment, about 27% of pathology specimens could not be characterized, particularly from patients treated during the earliest time period, which suggests that referring adult RMS patients to an expert center increases the chances of a better characterization of the disease [29].

Moreover, our study survival data reveals two major observations. First, patients with metastatic RMS were found to have an 18% survival rate 5 years from diagnosis with an apparent 12% survival past 15 years, which is unexpectedly high in this setting. Second, patients older than 50 years with localized RMS have a dismal 5-year OS of 13%. As this finding could not merely be explained

Table 4. Factors associated with progression-free survival and overall survival for patients with metastatic disease.

	No. of Pts (%)	Progression-free survival				Overall survival			
		Median (years)	Univariate P-value	Multivariate		Univariate P-value	Multivariate P-value	Hazard ratio	95% CI for HR
				Hazard ratio	95% CI for HR				
Gender									
Female	34 (45)	0.8	—	—	—	—	—	—	—
Male	42 (55)	1.0	0.541	—	—	—	—	—	—
Age									
<20	48 (63)	1.1	—	—	—	—	—	—	—
20–50	22 (29)	0.7	0.384	0.011	1.23–4.87	0.428	0.015	2.44	1.19–5.00
>50	6 (8)	0.5	0.037	0.139	0.79–5.61	0.036	0.158	2.02	0.76–5.36
Location									
Head and neck	15 (20)	1.8	—	—	—	—	—	—	—
GU	13 (17)	1.4	0.703	0.187	0.73–5.16	0.718	0.220	1.93	0.67–5.56
Trunk (chest wall/back)	9 (12)	1.0	0.035	0.013	1.35–11.85	0.014	0.006	5.06	1.60–16.02
Intraabdominal/pelvis	21 (28)	0.6	0.001	0.002	1.73–10.08	0.002	0.003	4.02	1.61–10.05
Extremities	18 (24)	0.8	0.073	0.008	1.40–9.36	0.072	0.010	3.90	1.39–10.96
IRS									
3	1 (1)	—	—	—	—	—	—	—	—
4	75 (99)	1.0	—	—	—	—	—	—	—
IRS group									
III	1 (1)	—	—	—	—	—	—	—	—
IV	75 (99)	1.0	—	—	—	—	—	—	—
Chemotherapy									
Doxorubicin, no ifosfamide	38 (50)	1.0	—	—	—	—	—	—	—
Ifosfamide, no doxorubicin	5 (7)	1.4	0.654	—	—	0.404	—	—	—
Doxorubicin plus ifosfamide	11 (14)	0.6	0.201	—	—	0.390	—	—	—
Actinomycin D, no doxorubicin/ifosfamide	13 (17)	0.9	0.792	—	—	0.750	—	—	—
Any regimen, no actinomycin D/ doxorubicin/ifosfamide	2 (3)	1.3	0.815	—	—	0.808	—	—	—
Sequential doxorubicin/ifosfamide	4 (5)	1.7	0.898	—	—	0.251	—	—	—
Unknown	3 (4)	0.8	—	—	—	—	—	—	—
Treatment modality									
Chemotherapy	18 (24)	0.6	—	—	—	—	—	—	—
Surgery plus chemotherapy or chemotherapy plus radiotherapy	39 (51)	1.0	0.051	0.131	0.32–1.16	0.059	0.060	1.00	0.27–1.03

Table 4. Continued.

	No. of Pts (%)	Progression-free survival				Overall survival				
		Median (years)	Univariate P-value	Multivariate		Univariate P-value	Multivariate			
				Hazard ratio	95% CI for HR		P-value	Hazard ratio	95% CI for HR	
Surgery plus chemotherapy plus radiotherapy	16 (21)	1.7	0.003	0.037	0.40	0.17–0.95	0.003	0.018	0.34	0.14–0.83
Unknown	3 (4)	–	–	–	–	–	–	–	–	–
Tumor Size										
0.00–5.00 cm	21 (28)	1.3	–	–	–	–	–	–	–	–
5.01–10.00 cm	29 (38)	1.0	0.447	–	–	–	0.404	–	–	–
>10.00 cm	23 (30)	0.5	0.142	–	–	–	0.172	–	–	–
Unknown	3 (4)	–	–	–	–	–	–	–	–	–

GU, genitourinary; IRS, intergroup Rhabdomyosarcoma Study.

by the natural aging of patients, metastatic patients of this age group did not share the similar long-term survival trend. This raises the question that patients over 50 years of age with localized disease may be treated less effectively than the younger patients.

While looking closer at the treatment disparities among age groups, the first aspect was that, in localized disease, the probability of not receiving chemotherapy increased with age, which may have reduced the probability of long-term survival. Meanwhile, patients older than 50 years of age with metastatic RMS were much more likely to receive chemotherapy only whereas their younger counterparts were more likely to be treated with approaches including surgery or radiotherapy. This finding confirms the recent study from Kojima et al. [30] that judicious use of local therapy is critical to survival of patients with metastatic disease. Moreover, chemotherapy regimens including ifosfamide are the mainstay of chemotherapy used in older patients with RMS; however, ifosfamide is often withheld or limited in its use due to associated toxicity in this population. This leads to speculation that perhaps alternative agents such as cyclophosphamide could be used more frequently.

Alternatively, RMS is possibly a different biological and clinical entity in patients aged 10 years or greater. As the subtype and translocation status have prognostic significance, the different pattern of histology across age, for instance the pleomorphic subtype uncommon in pediatric patients, may explain the difference in outcome. While few studies focused on the age-related biological and molecular differences of RMS [23], the cell of origin of RMS was investigated in recent works such as the European Pediatric Soft Tissue Sarcoma Study Group showing that the patient outcomes and gene expression signatures of fusion-negative ARMSs were very similar to those of ERMSs, and may help better understand the relationship between outcome and histological subtype [31].

Finally, a report based on SEER data showed that the outcome of children and adolescent younger than 15 years of age with RMS appeared to have improved between 1975 and 1982, reflecting the impact of management improvement as a result of clinical trial participation but had been stable since [32].

Possibly due to methodology, selection bias, or referral patterns, our work shows no quantifiable clinical improvement in the outcome of older patients over the last 50 years despite a better understanding of the disease biology, imaging, and treatment progress.

Despite its inherent limitations, this study highlights the need to improve the management of patients with RMS over 10 years of age. We should ensure that these patients benefit as much from clinical management and research progress as younger patients with RMS.

Conflict of Interest

None declared.

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IV-2 *PAX3/7-FOXO1* Fusion Status in Older Rhabdomyosarcoma Patient Population by Fluorescent *In Situ* Hybridization

This manuscript reports the analysis of *PAX3/7-FOXO1* translocation in adult patients with RMS. In pediatric alveolar RMS, the *PAX3-FOXO1* and *PAX7-FOXO1* gene fusions are prognostic indicators, while little is known concerning this disease in older patients. To determine whether *PAX3/7-FOXO1* fusion gene status correlates with outcome in adolescent, young adult, and adult RMS patients, the histological, immunohistochemical, and clinical characteristics of 105 patients followed at The University of Texas MD Anderson Cancer Center from 1957 to 2001 were evaluated. The samples were assembled into a tissue microarray, and fusion gene status was determined by Fluorescence in situ hybridization using *PAX3*, *PAX7*, and *FOXO1* loci-specific probes. The disease characteristics and specific gene fusion were correlated with patient outcomes using the log-rank test. Fifty-two percent of the samples exhibited a *PAX3-FOXO1* fusion, 15% the *PAX7-FOXO1* fusion, and 33% were negative for a rearrangement of these loci. The presence of *PAX3/7-FOXO1* translocation was significantly associated with a higher frequency of metastatic disease. Although a statistically significant correlation between the *PAX3/7-FOXO1* fusion gene status and overall survival was not identified, there was a trend toward better outcome for patients with fusion-negative RMS. Therefore, identification of a *FOXO1* fusion appears to be an interesting tool for predicting outcomes in older RMS patients and is worth further investigations in this rare subgroup of RMS population.

***PAX3/7-FOXO1* fusion status in older rhabdomyosarcoma patient population by fluorescent in situ hybridization**

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Abstract

Purpose In pediatric alveolar rhabdomyosarcoma, the *PAX3-FOXO1* and *PAX7-FOXO1* gene fusions are prognostic indicators, while little is known concerning this disease in older patients. To determine whether *PAX3/7-FOXO1* fusion gene status correlates with outcome in adolescent, young adult, and adult rhabdomyosarcoma patients, the histological, immunohistochemical, and clinical characteristics of 105 patients followed at The University of Texas MD Anderson Cancer Center from 1957 to 2001 were evaluated.

Methods The samples were assembled into a tissue microarray, and fusion gene status was determined by fluorescence in situ hybridization using *PAX3*, *PAX7*, and *FOXO1* loci-specific probes. The disease characteristics and specific gene fusion were correlated with patient outcomes using the log-rank test.

Results Fifty-two percent of the samples exhibited a *PAX3-FOXO1* fusion, 15% the *PAX7-FOXO1* fusion, and 33% were negative for a rearrangement of these loci. The presence of *PAX3/7-FOXO1* translocation was significantly associated with a higher frequency of metastatic disease. Although a statistically significant correlation between the *PAX3/7-FOXO1* fusion gene status and overall survival was not identified, there was a trend toward better outcomes for patients with fusion-negative RMS.

Conclusions Therefore, identification of a *FOXO1* fusion appears to be an interesting tool for predicting outcomes in older rhabdomyosarcoma patients and is worth further investigations in this rare subgroup of RMS population.

Keywords Rhabdomyosarcoma · Chromosomal rearrangement · *PAX-FOXO1* · Fluorescent in situ hybridization · Tissue microarray

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Introduction

Rhabdomyosarcoma (RMS) is a rare, highly malignant mesenchymal neoplasm. While it is the most common soft tissue sarcoma in children, RMS incidence declines with age, representing less than 0.1% of adult malignancies and about 10% of all adult soft tissue sarcomas in the United States (Parham and Ellison 2006; Jemal et al. 2006). Moreover, the Intergroup Rhabdomyosarcoma Study Group (IRSG) observed a relationship between age and survival in RMS patients (Raney et al. 2001; Maurer et al. 1988; Maurer et al. 1993; Crist et al. 1995; Crist et al. 2001). The 5-year overall survival for adult patients with primary disease is a dismal 20–40% (Little et al. 2002), whereas in children, it is between 60 and 80% (Crist et al. 1995). In adult patients with metastatic disease, 5-year survival is

less than 5% (Esnaola et al. 2001; Ferrari et al. 2003; Hawkins et al. 2001).

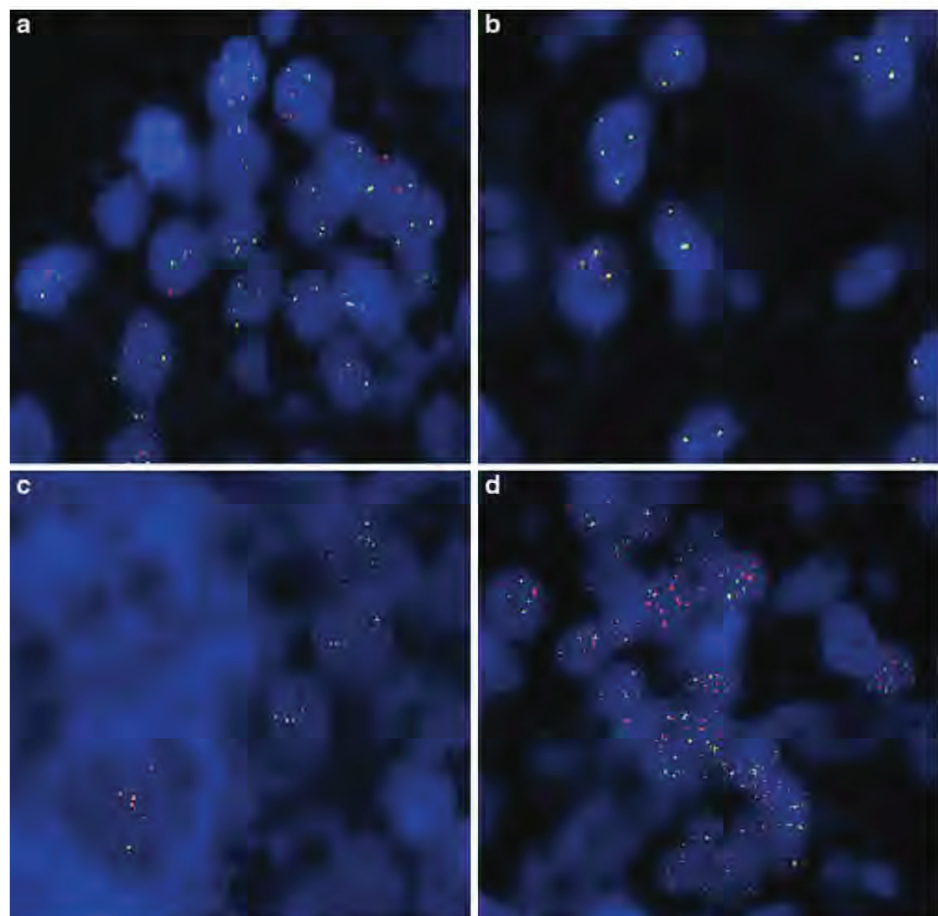
RMS is divided into different subtypes according to the histologic features. The main subtypes are alveolar RMS (ARMS), embryonal RMS (ERMS), and pleomorphic RMS. The specific histologic subtype is correlated with survival in both children and adults. Classically, ERMS has a better prognosis than either ARMS or pleomorphic RMS (Little et al. 2002; Raney et al. 2010). A recent study demonstrated that ERMS and pleomorphic RMS likely share a same continuum of disease regarding mutational profile (Rubin et al. 2011).

Because of overlapping morphologic features, particularly with ERMS and solid pattern ARMS, molecular analysis is often used as a complementary diagnostic tool (Kohashi et al. 2008; Asmar et al. 1994; Morotti et al. 2006). ERMS is the most common RMS subtype in adults and is characterized by a loss of heterozygosity in 11p15.5 (Scrabble et al. 1989). In contrast, most ARMS are characterized by chromosomal translocations $t(2;13)(q35;q14)$, resulting in the PAX3–FOXO1 fusion protein, or chromosomes 1 and 13, $t(1;13)(p36;q14)$, resulting in the PAX7–FOXO1 fusion (Sorensen et al. 2002; Davis et al. 1994). Furthermore, pediatric patients with fusion-positive (involving either PAX3 or PAX7)

ARMS have shorter overall survival than those with fusion-negative ARMS. Lastly, in metastatic pediatric patients, a translocation involving PAX3 is associated with shorter overall survival than a translocation involving PAX7 (4-year overall survival: 75% for PAX7–FOXO1 vs. 8% for PAX3–FOXO1; $P = 0.0015$) (Sorensen et al. 2002). Interestingly, according to the European Pediatric Soft Tissue Sarcoma Study Group (EpSSG), the patient outcomes and gene expression signatures of fusion-negative ARMSs are very similar to those of ERMSs (Williamson et al. 2010). Because the IRSG recommendations indicate that the pathology subtype may influence the aggressiveness of the treatment choice according to risk stratification guidelines (Raney et al. 2001), one of which is the PAX3/7–FOXO1 translocation in ARMSs, the translocation status is commonly assessed for pediatric patients with RMS (Barr et al. 2006).

The diagnosis and treatment strategy for Adolescent and Young Adult (AYA) as well as adult patients with RMS is challenging (Bleyer 2005; Van Gaal et al. 2011) and often emulated from the childhood RMS guidelines (Miettinen 1988). Indeed, only 27% of patients in IRSG III and IV studies were 10 years old or older. Attempts to justify this extrapolation frequently cite the unfortunate lack of specific studies

Fig. 1 Representative FISH analysis of the PAX3/7–FOXO1 fusion **a** FISH analysis with the FOXO1 break-apart probe set demonstrates split red and green signals indicative of a FOXO1 rearrangement. **b** The fused red/green signals are consistent with no FOXO1 rearrangement. **c** FISH analysis using PAX3–FOXO1 dual spanning probe set shows fused red/green signals indicative of a PAX3–FOXO1 fusion. **d** FISH analysis with a PAX7 break-apart probe showing split red and green signals and amplification (common with PAX7 rearrangements)



for AYA and adults with RMS (Sultan et al. 2009). This leads to several concerns regarding the appropriate management for this patient population. First, there are no data to support the use of the same diagnostic, prognostic, and treatment approaches for these two distinct age-related subgroups, with what may also be distinct biological and clinical disease entities. Second, *PAX3/7-FOXO1* fusion status has not been shown to be an important clinical or biological factor in adult patients; rather, there are limited data on translocation testing in this population and its clinical significance.

To determine whether *PAX3/7-FOXO1* fusion gene status is associated with outcomes in AYA and adult RMS patients, a large panel of specimens of older patients followed at our institution was characterized by fluorescence in situ hybridization (FISH) and these findings were correlated with specific clinicopathologic parameters.

Methods

Patient samples

One hundred and five formalin-fixed, paraffin-embedded tissue samples from a database of 251 patients with RMS followed at The University of Texas MD Anderson Cancer Center between 1957 and 2001 were available for histological, immunohistochemical, and clinical evaluation. Slides of tumor-tissue samples were stained with hematoxylin and eosin at our institution to confirm the diagnosis. Demographic and clinical data were abstracted from the patient records.

Histopathologic analysis

The formalin-fixed paraffin-embedded (FFPE) tissue samples from patients with RMS were assembled into a TMA. Viable tumor was selected according to morphologic features and formatted as two 0.6 mm-diameter tissue cores into a standard 45 × 20 mm recipient TMA paraffin block using a stainless steel stylet (Beecher Instruments, Silver Spring, MD). Finally, 4- μ m-thick sections were mounted on poly-L lysine-coated slides.

Fluorescent in situ hybridization

To assess the presence of *PAX3*, *PAX7*, and *FOXO1* rearrangements, break-apart probes for *PAX3*, *PAX7*, and *FOXO1* were employed as previously described (Bridge et al. 2000). Briefly, each probe was labeled by nick translation with either Spectrum-green or Spectrum-orange-deoxyuridine triphosphate which allowed the visualization of two colors by FISH (Fig. 1). Fused or split red and green signals indicate, respectively, absence or presence of *PAX* and *FOXO1* rearrangements.

Table 1 Clinical characteristics of patients with RMS

Characteristics	No. of pts (%)
<i>Age</i>	
≤10	16 (15)
>10	89 (85)
<i>Sex</i>	
Female	44 (42)
Male	61 (58)
<i>Histology</i>	
ERMS	62 (59)
ARMS	31 (30)
Pleomorphic	11 (10)
Unclassified	1 (1)
<i>Primary site</i>	
Head and neck	47 (45)
Genitourinary	11 (10)
Extremities	23 (22)
Other	24 (23)
<i>Invasion</i>	
Yes	64 (61)
No	35 (33)
Unknown	6 (6)
<i>Size</i>	
≥5 cm	56 (53)
<5 cm	42 (40)
Unknown	7 (7)
<i>Nodal involvement</i>	
Regional	10 (10)
No	95 (90)
<i>Metastatic</i>	
Yes	31 (30)
No	74 (70)

Statistical analysis

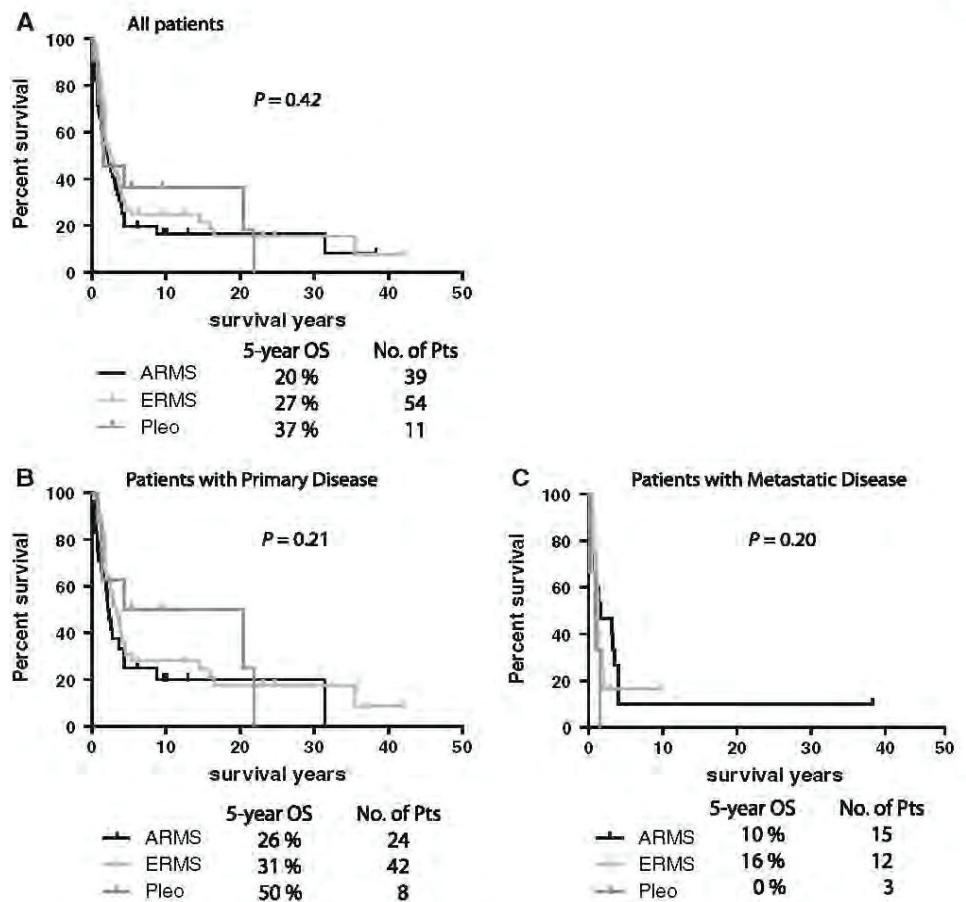
Survival data were retrieved from patient records, and overall survival was measured as the time from diagnosis until death or the date of last contact (censored). The histologic type and specific gene fusion were correlated with patient outcomes using the log-rank test, and comparisons of metastasis frequency and gene fusions were made using the Chi-square test. Prism software was used to generate the Kaplan–Meier curves.

Results

Patients and tumor characteristics

Of the 105 patients included in our study, 85% were older than 10 years at diagnosis, with a median age of 19 years

Fig. 2 Kaplan–Meier overall survival estimates according to histologic classification



(range 0.3–102 years; Table 1). The alveolar subtype was identified in 37% of cases, the embryonal subtype in 52% of cases, and the pleomorphic subtype in 11% of cases. Only one specimen remained unclassified. The majority of tumors were localized, with the head and neck being the most common location. The median overall survival was determined for patients with ARMS (26 months), ERMS (31 months), and pleomorphic RMS (18 months) (Fig. 2a). We analyzed separately patients with primary versus metastatic disease. Although the ERMS histology seemed to have a superior overall survival, we found no statistically significant difference in overall survival among the alveolar, embryonal, and pleomorphic subtypes (Fig. 2b, c).

Identification of *PAX3/7-FOXO1* translocations in formalin-fixed paraffin-embedded tumor specimens by tissue microarray

We next determined whether the *PAX7-FOXO1* or *PAX3-FOXO1* translocations could be detected by FISH in the TMA of samples from patients with RMS. From the two analyzed TMA slides, twenty-one (20%) samples were depleted or presented no tumor on the tissue microarray.

We were able to assess the result of the FISH experiments in 52 (63%) of the 83 adequate samples. The probes did not hybridize to 31 (37%) of the samples. The *PAX3-FOXO1* fusion was found in 26% of these 52 cases, the *PAX7-FOXO1* fusion in 8%, and no fusion in 65% (Table 2). Among the histologically defined ARMS specimens, 18 (67%) had a gene fusion, mostly *PAX3-FOXO1* (52%). No fusion was detected in the pleomorphic subtype.

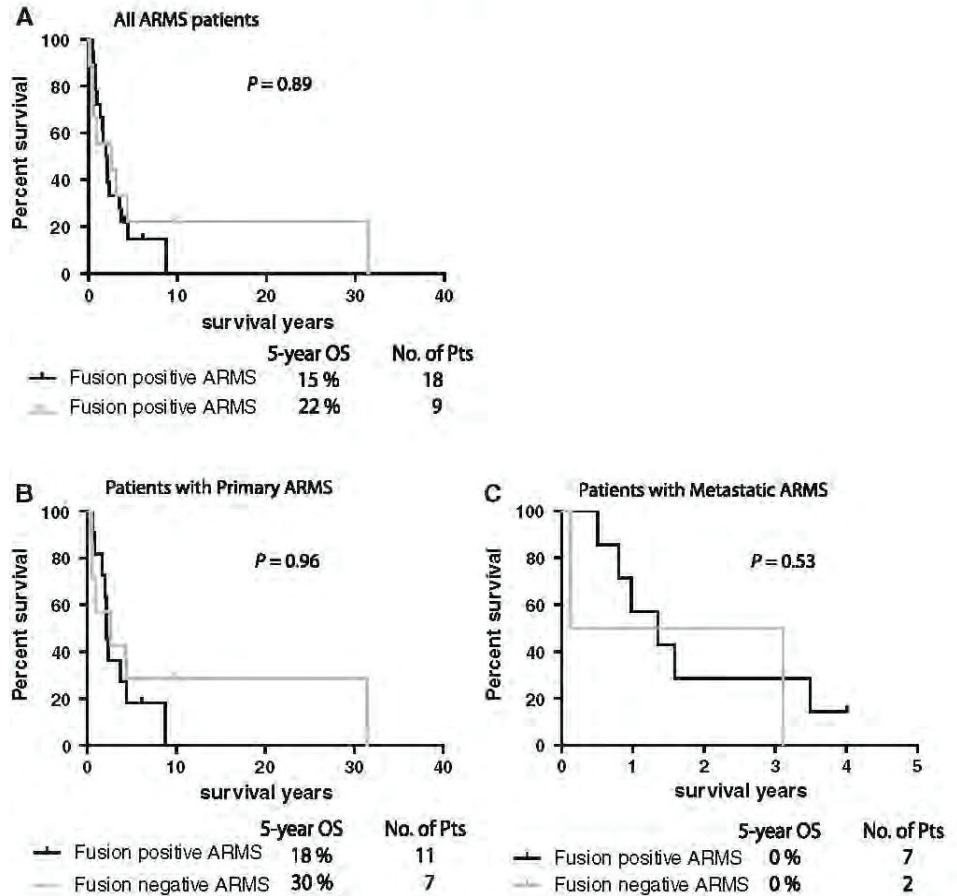
Outcomes of patients with fusion-positive RMS

We compared the group of patients with *PAX3/7-FOXO1* fusion-positive RMS to those with fusion-negative RMS. We found that the patients with fusion-positive RMS showed a trend toward worse survival than those patients whose tumors were fusion-negative, although this trend was not statistically significant, even in disease extension subgroups (Fig. 3). When we compared the overall survival of patients with RMS by specific translocation, *PAX3-FOXO1* versus *PAX7-FOXO1*, we found that the type of fusion was not correlated with survival, even when stratified by presence or absence of metastases, but the number of *PAX7-FOXO1* cases was low (Fig. 4).

Table 2 FISH results by histologic subtype

Histology diagnosis	Patient number	Number of FISH analyzed tumor samples	Fusion-negative n (%)	<i>PAX3-FOXO1</i> n (%)	<i>PAX7-FOXO1</i> n (%)
ARMS	39	27	9 (33%)	14 (52%)	4 (15%)
ERMS	54	20	20 (100%)	0 (0%)	0 (0%)
Pleomorphic	11	5	5 (100%)	0 (0%)	0 (0%)
Total	104	52	34	14	4

Fig. 3 Kaplan–Meier overall survival estimates for ARMS patients according to *PAX3/7-FOXO1* fusion status



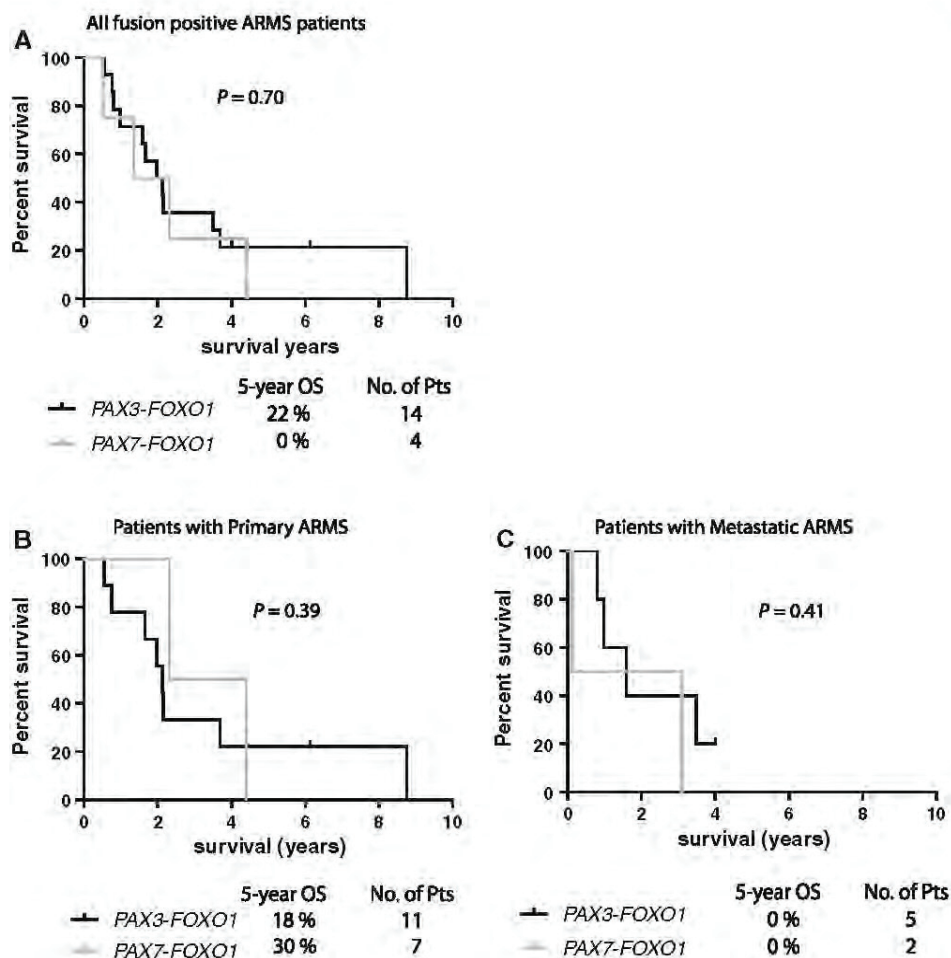
Patients with ERMS and fusion-negative ARMS have similar outcomes

To determine how fusion-positive ARMSs behave clinically, we compared the overall survival of patients with ERMS, fusion-positive ARMS, and fusion-negative ARMS tumors. We found no significant differences in overall survival between these 3 groups (Fig. 5). However, there was a trend toward shorter survival for those patients with a translocation, but this trend did not reach statistical significance ($P = 0.15$), possibly due to the small numbers. The fusion-negative ARMS and the ERMS groups showed similar outcomes, with several long-term survivors (Fig. 5a, b).

Metastatic disease frequency in fusion-positive patients

To evaluate the effect of *PAX3/7-FOXO1* translocations on risk of metastasis, we calculated the percentage of patients with metastatic disease at diagnosis for patients with fusion-positive, fusion-negative ARMS and ERMS. There was a significant increase in rate of metastatic disease for patients with fusion-positive ARMS (39%), compared with those whose tumor did not have a translocation ($P = 0.0081$, $\chi^2 = 9.6$; 2 degrees of freedom). ERMS and fusion-negative ARMS appeared similar in matter of metastatic disease frequency (22%; Fig. 6).

Fig. 4 Kaplan–Meier overall survival estimates for ARMS patients with *PAX3-FOXO1* or *PAX7-FOXO1* fusion



Discussion

RMS is a rare entity with limited data regarding molecular classification and prognosis for patients over 10 years old (Wolden and Alektiar 2010). Due to the sustained work of the IRSG within the past decade, treatment of childhood RMS is clearly defined according to risk stratification. Both histology subtype and fusion status are important criteria for classifying patients. IRSG guidelines recommend that RMS patients be followed at a specialized center and that the pathology should include the determination of the *PAX3/7-FOXO1* fusion status (Raney et al. 2001; Maurer et al. 1988; Maurer et al. 1993; Crist et al. 1995; Crist et al. 2001).

The present work studied the relevance of the fusion assessment in older patient population including a majority of AYA and adult RMS patients. We found that the presence of *PAX3/7-FOXO1* translocation was significantly associated with a higher frequency of metastatic disease. Additionally, we found that patients with fusion-negative ARMS trended toward better outcomes than those with fusion-positive ARMS. Hence, we found that patients with

fusion-negative ARMS tended to have overall survival times that were similar to those of patients with ERMS, which is in accordance with the recent EpSSG conclusions relative to childhood RMS that fusion-negative ERMS is clinically similar to fusion-negative ARMS (Williamson et al. 2010). The type of translocation *PAX3-FOXO1* or *PAX7-FOXO1* did not show any prognostic significance, even in the metastatic disease group.

Due to the rarity of RMS, we had to evaluate patients who had been followed from 1957 to 2001 and only 105 patients fit our criteria. The small number of patients clearly impacted the statistical significance of our analyses. Moreover, the age of the tissue samples appeared to affect the FISH technique resulting, which explains why we were unable to analyze close to one-third of the specimens (Chang et al. 2009). Ideally, less than 2–5 years after fixation in formalin and paraffin embedding is the threshold for a successful FISH analysis; in routine clinical practice, most will be tested within days to weeks of fixation. The majority of our samples were older than this suggested guideline. Performing FISH on a TMA is also technically more demanding than using whole sections from a single

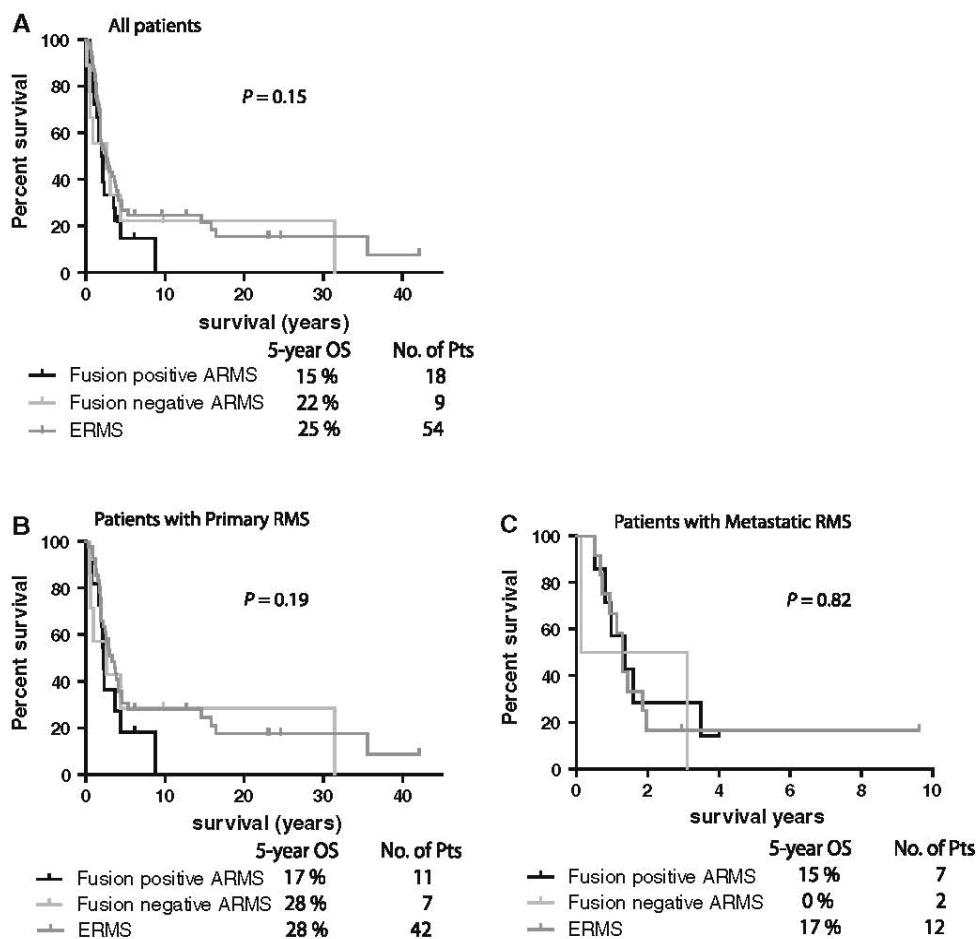


Fig. 5 Kaplan–Meier overall survival estimates according to ARMS PAX3/7–FOXO1 fusion status and ERMS

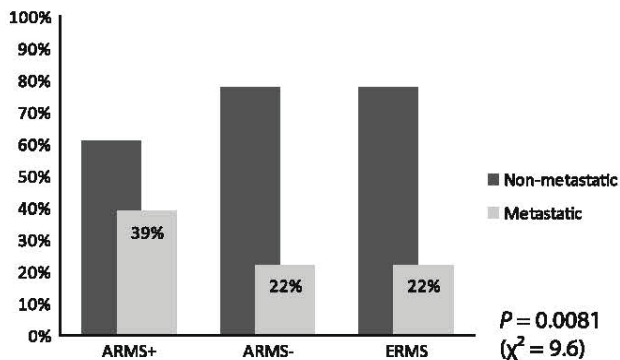


Fig. 6 Frequency of patients with metastatic disease. More patients with fusion-positive ARMS had metastatic disease than those with fusion-negative ARMS or ERMS ($P = 0.0081$)

case. Regarding a failure rate lower than 30% in these unfavorable specimen conditions, FISH analysis is a good option for identifying the PAX3/7–FOXO1 fusion in FFPE.

Our study emphasizes both the difficulties with retrospective molecular studies of this rare disease and the need

of specific studies regarding the relevance of PAX3/7–FOXO1 fusion assessment in AYA and adult RMS as a prognostic factor.

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Conflict of interest We declare that we have no conflict of interest.

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IV-3 Targeted polytherapy in small cell sarcoma and its association with doxorubicin

While a change of paradigm occurred in the last decade from chemotherapy to targeted therapy for cancer treatment, this work investigates the optimal combination of targeted agents with doxorubicin, sarcoma standard of care. This work aims to study targeted therapy combinations to offer a preclinical background to clinical trials. We sought to identify most active bi and tritherapy combinations of targeted agents and how to timely orchestrate them together and with standard chemotherapy doxorubicin in small cell sarcoma cell lines.

Three sarcoma cell lines were studied RD18 (RMS), A204 (undifferentiated RMS) and TC 71 (Ewing's sarcoma). Each cell line was exposed to increasing concentrations of vorinostat (HDAC inhibitor), 17-DMAG (Hsp90 inhibitor), abacavir (anti-telomerase) and sorafenib (tyrosine kinase inhibitor) alone, combined 2 by 2, then with doxorubicin. Viability was assessed by MTS assay. The Chou and Talalay combination index (CI) was used to determine additive (CI=1), synergistic (CI<1) or antagonistic effect (CI>1). Cell cycle analysis, measure of apoptosis by Annexin V and caspase 3/7 activity were studied using flow cytometry analysis and luminescent assays.

In monotherapy, the agents showed 30% to 90% decrease in viability but abacavir, which remained less active. Combination therapies with vorinostat, sorafenib and 17-DMAG showed strong synergism. Abacavir was found antagonistic with each drug. Either vorinostat or 17-DMAG synergized with doxorubicin, achieving 60% cell killing compared to doxorubicin alone 12%. However, no synergy was observed for sorafenib with doxorubicin. The triple therapy vorinostat, 17 DMAG and Doxorubicin did not show synergism but

transiently increased the subG1 population at 24H, 70% compared to 30% in monotherapy with an increase in early caspase-independent apoptosis.

This work provides preclinical evidence of synergism of dual targeted therapy combinations. Indeed, a synergism is achieved with biotherapies including vorinostat, 17 DMAG and sorafenib in small cell sarcoma cell lines. Targeted tritherapy not including doxorubicin does not achieve synergism and suggests more side effects for a modest cell viability reduction. In adjunction to doxorubicin, the targeted bitherapy combinations enhance doxorubicin cytotoxicity at therapeutically relevant concentrations, allowing to reduce its dose or prolong its administration. The most efficient combination revealed by our screening was doxorubicin associated to the targeted bitherapy vorinostat and 17 DMAG who achieved a synergistic induction of caspase-mediated and -independent apoptosis. According to our data, the most promising combination had also to include the HDAC inhibitor vorinostat. Our results do not support the use of targeted-only polytherapy as the triple combination of agents synergistic 2 by 2 did not achieve synergism.

Material and Method

Cell culture

TC71 human Ewing's sarcoma cells were cultured in RPMI1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine. A204 human undifferentiated RMS cell line was cultured in Mc Coy Media with 10% fetal bovine serum, 2 mmol/L L-glutamine. RD18 human RMS cell line was cultured in modified Dulbecco's Modified Eagle's Medium (DMEM) 12 media,

supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. All cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). They were negative for Mycoplasma, as determined by UT-MD Anderson CCSG Characterized Cell Line Core and were maintained at 37 °C in a humidified incubator, with 5% CO₂. Cell lines are described in Table 1.

Chemicals

Vorinostat or suberoylanilide hydroxamic acid (SAHA) was provided by Merck & Co. Inc., (Whitehouse Station, NJ, USA), doxorubicin HCl was obtained from Ben Venue lab. (Benford, OH) and 17 DMAG was purchased from Invivogen (San Diego, CA). Sorafenib and Abacavir were purchased from the University of Texas—MD Anderson Cancer Center Pharmacy. The drugs were dissolved in dimethyl sulfoxide (DMSO) (Fisher Bioreagents) at 10 mmol/l and filtered through 0.22 micron filters, and aliquots were stored in –20°C, protected from light.

Viability assays

Cells were cultured in 100 mm dishes (Corning Life Sciences, Corning, NY) then seeded in 96-well plates at a concentration of 5 x10 cells per well and incubated for 24h, 48h, 72 h with increasing concentrations of SAHA, 17-DMAG, abacavir and sorafenib, initially as monotherapy, then in combination. Based on those first results, each agent and the succeeding combinations was associated with doxorubicin. At the end of incubation, the reduction in cell viability compared with untreated cells was determined by MTS assay through the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Absorbance at 490

nm reflecting the number of living cells in culture was measured using KC Junior software and microplate reader (Bio-Tek Instruments, Winooski, VT). Relative cell viability (%) was calculated as the mean absorbance of replicate treatment-wells minus the mean absorbance of replicate background wells, divided by the mean absorbance of replicate DMSO-treated wells minus the mean absorbance of replicate background wells, multiplied by 100. Direct cell counting were done by counting nuclei (5–25 μm) using an automated Vi-Cell Analyzer (Beckman Coulter) as described previously or Mozi Z cell counter (Orflo)

Cell cycles, apoptosis and caspase 3/7 activity assay

Cell cycle assay was performed by propidium iodide (PI) staining (Roche, Indianapolis, IN, USA) and analysed by a FACS Canto II flow cytometer and FACS Diva 6.1 software (BD Biosciences, San Jose, CA) as previously described¹⁸⁶ Cells was stained by alexa fluor 488 annexin V and PI (Invitogen, Carlsbad, CA USA) to assess apoptosis by flow cytometry and caspase 3 and 7 activities were measured using the Apo-One homogeneous Caspase-3/7 kit (Promega Inc., Madison, WI, USA) according to manufacturer's instructions.

Synergy analysis

The combination indices (CI) method of Chou and Talalay was used to determine whether the combinations were synergistic (<1), additive ($=1$), or antagonistic (>1). Briefly, CIs were determined by isobologram analysis of synergy (Chou-Talalay method). Representative normalized isobolograms and Fraction affected (Fa)-CI plots, graphically depict the growth-inhibitory interactions between the 2 drugs tested. Fa was measured cell viability and apoptosis assays, and CIs were generated using CalcuSyn software (Biosoft, Cambridge,

UK). The combinations were considered synergistic if the CI was inferior to 1, additive for a CI equal to 1, or antagonistic for a CI superior to 1.

Chou and Talalay formula and qualitative correlation of the CI value

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_1(D)_2}{(D)_1(D)_2}$$

<0.1	very strong synergism
0.1–0.3	strong synergism
0.3–0.7	synergism
0.7–0.9	moderate to slight synergism
0.9–1.1	nearly additive
1.1–1.45	slight to moderate antagonism
1.45–3.3	antagonism
>3.3	strong to very strong antagonism

Results

Bi targeted therapies with vorinostat, sorafenib and 17-DMAG result in a synergistic decrease in viability in small cell sarcoma cell lines.

We first determined the IC₅₀ of each targeted therapy drug as a single agent in each cell line. The abacavir IC₅₀ was very high and not even reached in RD18 cell line. We based our combination data on the IC₂₅ of each drug and then determined an IC₅₀ of each targeted agent. We estimated the CI using Chou and Talalay method as reported in Table 9. Abacavir was found antagonistic with each drug. However, vorinostat, sorafenib and 17-DMAG had a synergistic activity, which was consistent across the different cell lines except in RD18 for the 17 DMAG plus vorinostat combination. Vorinostat plus 17 DMAG achieved the best synergy. Abacavir was not tested further.

Table 9. IC50 of monotherapy and combinations and their CI - additive (CI=1), synergistic (CI<1) or antagonistic effect (CI>1).

	RD 18		A204		TC71	
Monotherapy	IC50 (uM)					
sorafenib	5		5		5	
17-DMAG	10		10		3	
abacavir	Not reached		750		375	
vorinostat	3		1		2	
Combination	IC50 (uM)	CI	IC50 (uM)	CI	IC50 (uM)	CI
sorafenib	2.5	0.8	2.5	0.7	2.5	0.5
17-DMAG	5		10		5	
sorafenib	2.5	1.1	5	1.4	2.5	1.1
abacavir	375		188		188	
sorafenib	5	0.7	5	0.9	2.5	0.8
vorinostat	1.5		3		3	
17-DMAG	10	1.1	5	1.2	5	1
abacavir	188		750		188	
17-DMAG	1.5	3	1.5	0.7	1.5	0.6
vorinostat	0.75		3		0.75	
abacavir	750	1.9	750	3.7	188	1.4
vorinostat	3		3		3	

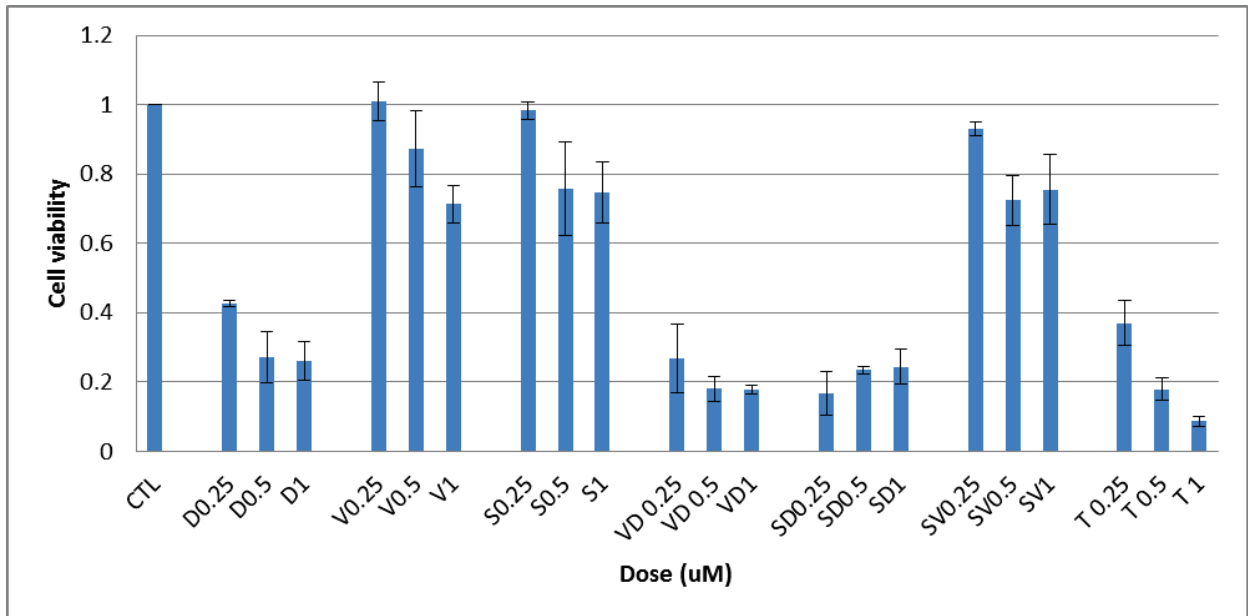
Targeted tritherapy does not reach synergism compared to a bitherapy

Drugs that were synergistic 2 by 2 were combined together. Cells were exposed to vorinostat, 17 DMAG and sorafenib at the doses of 0.25, 0.5 and 1 uM for homogeneity. Cell viability revealed by MTS assay showed that the lowest viability was achieved by the triple therapy (Figure 7A and B). However, the action of the 3 drugs together was either additive or antagonistic compared to targeted bitherapies in all 3 cell lines (mean of CIs = 1.2).

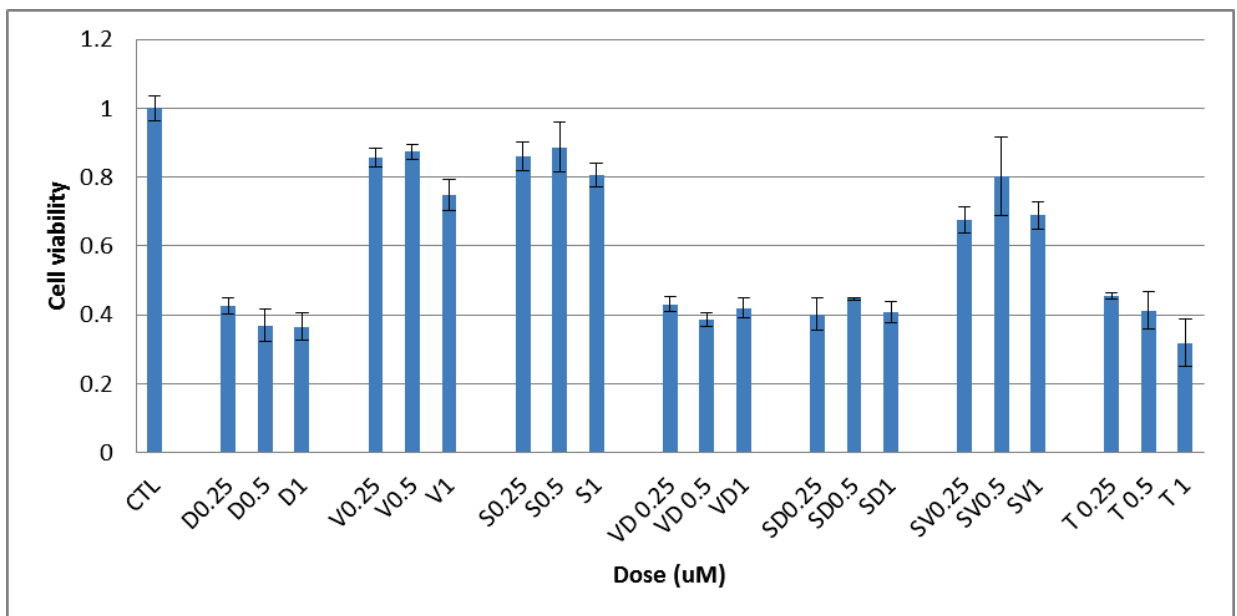
Figure 7. Cell viability of cell lines with triple therapy (A) TC71 and (B) RD18.

S=sorafenib, D=17 DMAG, V=vorinostat

A



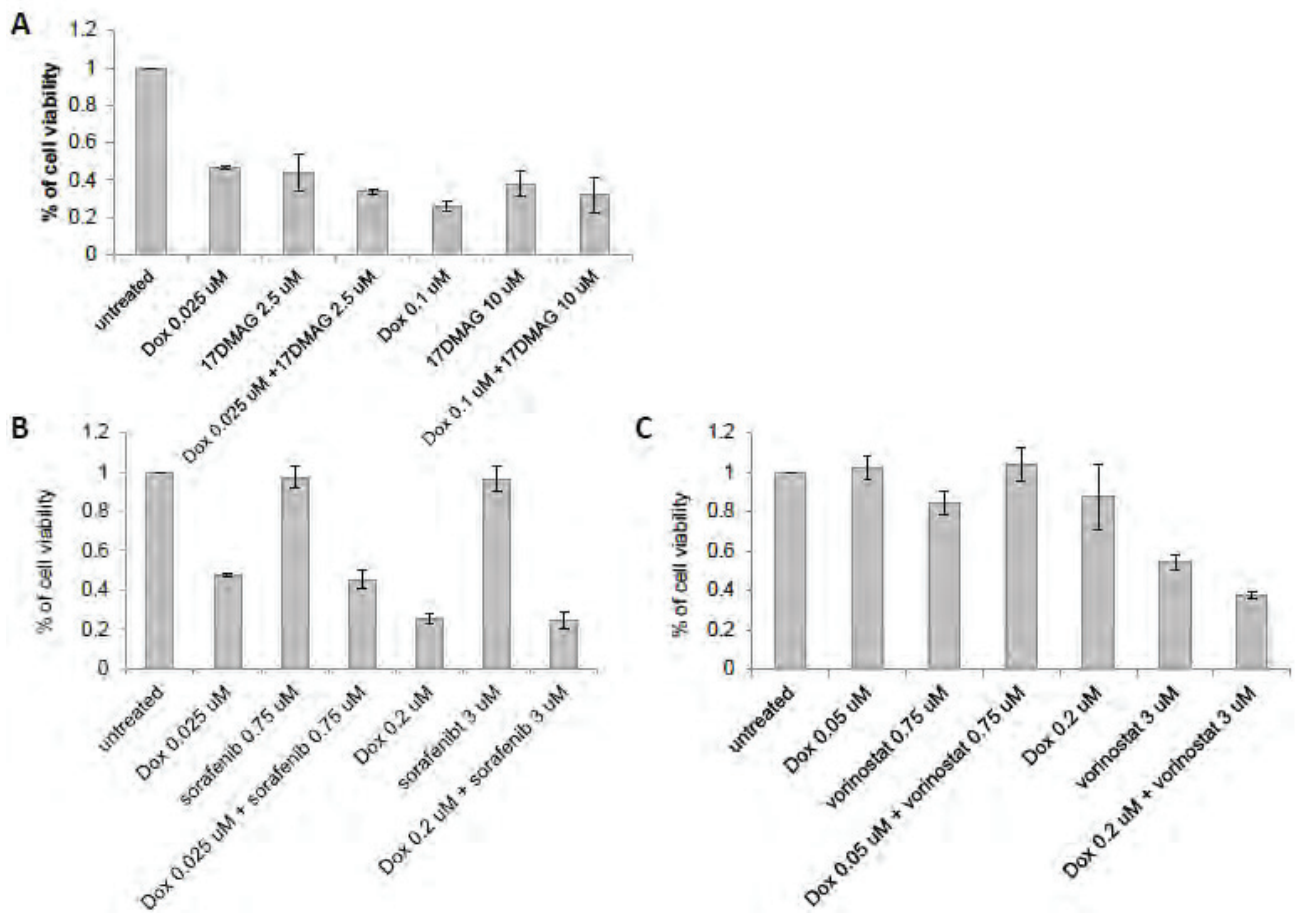
B



Doxorubicin plus a targeted bitherapy

In order to identify an optimal combination, we first tested doxorubicin with each 3 targeted agent: vorinostat, 17 DMAG and sorafenib. (Fig 8). We obtained a reduction of cell viability by the combination of doxorubicin that was moderate for 17 DMAG (Fig. 8A), with a mild synergism (CI: 0.8), almost none observed with sorafenib (Fig. 8B) and more substantial with vorinostat (Fig. 8C) with a synergistic CI of 0.7. We thus tested the combination therapy consisting of doxorubicin with either vorinostat plus 17 DMAG (Fig 9A) or sorafenib plus 17 DMAG (Fig 9B). A modest reduction in cell viability was observed with the combination of doxorubicin plus a bitargeted therapy vorinostat plus 17 DMAG while no effect was observed for doxorubicin added to sorafenib plus 17 DMAG (Fig 9 A and B). Both CIs were above 1 for these triple combinations.

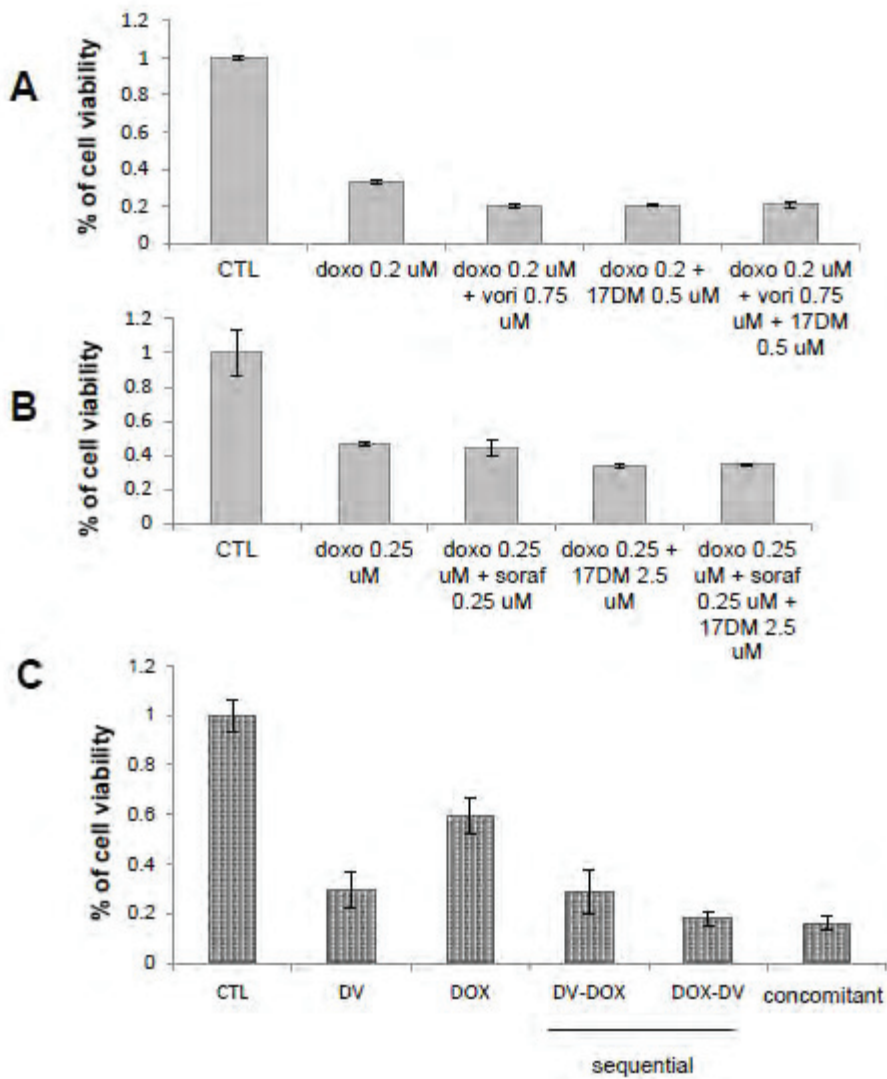
Figure 8. Cell viability of doxorubicin plus either (A) 17 DMAG (RD18), (B) sorafenib (RD18) or (C) vorinostat (TC71)



Timing of combination

To try to understand the mechanism of action and the interactions between targeted therapies and standard chemotherapy agents like doxorubicin, we studied further the most successful triple combination consisting of doxorubicin, vorinostat and 17 DMAG. Using different treatment sequences, we tested cell viability to assess the optimal timing to use each therapeutic class. Our results show first that to treat first cells with the bitargeted therapy seems more active than a sequence starting by chemotherapy. Furthermore, a sequencing treatment was not superior to a concomitant one. (Fig 9C)

Figure 9. Cell viability of doxorubicin plus 17 DMAG and (A) vorinostat (TC71) or (B) sorafenib (RD18) and (C) the sequential or concomitant administration of doxorubicin plus vorinostat-17 DMAG (RD18).



Apoptosis of Doxorubicin plus vorinostat-17 DMAG bitargeted therapy

The triple therapy vorinostat, 17 DMAG and doxorubicin did not show synergism (CI:1.2) but transiently increased the subG1 population at 24H, 70% compared to about 30% in monotherapy (Fig 10 A and B). Mechanism of action was further investigated and showed an increase in caspase 3 dependent apoptosis with the combination (Fig 11) and an early caspase-independent apoptosis (11% to 36%). (Fig 12)

Figure 10. Cell cycle distribution of TC71 cells treated with vorinostat, doxorubicin and 17 DMAG as (A) monotherapy and (B) in combination.

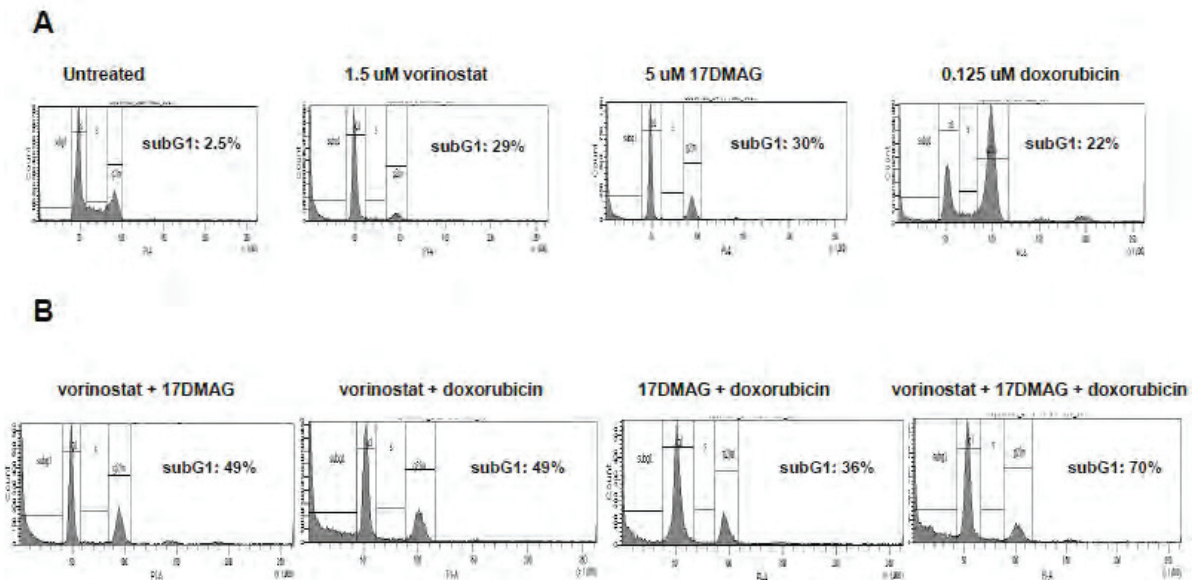


Figure 11. Measure of apoptosis by Caspase 3/7 activity of doxorubicin, vorinostat and 17 DMAG combination in TC71 cell line

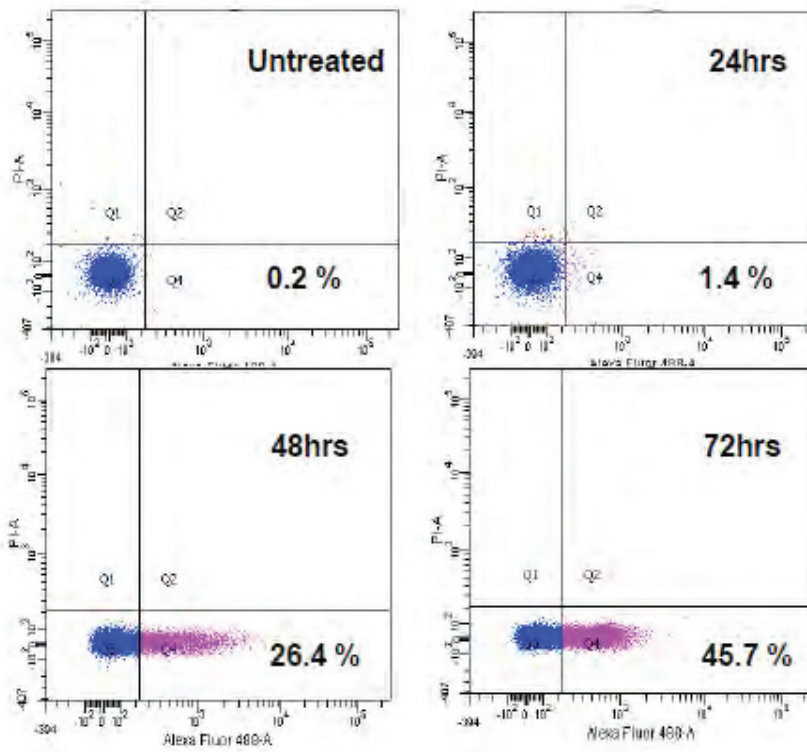
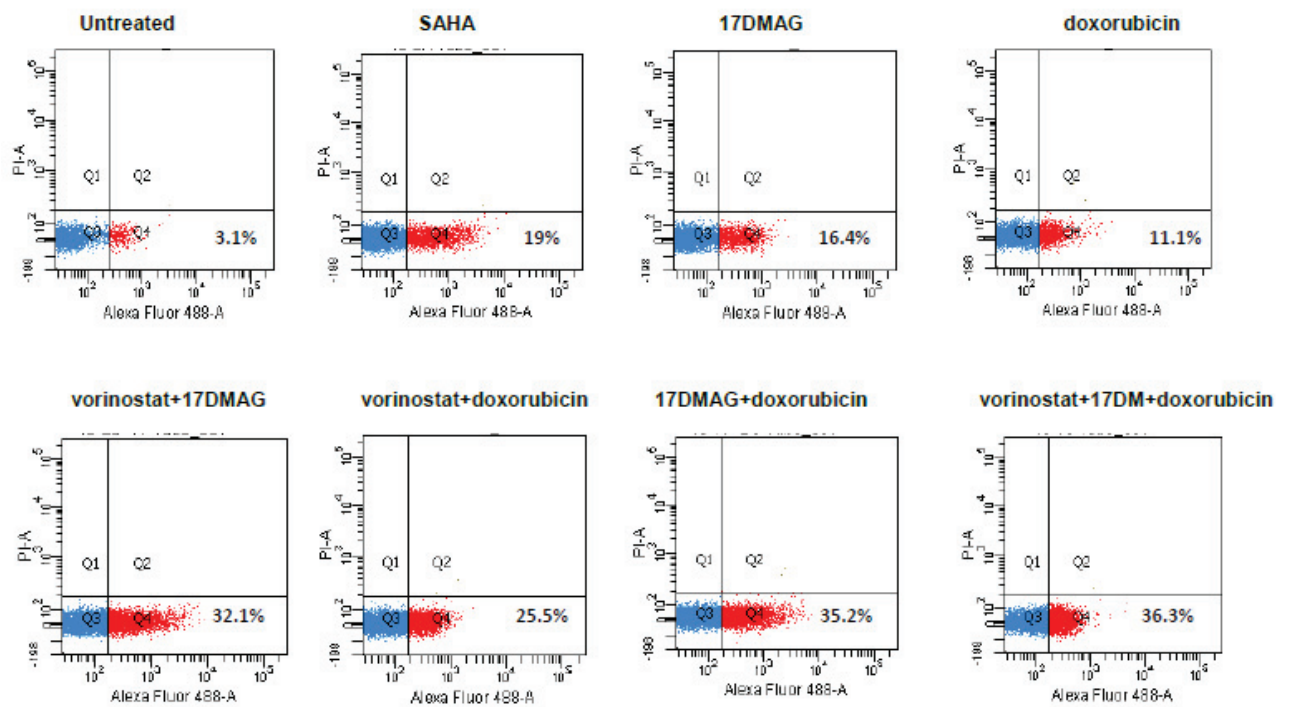


Figure 12. Apoptosis measured with Alexa Fluor 488 Annexin V and PI staining of doxorubicin, vorinostat and 17 DMAG combination in TC71 cell line



V- DISCUSSION

V-1 RMS, the need to find new drugs

RMS is a poor prognosis disease in adult patients. Due to the sustained work of the IRSG within the past decade, treatment of childhood RMS is clearly defined according to risk stratification. A report based on SEER data showed that the outcome of children and adolescent younger than 15 of age with RMS appeared to have improved between 1975 and 1982, reflecting the impact of management improvement but had been stable since.¹⁸⁷

Our study revealed no improvement of outcome over the past 50 years for the adult patient population we studied. Patients with metastatic RMS were found to have a 18% survival rate at 5 years from diagnosis with an 12% survival past 15 years. This outcome was even poorer for patients over 50 of age, even with localized disease (5-year OS of 13%). No clinical or natural history of the disease can explain these age-differences in outcome as the clinicopathologic pattern of adult and pediatric RMS seems similar according to our data. The major observation on management differences is that younger patients are treated more aggressively and are more likely to receive multidisciplinary therapy than their older counterparts. It is clear from this study that there is a need to improve the management of older patients with RMS. We should ensure that these patients benefit as much from research progress and clinical management as younger patients with RMS.

Both histology subtype and fusion status are important criteria for classifying patients. IRSG guidelines recommend that RMS patients be followed at a specialized center and that the pathology should include the determination of the *PAX3/7-FOXO1* fusion status.

Targeted therapies represent a new paradigm with regards to drug development and how to orchestrate them together and to optimally use them with standard chemotherapy. Preclinical studies such as ours offer some background to test targeted bitherapy in association with doxorubicin in small cell sarcoma. While standard treatment of small cell sarcoma still involves chemotherapy agents, targeted therapies represent a new field to explore to enrich the therapeutic palette of these poor prognosis diseases. This work provides preclinical evidence of synergism of dual targeted therapy combinations. Indeed, a synergism is achieved with biotherapies including vorinostat, 17 DMAG and sorafenib in small cell sarcoma cell lines. Targeted tritherapy not including doxorubicin does not achieve synergism and suggests more side effects for a modest cell viability reduction. In adjunction to doxorubicin, the targeted bitherapy combinations enhance doxorubicin cytotoxicity at therapeutically relevant concentrations, allowing to reduce its dose or to prolong its administration. The most efficient combination revealed by our screening was doxorubicin associated to the targeted bitherapy vorinostat and 17 DMAG who achieved a synergistic induction of caspase-mediated and -independent apoptosis. According to our data, the most promising combination had also to include the HDAC inhibitor vorinostat. Our results do not support the use of targeted-only polytherapy as the triple combination of agents synergistic 2 by 2 did not achieve synergism. This conclusion echoes with the recent failure of early phase trials of targeted therapies as single agent. Phase III trials are now investigating adjunction of one targeted therapy to standard chemotherapy.⁹⁶

V-2 Limits of this work

Sarcoma biggest issues are its heterogeneity and its rarity. We used RMS as model to elaborate rationale for a targeted polytherapy. As an even rarer tumor, it might not be the best model to represent sarcomas. However, its link to pediatric cancers is interesting. Indeed, pediatric scientific societies have always been facing the issue of rare diseases and their collaborative effort is a model to be adapted in adult patients, such as the much higher inclusion of patients in clinical trials and the systematic referral to expert center.

However, the small number of RMS patients raised some concerns regarding our data.

First, we had to evaluate patients who had been diagnosed from more than 60 years ago. The small number of patients clearly impacted the statistical significance of our analysis and the age of the tissue samples appeared to have affected the FISH technique quality.

Moreover, *in vitro* studies present inherent limitations. Indeed, the cell lines may not represent the real spectrum of small cell sarcoma, exemplified by their p53 status. But cell line model allows a tremendous amount of experimentations in a timely manner which is not the case with mice experimentation. Due to the many disappointing early phase clinical trials, we cannot spare such a model to increase our knowledge to push further combination of targeted agents in clinic.

A screening has inherent bias. Indeed, it is a binary answer that is expected: yes or no the drug is active, whereas the threshold to consider a drug active is yet to be defined. It is

currently discussed in phase II clinical trials like the growth modulation index¹⁸⁸, but this question is equally relevant in preclinical works.¹⁸⁹

Current methodology of clinical trials follows the same binary course from Phase I to III. Evidence based medicine is nowadays the best way to insure quality and good practice but it may not be the best creative environment for basic or translational research. Indeed, science asks Nature to answer by yes or no to our questions and may prevent some new paths, new drugs or other options to be discovered.¹⁹⁰ As opposed to this vision, we chose an empirical approach to the translational part of this work rather than a screening of sarcoma active proven agents. A drug such as abacavir, regarding its mechanism of action as anti-telomerase would have made a good candidate for early phase clinical trial but was proved not to have effect in monotherapy. However, an activity might have been seen in combination as no decrease in viability may not absolutely mean no synergism with other agents.¹⁹¹ Therefore abacavir was also tested in combination but failed to potentiate its partner agents which led us to abandon the drug for further testing and not to retain it for clinical trial in small cell sarcoma.

V-3 How to find new drugs

In the new era of personalized medicine, targeted therapies may represent hope for a more accurate and relevant approach but a better understanding of the biologic mechanism of the tumor itself is also needed. Many sarcoma have a specific chromosomal translocation such as small cell sarcoma: PAX3 or PAX7-FOXO1 in alveolar RMS⁴⁵, loss of heterozygosity in

chromosome 11⁴⁸, FLT1-EWS1 in Ewing's sarcoma.^{48, 192-194} These translocations or their downstream signaling pathways might be targetable in the future.

The key tumor suppressor gene p53 might also be of interest in tumor response to targeted agents. The majority of Ewing sarcomas are wild type for p53^{195, 196}, and studies have demonstrated that the chimeric protein EWS-FLI1 silences p53 activity^{197, 198}. Recent data suggest that the molecular mechanism about the abrogation of p53 by EWS-FLI1 involves a decrease of acetylation of p53 and increase of mdm2-mediated p53 degradation¹⁹⁹. In this perspective the use of HDAC inhibitors may re-acetylate p53 and results in an increase of p53 stability and activity. In our study, TC71 cell line is mutated for *P53* but was sensitive to vorinostat and was the cell line that reached the best viability reductions in combination therapies. In contrast with Ewing's sarcoma, *P53* is mutated in the majority of RMS²⁰⁰. *In vitro*, the wild-type *P53* A204 cells was sensitive to different agents in monotherapy but the combinations failed to achieve synergism compared to other cell lines. Finally, RD18 was mutated for *P53* and was sensitive to both monotherapies and combinations. The concept of tumor heterogeneity discussed in numerous recent articles stress the need of combination of therapies.^{153, 201, 202} Indeed, failure of targeted therapy in clinical trials may be due to the classic monotherapy scheme used to develop classic drugs. The phase I process has the objective to reach the maximal tolerated dose (MTD) and might not be appropriate for targeted therapy. So far, no new design has been developed such as whether the drug reaches its target or whether the pathway is inhibited, even if it is often reported as secondary objective. Moreover, combining targeted agents is challenging in clinic due to a high toxicity often observed with biotherapies in phase I trials^{131, 177}. However, the idea was to find the lowest dosage of each agent to observe an activity. In a phase I trial design, the goal would

be to get the minimal targeting dose, the minimal dose allowing the targets of the agents to be inhibited instead of the MTD. Furthermore, the introduction of a second or third targeted agent might overcome resistance and therapeutic failure due to clones emerged by Darwinian selection but the whole drug development should be remodelled to adapt to targeted polytherapy clinical investigation.²⁰²

Finally, progress, according to history of science has often been due to revolutions, random finding that completely change a paradigm rather than a step-to-step progression of knowledge.²⁰³ The image of trench war versus a battle that would make a big difference has also been evoked in oncology clinical research.²⁰⁴ The empiric approach presents the advantage to consider other pathways and therapeutic classes and opens to creativity rather than expect an answer to what is thought to be the next step.

VI PERSPECTIVES

As a result of this work, we can speculate that age plays a role on a genetic or molecular level on RMS natural history. Indeed, age has been identified in other studies as a continuous variable predicting of poor survival in multivariate analysis.²⁰⁵ One hypothesis is genomic instability that increases with age can trigger adverse prognosis such as metastatic spreading, as it had been demonstrated in synovial sarcoma.¹⁵⁷ The same genetic instability can lead to more frequent genetic abnormalities such as translocations. Therefore, a future research work that might be conducted is the investigation of other translocations, particularly on *PAX3/7-FOXO1* fusion-negative RMS at a DNA level through comparative genomic hybridization CHG array and via transcriptome study using RNA-sequencing. This work may lead to discover new translocations with potential prognostic implication.

Moreover, based on the targeted polytherapy screening, a phase I trial using a 3+3 design can be conducted using vorinostat plus 17DMAG to assess its toxicity profile. A window phase II study might be designed with the same targeted bitherapy before standard VAC and would investigate the concept *in vivo* of drug sequencing.

Targeted therapies offer a new paradigm to laboratory, translational and clinical research in oncology. In sarcoma, imatinib was the first TKI used to effectively targeted a molecular abnormality (activating mutation of KIT) in GIST patients.²⁰⁶ As single agent, it delays relapse or progression of GIST, but is incapable of achieving cure.^{207, 208} The similitude between oncology and infectious diseases is even stronger as HIV is a disease treated but not cured by a combination of targeted antiretroviral agents.²⁰⁹ In cancer, we can speculate that

polytargeted therapies will be used in the future to treat patients but adjunction of chemotherapy or other class of drug is very likely to be needed to obtain definitive cure.

It is anticipated that a complete change of the tumor nomenclature will occur with the more and more growing number of biomarkers and corresponding target drugs. Indeed, a biomarker like ALK in RMS and non-small cell lung cancer (EMLA4-ALK) will lead to use the same drug, crizotinib, no matter the histology, the organ of the disease. Her2 amplification in breast and gastric cancer is another example. Pilot programs have been launched called AcSé for securised access to therapeutic innovation and offers molecular testing and opening of phase II trials when an histology a biomarker is found. Currently, two drugs are investigated crizotinib and vémurafenib. The era of targeted therapies is likely to break the organ barrier of oncology and may lead to disregard the tissue of origin, at least partly.

Since 2004, cancer is the first mortality cause in front of cardiovascular diseases. Oncology is a relatively new specialty that started its development late compared to other disciplines. In fact, no response to treatment could be assessed before the CT-scan revolution in 1970s'. Compared to haemopathies, were the number of blasts could be given from a blood sample. The decreased size of the tumor traduced in RECIST or Choi criteria is the only sign of tumor response to therapy. For profound tumors, only CT-scan and now MRI would accurately predict the patient benefit to stay on the current therapy. This delayed development of the oncology translates today by the 1st rank as mortality cause like

infectious diseases before penicillin discovery at the beginning of the last century. We could hope for the same tremendous improvement over the 21st century for oncology that infectiology had experienced over the past century. However, this implies sustained effort for research and investment of drug companies. As an oncologist, to apply up-to-date guidelines remains to use current therapeutic weapons that are far from being always curative. In this matter, pediatric cancer care is a model of activism relative to clinical research that we as oncologists for adult patients may follow. During the medical oncology training, no internship in a laboratory is mandatory and scientific cursus is only offered in parallel to the residency. Research is only an option for an oncologist while it is ethically required in our specialty still lacking knowledge and optimistic results for patients.

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APPENDIX

RESUME SUBSTANTIEL EN FRANCAIS

Introduction

Le rhabdomyosarcome de l'adulte est une tumeur rare dont le pronostic est plus défavorable que celui des patients pédiatriques. Cette différence peut être due à la nature même de la maladie, définissant ainsi deux entités distinctes, répondant à des caractéristiques cliniques, moléculaires propres. Cependant, des différences de prise en charge peuvent également être en cause et ouvrent des pistes d'amélioration des pratiques. Afin d'élucider ce point, le présent travail propose d'étudier les caractéristiques cliniques et moléculaires ainsi que les différences de prise en charge des adolescents et adultes atteints de rhabdomyosarcome. A l'image des sarcomes des tissus mous, les rhabdomyosarcomes présentent, de part leur rareté et leur hétérogénéité, un défi en matière de recherche clinique et préclinique. En effet, les essais cliniques se focalisant sur un sous-type particulier souffrent d'un manque de puissance du fait d'un nombre très restreint de patients tandis qu'en rassemblant différentes histologies afin d'avoir plus de patients, les résultats se trouvent difficilement interprétables du fait d'histoire naturelle et de biologie distinctes. Ainsi, une meilleure compréhension des caractéristiques cliniques et moléculaires de chaque type de sarcome semble cruciale. Cette problématique explique le fait que le sarcome est à ce jour un exemple de collaboration internationale en matière de recherche, de base de données et d'essais cliniques.

Les RMS présentent une hétérogénéité en fonction de l'âge, ce dernier étant inversement proportionnel à la survie. Cependant, peu d'études se penchent sur les spécificités de cette population de patients porteurs de RMS. Du fait du peu de littérature dédiée à ce type de

patients, la prise en charge est une transposition soit de la prise en charge des RMS de l'enfant ou des sarcomes de l'adulte. La question première de ce travail consiste à décrire les caractéristiques cliniques des patients adolescents et adultes porteurs d'un RMS ainsi que leur prise en charge à travers l'étude rétrospective de 239 patients âgés de 10 ans ou plus, suivis au MD Anderson Cancer Center dans l'idée d'identifier les distinctions cliniques liées à l'âge, aussi bien que les différences de prise en charge pouvant expliquer le pronostic défavorable de ces patients comparé aux patients pédiatriques.

Sur le plan moléculaire, certains RMS alvéolaires (ARMS) présentent une translocation entre soit le chromosomes 2 et 13, $t(2;13)(q35;q14)$, *PAX3-FOXO1* ou entre le chromosomes 1 et 13, $t(1;13)(p36;q14)$, résultant en la fusion *PAX7-FOXO1*, mettant, ainsi, en contact deux facteurs de transcription, donnant un produit de fusion fonctionnant comme un super facteur de transcription dont les gènes cibles sont impliqués dans l'oncogénèse. Les patients pédiatriques ayant un ARMS avec fusion positive, que celle-ci concerne *PAX3* ou *PAX7* ont une survie inférieure à ceux ayant un ARMS à fusion négative. De même, chez les patients pédiatriques métastatiques, une fusion impliquant *PAX3* est associée à un moins bon pronostic que ceux porteurs de la translocation impliquant *PAX7* (OS à 4 ans: 75% pour *PAX7-FOXO1* contre 8% pour *PAX3-FOXO1*; $P = 0.0015$).⁴⁵ De plus, selon l'European Pediatric Soft Tissue Sarcoma Study Group (EpSSG), la survie des patients porteurs d'ARMS à fusion négative serait similaire à celle des ERMS.

Du fait de l'implication pronostic du sous-type histologique en matière d'agressivité du traitement, le statut fusionnel est systématiquement réalisé chez les patients pédiatriques. La seconde partie de ce travail étudie l'incidence et la valeur pronostique de la recherche de

PAX3/7-FOXO1 chez l'adolescent et l'adulte en matière de survie à travers l'étude par hybridation *in situ* en fluorescence (FISH)

La prise en charge des rhabdomyosarcomes n'a que peu évolué ces dernières décennies en matière de chimiothérapie, toujours prédominée par la doxorubicine, la vincristine et l'ifosfamide. Or, la chimiothérapie, tout protocole confondu ne parvient à obtenir qu'une survie à 5 ans de moins de 10% chez les sarcomes métastatiques dont les RMS. De nombreux essais de phase II de thérapies ciblées en monothérapies se sont révélés négatifs, exigeant des rationnels précliniques solides avant d'envisager un essai précoce.

Une stratégie possible afin de potentialiser l'utilisation de thérapies ciblées et atteindre un impact clinique est de combiner les agents entre eux. L'association de 2 ou d'une polythérapie ciblée pourrait prévenir les mécanismes de résistance et les rechutes observées en clinique en monothérapie. De plus, les thérapies ciblées seraient susceptibles de potentialiser les agents de chimiothérapie standard, permettant d'en réduire les doses ou de pouvoir en prolonger l'utilisation, à l'image de la cardiotoxicité limitante des anthracyclines. Enfin, est-il possible de transposer le modèle des polythérapies utilisées dans le VIH au cancer en éliminant la chimiothérapie standard de la palette thérapeutique ?

La troisième partie de ce travail a consisté à étudier des combinaisons de thérapies ciblées et leur intégration à la doxorubicine, chimiothérapie de référence dans le sarcome sur lignées cellulaires de sarcomes à petites cellules, rhabdomyosarcomes et sarcome d'Ewing *in vitro*. 4 agents ont été testés, en monothérapie, puis 2 à 2 afin d'identifier une potentielle synergie entre eux. Parmi les agents synergiques, des associations de 3 agents ont été étudiées. Enfin, l'intérêt de l'association à la doxorubicine a été exploré, ainsi que la séquence optimale

d'utilisation. Les agents testés étaient le vorinostat, histone deacetylase inhibitor, le sorafenib, multikinase inhibiteur, le 17-DMAG, inhibiteur de la protéine de choc thermique HSP 90 et l'abacavir, inhibiteur de télomérase.

Le sorafenib est un inhibiteur multikinase qui cible le récepteur 1 du vascular endothelial growth factor (VEGF) et du fibroblast growth factor (FGFR), le Flt-3, c-kit, Raf-1 et les récepteurs alpha and beta du platelet-derived growth factor receptors (PDGFR). Ces voies de signalisation semblent être impliquées dans le RMS. En effet, l'expression du PDGFR alpha et beta est associée à une survie inférieure dans les RMS. De plus, RAF est activée par RAS dont certaines mutations activatrices sont présentes dans 35–50% of ERMS. Enfin, l'expression du FGFR1 a été décrite dans le RMS et associée à des modifications épigénétiques. L'inhibition de ces différentes voies conduit à une activité anti-tumorale, par inhibition de la prolifération cellulaire et de l'angiogenèse. Une action du sorafenib a été démontrée dans les synoviosarcomes *in vitro*. Il a été testé en phase II en monothérapie dans les sarcomes métastatiques ou en rechute. Une activité notable a été observée dans les angiosarcomes mais était minime sur les autres types de sarcomes, ouvrant la voie aux inhibiteurs de multikinases tels que le Pazopanib approuvé en monothérapie dans le STS.

Les inhibiteurs d'HDAC représentent une nouvelle classe thérapeutique en développement. Des modifications d'HDAC sont observées dans de nombreuses tumeurs. En effet, les inhibiteurs d'HDAC induisent la différenciation, un arrêt du cycle cellulaire, l'apoptose et inhibent la croissance tumorale dans les essais précliniques. Parmi les agents les plus avancés dans leur développement, le vorinostat ou Suberoylanilide Hydroxamic Acid exerce une action anti-tumorale en synergie avec la doxorubicine, des inhibiteurs de kinase et les rayonnements ionisants en préclinique. Des résultats prometteurs ont été observés sur des

modèles *in vivo* de prostate et d'hémopathies malignes. Le vorinostat est à son autorisation de mise sur le marché dans le lymphome cutané à cellules T. Des études *in vitro* récentes suggèrent une activité du vorinostat dans les sarcomes à petites cellules, notamment les ostéosarcomes et sarcomes d'Ewing. De plus, chez les souris mutées pour *Ptch* porteuses de RMS, la combinaison du 5-aza-2'deoxyctidine, un agent hypométhylant et du valproate de sodium, une des premiers inhibiteurs d'HDAC découvert permettait d'observer une activité anti-tumorale.¹³⁹ Plusieurs études de phase I ont été réalisées avec des inhibiteurs d'HDAC dont dans des patients pédiatriques porteurs de tumeurs solides réfractaires. Ils ont également été testés en combinaison avec le sorafenib en phase I et le temsirolimus.

L'HSP 90 est un membre de la famille des protéines de choc thermique (HSP) dont l'expression augmente dans la cellule, en lien avec un stress telle qu'une augmentation de température. Les HSP sont fortement exprimées dans les cellules tumorales, du fait de l'hypo-oxygénation ou du stress post-chimiothérapie. De plus, l'HSP 90 joue un rôle pivot dans la survie de la cellule tumorale et la croissance de différents types de cancers. L'inhibition d'HSP90 sur lignées cellulaires de RMS a montré un blocage de la prolifération et de la migration ainsi que l'induction de l'apoptose *in vitro* ; ce qui a été confirmé *in vivo* sur modèle murin. L'antagoniste d'HSP 90, le 17-DMAG est actuellement en essais cliniques et semble prometteur dans les sarcomes.

L'activité télomérase est augmentée dans de nombreux cancers et induit une protection des cellules tumorales du vieillissement et de la mort cellulaire. L'analogue nucléosidique antirétroviral abacavir (Ziagen) présente une puissante activité anti-télomérase dans de nombreux modèles cellulaires et animaux. Dans les tumeurs solides, notamment sur lignées

de cancer de prostate, une réduction de la prolifération et de la sénescence. Il n'a pas été testé sur le sarcome jusqu'à présent.

De nombreuses interactions ont été découvertes entre les différentes voies inhibées par ses 4 agents. En effet, sur les tumeurs stromales gastro-intestinales, l'HSP90 a été identifiée comme étant une cible du vorinostat, qui l'acétyle et induit ainsi l'apoptose. De ce fait, plusieurs essais cliniques précoces sont en cours afin de tester des combinaisons de thérapies ciblées telles que le sorafenib et le vorinostat chez les patients porteurs de tumeurs solides.

Patients et méthode

Patients

Deux cent trente-neuf patients consécutifs âgés de 10 ans et plus, furent diagnostiqués pour un RMS et traités au MD Anderson entre 1957 et 2003. Les données démographiques, les caractéristiques cliniques et les éléments de survie ont été revus rétrospectivement. Les tumeurs ont été classées selon leur degrés d'extension, entre maladie localisée et métastatique. L'envahissement des ganglions lymphatiques locorégionaux a été inclus dans le groupe non-métastatique tandis que les ganglions distaux étaient considérés dans le groupe métastatique. Les sites anatomiques furent d'abord définis selon la classification favorable/défavorable de l'IRS mais furent ensuite élargie devant l'absence de signification pronostique. Les sites de la tumeur primitive ont été classés en tête et cou, génito-urinaire, tronc, intra-abdominal/pelvis et extrémités. La taille de la tumeur correspond à son plus grand diamètre sur le scanner prétraitement et définie 3 groupes >10 cm, 5.01-10 cm and <5cm. L'envahissement ganglionnaire a été évalué par examen clinique, les examens d'imagerie et/ou sur prélèvement chirurgical. L'infiltration tumorale et la présence de métastases ont été définies par imagerie et/ou lors d'un temps chirurgical.

Anatomopathologie

Le diagnostic histologique a été réalisé au moment du diagnostic ou refait lors de la première visite du patient au MD Anderson puis une nouvelle fois au moment de l'inclusion dans l'étude par deux anatomopathologistes différents. Une étude immunohistochimique incluant entre autre desmine, myoglobine, and actine était faite systématiquement ainsi que l'étude des réarrangements classiques des sarcomes si nécessaires. Les sous-types histologiques furent divisés en ERMS, ARMS, PRMS et indifférenciés.

Les patients ayant du matériel tumoral en quantité suffisante ont été sélectionnés pour l'étude de la translocation PAX3/7-FOXO1. Cent cinq prélèvements fixés dans le formol et inclus en paraffine de patients furent assemblés en tissu micro-array (TMA). Les emplacements comportant de la tumeur viable furent sélectionnés et deux carottes de 0.6 mm de diamètre furent inclus dans un bloque de paraffine standardisé de 45 x 20 mm en utilisant un stylet en acier inoxydable (Beecher Instruments, Silver Spring, MD). Enfin, une section de 4 um a été montée sur lames, recouvertes de poly-L lysine.

Hybridation *In Situ* en Fluorescence

Pour évaluer la présence de réarrangement impliquant *PAX3*, *PAX7*, and *FOXO1*, des sondes sécables furent employées tel décrit précédemment.²¹⁰ En bref, chaque sonde a été marquée par nick translation avec un marqueur fluorescent vert (Spectrum-green) ou rouge (Spectrum-orange-deoxyuridine triphosphate) permettant la visualisation de 2 couleurs par FISH (Figure 2). Les points rouge et vert fusionnés ou séparés indiquaient respectivement l'absence ou la présence de réarrangement de *PAX* and *FOXO1*.

Analyse Statistique

La survie des patients a été évaluée en fonction des paramètres cliniques et histologiques suivants: âge, sexe, infiltration tumorale, localisation et taille de la tumeur, présence de ganglions lymphatique envahis, classification IRS et présence d'une fusion PAX3/7-FOXO1. La prise en charge a été analysée en fonction du recours multidisciplinaire (chirurgie, chimiothérapie, radiothérapie) ainsi que du type d'agent de chimiothérapie. La survie a été mesurée à partir du diagnostic à la date du décès ou du dernier contact avec le patient.

La méthode de Kaplan et Meier a été employée pour estimer la survie médiane globale (overall survival OS), la survie sans rechute (recurrence-free survival RFS) chez les patients non métastatiques et la survie sans progression (progression-free survival PFS) pour les patients métastatiques. Une régression de Cox a été utilisée pour modéliser l'OS, la RFS et la PFS et estimer les hazard ratios pour des facteurs pronostic potentiels en analyse univariée. Nous avons ensuite inclus en analyse multivariée tous les facteurs trouvés significatifs à un niveau de 0.25 en analyse univariée et avons utilisé une élimination inverse des facteurs un à un jusqu'à obtenir pour tous les facteurs restants un niveau de significativité de 0.05. Toutes les analyses ont été faites grâce au logiciel SAS 9.1 pour Windows (Copyright © 2002-2003 by SAS Institute Inc., Cary, NC). La fréquence des métastases comparée à la présence de réarrangement a été réalisée en utilisant le test du Chi2.

Culture cellulaire

Les cellules de la lignée TC71 de sarcoma d'Ewing ont été cultivées en milieu RPMI1640 avec 10% serum foetal bovin (FBS) et 2 mmol/L de L-glutamine. La lignée A204 de RMS indifférencié humain a été cultivée dans du milieu de culture Mc Coy avec 10% de FBS et 2

mmol/L L-glutamine. Les cellules RD18 de RMS humain étaient cultivées dans du Dulbecco's Modified Eagle's Medium (DMEM) 12 supplémenté par 1% de pénicilline et streptomycine et 10% de FBS. Toutes les lignées ont été achetées auprès de l'American Type Culture Collection (ATCC; Manassas, VA). Elles ont été testées et étaient dépourvues de contamination par mycoplasme et étaient maintenues à 37 °C en incubateur humidifié avec 5% de CO₂.

Agents

Le vorinostat a été obtenu par Merck & Co. Inc. (Whitehouse Station, NJ, USA), la doxorubicine HCl, par Ben Venue lab. (Benford, OH) et le 17 DMAG a été acheté via Invivogen (San Diego, CA). Le sorafénib et l'abacavir ont été achetés à la pharmacie l'UT-MDACC. Les agents ont été dissous dans du diméthyl sulfoxyde (DMSO) (Fisher Bioreagents) à la concentration de 10 mmol/l et filtrés à travers un filtre de 0.22 micron filters et des aliquotes ont été conservés à -20°C, protégés de la lumière.

Tests de Viabilité

Les cellules ont été cultivées dans des boîtes de Petri de 100 mm de diamètre (Corning Life Sciences, Corning, NY) puis ensemencées dans des plaques de 96 puits à la concentration de 5 x10⁴ cellules par puit et incubées pendant 24h, 48h, 72 h avec des doses croissantes de vorinostat, 17-DMAG, abacavir et sorafénib, initialement en monothérapie, puis en combinaison. Sur la base de ces premiers résultats, chaque agent et les combinaisons ayant révélé une activité synergique ont été associées à la doxorubicine. À la fin de l'incubation, la réduction de la viabilité cellulaire était mesurée en comparaison avec les cellules non traitées, par test MTS à l'aide du kit CellTiter 96 Aqueous Non-Radioactive

Cell Proliferation Assay (Promega Corporation, Madison, WI) utilise selon les instructions du fabricant. L'absorbance à 490 nm reflétant le nombre de cellules vivantes en culture a été mesurée à l'aide du programme KC Junior et d'un lecteur de microplaques (Bio-Tek Instruments, Winooski, VT). La viabilité cellulaire relative a été calculée par la moyenne des absorbances des essais fait en triplet moins l'absorbance de la plaque seule, divisée par la moyenne de l'absorbance des triplets de cellules traitées par DMSO moins l'absorbance de la plaque seule multipliée par 100. Un comptage cellulaire a été fait par dénombrement des noyaux (5–25 μm) grâce au lecteur automatique Mozi Z cell counter (Orflo)

Cycle cellulaire, apoptose et test d'activité de caspase 3/7

Le cycle cellulaire a été analysé par coloration par propidium iodide (PI) (Roche, Indianapolis, IN, USA) par cymomètre de flux FACS Canto II et le logiciel FACS Diva 6.1 (BD Biosciences, San Jose, CA). Les cellules ont aussi été colorées par by Alexa fluor 488 annexin

V et PI (Invitogen, Carlsbad, CA USA) afin de quantifier l'apoptose par cryométrie de flux. Enfin, l'activité des caspases 3 et 7 ont été mesurées à l'aide du kit Apo-One homogeneous Caspase-3/7 (Promega Inc., Madison, WI, USA) selon les instructions du fabricant.

Analyse de la synergie

Les indices de combinaison (CI), calculés selon la méthode de Chou and Talalay ont permis de déterminer le caractère synergique, additif, ou antagoniste. En bref, les CI ont été calculés en fonction de la fraction de cellules affectées (Fa) par la dose, mesurée par viabilité cellulaire et le teste d'apoptose, et les CI ont été générés en utilisant le logiciel CalcuSyn (Biosoft, Cambridge, UK). Les combinaisons étaient considérées synergiques lorsque le CI était inférieur à 1, additive lorsque le CI était égal à 1, et antagoniste si le CI était supérieur à 1.

Résultats

Caractéristiques des Patients

La cohorte représentait 239 patients âgés de 10 ans ou plus, suivis et traités au MD Anderson pour un diagnostic de RMS entre 1957 et 2003, ce qui correspond à seulement 1.2% des 19,708 patients suivis pour sarcome durant la période de l'étude. L'âge médian des patients était de 19 ans avec un intervalle entre 10 et 102 ans. 80% des patients étaient âgés de 15 ans ou plus. On comptait 97 femmes (40.6%) pour 142 hommes (59.4%) (Sexe ratio 1.46) (Tableau 2). Le sous-type embryonnaire était le plus représenté et tendait à avoir une survie plus longue comparée aux autres sous-types (Fig.3A). Afin de refléter l'évolution de la prise en charge des RMS, durant les 45 ans constituant la période de l'étude, la survie a été comparée entre 3 différentes périodes: 1957-1979, 1980-1989 and 1990-2003. Pas de différence de survie à 5 ans n'a été observée et celle-ci restait stable autour de 33% (Fig. 3B). De plus, l'incidence de l'envahissement ganglionnaire était utilisée comme *surrogate* du progrès de l'imagerie mais, de même, la détection de ganglions envahis est restée stable avec un taux de 7% des patients.

Identification de la translocation de *PAX3/7-FOXO1* par FISH sur Tissue Microarray

Des 2 lames de TMA analysées, 21 (20%) spécimens étaient trop pauvres ou dépourvus de tissu tumoral. Nous avons été en mesure d'obtenir un résultat en FISH dans 52 (63%) des 83 spécimens adéquats. Les sondes ne se sont pas hybridées pour 31 spécimens (37%). La fusion *PAX3-FOXO1* a été trouvée dans 26% de ces 52 cas, tandis que la fusion *PAX7-FOXO1* a été trouvée dans 8% des cas et aucune des 2 types de fusion n'a été trouvée dans 65% des cas (Tableau 3). Parmi les RMS définies de sous-type alvéolaire, 18 (67%) avaient une fusion de gène, principalement *PAX3-FOXO1* (52%). Une translocation *PAX3/7-FOXO1* a été trouvée dans 8 RMS définies histologiquement comme étant embryonnaires qui ont

ensuite été reclassés alvéolaires. Aucune fusion n'a été trouvée dans les spécimens classés pléomorphes.

Survie des Patients ayant un ARMS à fusion positive

Nous avons comparé les groupes de patients porteur d'une fusion de *PAX3/7-FOXO1* soit les ARMS à fusion positive aux patients porteurs d'ARMS avec absence de fusion (à fusion négative). Il apparaît que les patients à fusion positive présentaient une tendance à avoir une survie inférieure à ceux ayant une fusion négative, bien que la significativité ne soit pas atteinte, que ce soit parmi les patients dont la maladie était localisée ou métastatique. (Figure 4). Lorsque nous avons comparé la survie globale en fonction du type de translocation, soit *PAX3-FOXO1* vs. *PAX7-FOXO1*, nous n'avons pas observé de corrélation avec la survie mais le nombre de cas porteur de la fusion *PAX7-FOXO1* était faible. (Figure 5)

Les Patients ayant un ERMS et ceux ayant un ARMS à Fusion-Négative tendent à avoir une évolution similaire

Afin d'évaluer comment les ARMSs à fusion-positive se comportent cliniquement, nous avons comparé leur survie avec celle des ERMSs, des ARMSs à fusion-positive et des ARMSs à fusion-négative. Nous n'avons pas observé de différence significative ($P = 0.15$) entre ces 3 groupes de patients (Figure 6). Cependant, une tendance vers une survie plus courte était suggérée pour les patients porteurs d'ARMS à fusion positive, tandis que les groupes de porteur d'ERMS et d'ARMS à fusion négative montrait une évolution semblable, avec plusieurs survivants à long terme (Figure 6A and 6B)

Fréquence augmentée de maladie métastatique chez les patients à ARMS a fusion positive

Afin d'évaluer l'effet des translocations *PAX7/3-FOXO1* sur le risque de métastase, nous avons calculé le pourcentage de patients avec une maladie métastatique au diagnostic, en fonction du statut fusionnel, soit des ARMS a fusion-positive, fusion-négative and ERMS. Une augmentation significative du taux de maladie métastatique était observé parmi les patients avec un ARMS a fusion-positive (39%), compare aux patients dont la tumeur ne présentait pas de translocations ($P = 0.0081$, $\chi^2 = 9.6$; 2 degrés de liberté). Une fois encore, les ERMSs et les ARMSs semblaient avoir un taux identique en matière de fréquence de maladie métastatique (Figure 7).

Maladie localisée

Données démographiques

Cent soixante-trois patients (68%) ne présentaient pas de métastase et incluait 63 femmes (38.7%) et 100 hommes (61.3%). Leur âge médian était de 22 ans (10 et 102 ans). Tandis que l'âge moyen était de 28.6 ans avec une déviation standard de 18.1.

Caractéristiques tumorales

Soixante-deux pourcents des tumeurs étaient considérées comme invasives lors de la chirurgie ou à l'imagerie. La localisation tumorale la plus importante concernait la tête et le cou (44%), suivi par le tractus génito-urinaire (GU) (20%) et les extrémités (18%). Les sites du tronc, intra-abdominal et pelvis représentaient chacun moins de 10% des patients. Quarante-vingt pourcent des patients n'avaient pas d'envahissement ganglionnaire (Tableau 2).

La majorité des tumeurs mesuraient moins de 5 cm (41%). Trente-sept pourcent étaient composés entre 5 et 10 cm et 17% étaient supérieures à 10 cm. Dans 8% des cas, la taille de la tumeur n'était pas spécifiée. Selon la classification IRS, 44% des patients étaient classés stage 3, 36% stage 1 et 20% stage 2. D'après la classification IRS en groupes décrivant le statut post-chirurgical, la plupart des patients étaient classés au sein du groupe III (53%), tandis que le groupe I représentait 32% des patients, le groupe II 8%, et était inconnu pour 6% des cas.

Traitement

Peu de patients ont reçu une prise en charge locale seule (9%) tandis que 6% seulement n'ont eu que de la chimiothérapie seule. Trente-sept pourcent des patients ont reçu une prise en charge multimodale triple, correspondant à de la chirurgie, chimiothérapie et radiothérapie, tandis que 36% bénéficièrent d'une thérapie bimodale comprenant de la chimiothérapie associée soit à de la chirurgie, soit à de la radiothérapie. Les patients âgés de plus de 50 ans avaient plus tendance à bénéficier d'une approche triple que les patients plus jeunes mais le taux de stratégies basées sur la chimiothérapie était stable dans les différents groupes d'âge chez ces patients à RMS localisés. (Fig. 7A)

Un protocole de chimiothérapie incluant de l'actinomycine D a été donné à 23% des patients. Cependant, la majorité des patients recevant de l'actinomycine D avaient moins de 20 ans. De la doxorubicine a été administrée à 54% des patients et de l'ifosfamide à 18% des patients. 80% des patients recevant de la doxorubicine et de l'ifosfamide étaient âgés entre 20 et 50 ans. Les patients plus âgés avaient tendance à être traités avec moins d'ifosfamide que les patients d'âge compris entre 20 et 50 (Tableau 5). Quarante-huit pour cent des patients ont reçu au moins l'une de ces 3 drogues.

Evolution clinique

Les analyses de RFS et OS sont résumées dans le Table 4. Cent douze des 163 patients eurent une rechute de leur maladie ou décédèrent. Le suivi médian des 163 patients était de 3.3 ans avec un écart compris entre 0.3 et 42.7 ans. Pour les 64 patients qui restèrent vivants, la durée médiane de suivi était de 12.1 ans (de 0.6 à 42.7 ans), tandis que les 99 patients décédés eurent un suivi de 1.8 ans (de 0.3 à 31.9 ans). La RFS médiane était de 1.9 années avec un intervalle de confiance à 95% (95% CI) compris entre 1.3 et 2.8 ans. La RFS à 1 an était de 0.67 avec un 95% CI de 59% à 73%. La RFS à 2 ans était de 47% avec un 95% CI entre 39% et 54%, et la RFS à 5 ans était de 36% avec un 95% CI compris entre 29% et 43%. Treize patients eurent une rechute mais étaient vivants au dernier contact. En analyse univariée de la RFS, l'âge augmentant, l'invasion, et le fait de ne pas avoir reçu de chimiothérapie étaient associés significativement avec une RFS plus courte, tandis qu'une localisation génito-urinaire ou le fait d'avoir reçu de l'actinomycine D sans ni doxorubicine ni ifosfamide étaient associés avec une RFS plus longue. Pourtant, en analyse multivariée, seul l'âge croissant et l'invasion étaient associés conjointement à une RFS plus courte.

La OS médiane pour tous les patients ayant une maladie localisée était de 3.8 ans avec un CI 95% compris entre 2.8 et 7.6 ans. Quatre-vingt-dix-neuf des 163 patients sont décédés. La OS à 1 an était de 84% avec un 95% CI entre 78% et 89%. La OS à 2 ans était de 66% avec un 95% CI entre 58% et 72% et une OS à 5 ans à 44% avec un 95% CI intervalle compris entre 36% to 52%. En analyse univariée, l'âge croissant, l'invasion et le stage 3 de la classification IRS était associé avec une OS plus courte tandis que la localisation urogénitale, le fait d'avoir reçu de l'actinomycin D sans doxorubicine ni ifosfamide et avoir bénéficié d'une bi modalité incluant la chimiothérapie était significativement associé avec une OS prolongée. En analyse multivariée, seuls l'âge, l'envahissement et une thérapie bimodale étaient associés conjointement à l'OS. (Table 4) La courbe de survie de Kaplan-Meier présentait un point d'inflexion notable à 5 ans suivi d'un plateau pour les différents groupes d'âge. Les longs survivants (à 15 ans d'OS) représentaient 55% des patients âgés de moins de 20 ans, 31% des patients dont l'âge était compris entre 20 and 50 ans, et moins de 10% chez les patients de plus de 50 ans (OS à 5 ans à 13%, OS médiane de 1.7 ans). (Fig.3C)

Maladie Métastatique

Soixante-seize patients étaient métastatiques au diagnostic, incluant 34 femmes (44.7%) et 42 hommes (55.3%). L'âge médian était de 18 ans avec une variation comprise entre 10 et 67 ans. L'âge moyen était de 23.7 avec une déviation standard de 13.9.

Caractéristiques tumorales

Le primitif était situé en région abdominal ou pelvienne 28% des patients. Vingt-quatre pourcents des tumeurs étaient localisées aux extrémités, 20% au niveau de la tête et du cou et 17% au niveau de la sphère uro-génitale. Le tronc représentait 12% des locations tumorales primitives. Deux tiers des tumeurs primitives mesuraient plus de 5 cm (68%). (Table 6)

Traitement

Tous les patients métastatiques reçurent de la chimiothérapie. 39% bénéficièrent également soit d'une chirurgie, soit d'une radiothérapie tandis que 21% reçurent une stratégie incluant les 3 modalités de traitement. 17% des patients reçurent de l'Actinomycin D, 70% de la doxorubicine et 26% de l'ifosfamide. Seuls 2 patients ne reçurent aucun de ces 3 agents de chimiothérapie standards (3%). Le nombre de long survivants était plus important lorsque les 3 modalités étaient intégrées ensemble. En effet, les patients ayant une maladie métastatique traités avec un traitement incluant chimiothérapie avec soit la chirurgie, soit la radiothérapie avaient une survie à 14 ans à 14.4% tandis que les patients traités avec les 3 modalités, chimiothérapie, chirurgie et radiothérapie avaient une survie à 14 ans à 37.5%. Cependant, l'utilisation d'une bi ou triple modalité de traitement diminuait chez les patients

métastatiques âgés de plus de 50 ans, comparés aux patients plus jeunes (Fig. 7B), et les protocoles de chimiothérapie incluaient moins souvent de l'ifosfamide que les patients entre 20 et 50 ans. (Table 5)

Evolution clinique

Les analyses de PFS et OS sont résumées dans le Tableau 6. Le suivi médian des 76 ayant une maladie métastatique était de 1.4 ans. Pour les 14 patients vivants à la fin de l'étude, le suivi médian était de 8.9 ans tandis que les 62 patients décédés ont été suivis une médiane de 1.1 an.

La PFS médiane était de 0.9 ans avec un 95% CI entre 0.7 et 1.3 ans. Soixante-sept de ces 76 patients ont vu leur maladie progresser ou sont décédés. Cinq patients parmi ceux ayant progressé étaient vivants au dernier contact. La PFS à 1 an était de 45% avec un 95% CI entre 33% et 55%. La PFS à 2 ans était de 22% avec un 95% CI 14% et 32% et la PFS à 5ans était de 13% avec un 95% CI 7% et 22%. En analyse univariée, l'âge croissant et une localisation du primitif au niveau du tronc ou en région intra-abdominale ou pelvienne était significativement associés avec une PFS plus courte tandis qu'une stratégie associant les 3 modalités était associée avec une PFS plus longue. En analyse multivariée, ces mêmes facteurs ont été trouvés associés à la PFS, ainsi qu'une localisation au niveau des extrémités. (Table 6)

L'OS médiane pour les patients métastatiques était de 1.4 ans avec un 95% CI entre 1.0 to 1.8 ans. Soixante-deux des 76 patients sont décédés. L'OS à un an était de 60% avec un 95%

CI entre 49% et 70%. L'OS à 2 ans était de 32% (95% CI 22% et 43%) et une OS à 5 ans à 18% (95% CI 10% et 28%).

En analyse univariée, l'âge augmentant et la localisation au niveau du tronc ou intra-abdominal et pelvien étaient associés significativement avec une OS plus courte. Cependant, l'utilisation d'une triple modalité (Sx+Cx+XRT) était significativement associée avec une OS plus longue. Ces 3 facteurs restaient significatifs en analyse multi variée pour l'OS. (Table 6)

Les courbes de survie de Kaplan-Meier montrent un point d'inflexion autour de 3 ans, précédant un plateau chez les patients âgés de moins de 50 ans suggérant un taux de guérison entre 15% et 18% pour ces patients métastatiques.

Les bithérapies comportant du vorinostat, sorafenib et du 17-DMAG induisent une réduction synergique de la viabilité sur lignées de sarcome à petites cellules

Nous avons d'abord déterminé la concentration inhibitrice médiane (IC50) de chaque agent en monothérapie sur chaque lignée. L'IC50 de l'abacavir était très haute et non atteinte dans la lignée RD18. En combinaison, l'IC 25 de chaque drogue a été utilisée. L'abacavir s'est révélé antagoniste avec chacune des autres drogues. Cependant, le vorinostat, le sorafenib et le 17-DMAG avaient une activité synergique 2 à 2, et ce, de façon consistante à travers les différentes lignées cellulaires, sauf pour la combinaison du 17 DMAG plus vorinostat sur les cellules RD18. Le vorinostat associé au 17 DMAG obtinrent la meilleure synergie. L'abacavir n'a pas été étudié plus en avant.

Les thérapies ciblées n'agissent pas de façon synergique en trithérapie ciblée contrairement aux bithérapies

Les agents synergiques 2 a 2 ont ensuite été combinés ensemble. Ainsi, les cellules ont été exposées au vorinostat, 17 DMAG et sorafenib aux doses de 0.25, 0.5 and 1 uM dans un souci d'homogénéité. La viabilité cellulaire la plus basse par MTS a été atteinte par la triple thérapie mais à l'action des 3 agents ensemble étaient plutôt soit additive soit antagoniste comparée aux bithérapies ciblées sur les 3 lignées (moyenne des Cis = 1.2).

Doxorubicine associée a une bithérapie ciblée

Afin de déterminer une combinaison optimale, nous avons d'abord testé la doxorubicine avec chacune des 3 thérapies ciblées : vorinostat, 17 DMAG and sorafenib. Nous avons obtenu une réduction de la viabilité cellulaire modérée pour l'association doxorubicine et 17 DMAG avec un synergisme faible (CI: 0.8), tandis que peu d'activité a été observée avec le sorafenib. En revanche, l'association de la doxorubicine avec le vorinostat était plus importante ainsi qu'une synergie CI a 0.7. Nous avons ensuite testé les bithérapies ciblées associées a la doxorubicine consistant à la doxorubicine associée soit au vorinostat plus 17 DMAG, soit au sorafenib plus 17 DMAG. Une réduction modeste de la viabilité était observée avec la combinaison de doxorubicine plus la bithérapie ciblée vorinostat plus 17 DMAG tandis qu'aucun effet n'a été observée pour l'association de la doxorubicine avec le sorafenib plus le 17 DMAG. Les CIs étaient supérieurs à 1, soit antagonistes, pour chacune de ces combinaisons.

Séquence des associations

Afin de mieux comprendre le mécanisme d'action et les interactions entre thérapies ciblées et la doxorubicine, nous avons étudié la combinaison offrant les meilleurs résultats en matière de réduction de viabilité et de synergie: doxorubicine, vorinostat et 17 DMAG. En utilisant différentes séquences de traitement, nous avons testé la réduction de viabilité en fonction de différentes séquences d'administration. Les résultats montrent que l'administration première de la combinaison de thérapies ciblées avant la doxorubicine semble plus active que l'inverse, tandis qu'un traitement concomitant semble plus actif que l'un ou l'autre des traitements séquentiels.

Etude de l'apoptose de l'association doxorubicine plus la bithérapie ciblée : vorinostat-17 DMAG

L'association doxorubicine plus la bithérapie ciblée vorinostat-17 DMAG ne montrait pas de synergisme (CI:1.2) en terme d'induction de l'apoptose. En revanche, une augmentation transitoire à 24h de la population de cellules en subG1 était quantifiée à 70% compare aux 30% retrouvés en monothérapie. Le mécanisme d'action a été plus approfondie et a montré une augmentation de l'apoptose dépendante des caspases avec les combinaisons ainsi que de l'apoptose indépendante des caspases (11% to 36%).

Conclusion

Deux cent trente-neuf patients furent inclus sur cette période. Parmi ces patients, 163 avaient une maladie localisée et présentaient une médiane de survie globale (OS) de 3.8 années. Un âge supérieur à 50 ans et une infiltration tumorale étaient significativement associés à une survie plus courte pour ces patients non-métastatiques tandis que l'emploi d'une stratégie

intégrant chimiothérapie avec un contrôle locale par chirurgie ou radiothérapie était significativement associée à un meilleur pronostic. Les patients de plus de 50 ans avaient une OS à 5 ans de 13 % (la médiane OS 1.7 ans) en dépit d'une maladie localisée. Soixante-seize patients étaient métastatiques au diagnostic. Leur OS médiane était de 1.4 années. Approximativement 13 % de patients métastatiques de moins de 50 ans ont eu une survie prolongée de plus de 15 ans. L'utilisation d'une stratégie thérapeutique triple, intégrant chirurgie, chimiothérapie et radiothérapie était significativement associée à une survie prolongée. Par ailleurs, l'emploi de thérapie multimodale ainsi que l'utilisation d'ifosfamide étaient moindre chez les patients de plus de 50 ans comparé aux groupes de patients plus jeunes, parmi ces patients métastatiques. Au niveau moléculaire, 52% des patients présentaient une fusion de type *PAX3-FOXO1*, 15% la fusion *PAX7-FOXO1*, tandis que 33% des patients n'étaient pas porteurs d'aucune fusion. La présence du transcrit de fusion *PAX3/7-FOXO1* était significativement liée à un risque accru de maladie métastatique à l'image des données pédiatriques. Bien que la significativité statistique ne soit pas atteinte, une tendance à une survie plus longue était observée chez les patients négatifs pour la recherche de la fusion. L'étude *in vitro* de thérapies ciblées a permis d'identifier que les 4 agents à l'exception de l'abacavir étaient synergétiques 2 à 2 mais qu'une triple thérapie de ces agents ne permettait pas d'obtenir des résultats supérieurs aux bithérapies de thérapies ciblées. La combinaison du vorinostat plus le 17DMAG associée à la doxorubicine obtenait une meilleure efficacité que les bi ou tri thérapies. En termes de séquence de traitement, une administration concomitante était supérieure en efficacité à un traitement séquentiel.

Les caractéristiques cliniques et moléculaires dont le statut *PAX-FOXO1* du rhabdomyosarcome de l'adolescent et de l'adulte sont proches de celles de l'enfant,

notamment au niveau du caractère péjoratif de la translocation *PAX3/7-FOXO1*. La prise en charge du rhabdomyosarcome de l'adolescent et de l'adulte semble souffrir d'une approche moins agressive en matière de recours à un traitement local ou dans le choix d'agent de chimiothérapie, ce qui peut expliquer son pronostic péjoratif comparé au rhabdomyosarcome pédiatrique. De plus, des combinaisons de thérapies ciblées peuvent être intégrées aux protocoles de chimiothérapies standards.

CONTRIBUTION OF THE APPLICANT TO THE PRESENT WORK

On the 1st work, the applicant updated and collected the missing data. She designed the figures and tables and realized the Kaplan Meier curves. She interpreted the data, wrote, submitted and reviewed the manuscript.

On the second work, she collected the data of the FISH results and updated the clinical data. She designed, performed and interpreted the statistical analysis, drew the figures and tables, wrote and submitted the manuscript.

On the last work, she performed all the cell culture work and experiments, designed the tables and figures, wrote and submitted the manuscript.

Targeted polytherapy in small cell sarcoma and its association with doxorubicin

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Abstract

BACKGROUND: While a change of paradigm occurred in the last decade from chemotherapy to targeted therapy for cancer treatment, this work investigates the optimal combination of targeted agents with doxorubicin, sarcoma standard of care.

METHODS: Three sarcoma cell lines were studied RD18 (rhabdomyosarcoma), A204 (undifferentiated sarcoma) and TC 71 (Ewing's sarcoma). Each cell line was exposed to increasing concentrations of vorinostat (HDAC inhibitor), 17-DMAG (Hsp90 inhibitor), abacavir (anti-telomerase) and sorafenib (tyrosine kinase inhibitor) alone, combined 2 by 2, then with doxorubicin. Viability was assessed by MTS assay. The Chou and Talalay combination index (CI) was used to determine additive (CI=1), synergistic (CI<1) or antagonistic effect (CI>1). Cell cycle analysis, measure of apoptosis by Annexin V and caspase 3/7 activity were studied using flow cytometry analysis and luminescent assays.

RESULTS: In monotherapy, the agents showed 30% to 90% decrease in viability but abacavir, which remained less active. Combination therapies with vorinostat, sorafenib and 17-DMAG showed strong synergism. Abacavir was found antagonistic with each drug. Either vorinostat or 17 DMAG synergized with doxorubicin, achieving 60% cell killing compared to doxorubicin alone 12%. However, no synergy was observed for sorafenib with doxorubicin. The triple therapy vorinostat, 17 DMAG and Doxorubicin did not show synergism but transiently increased the subG1 population at 24H, 70% compared to 30% in monotherapy with an increase in early caspase-independent apoptosis.

CONCLUSION: This work provides evidence of synergism of dual combinations of vorinostat, 17 DMAG and sorafenib. In adjunction to doxorubicin, these combinations enhance doxorubicin cytotoxicity at therapeutically relevant concentrations.

Introduction

Soft tissue sarcoma (STS) is rare and a heterogeneous group of tumor. These two fundamental characteristics are major issues when trying to improve the standard of care.¹⁵⁴ Indeed, clinical trials suffer from inhomogeneous disease natural history or lack of power due to small number of patients in over specific subtypes.¹⁵⁵ As a result, sarcoma research has been an example for global collaboration for clinical trials¹⁵⁶ but also for the need to have a strong preclinical rationale before pushing further drugs or combinations.

Small cell sarcoma such as rhabdomyosarcoma and Ewing's sarcoma are chemosensitive tumors but the relapse rate is fairly high with a poor median survival in metastatic setting.¹⁴⁵ Despite its heterogeneity, doxorubicin and ifosfamid are the standard of care in most sarcoma, but this regimen confers only less than 10 % survival at 5 years in metastatic sarcoma.¹⁴⁶⁻¹⁵⁰ The development of targeted therapies has changed the landscape of sarcoma research, bringing hope to patients and clinicians to achieve better response rates and survival.^{151, 152} Yet, many early phase clinical trials have failed with targeted therapies as single agents.

Combination therapy is assumed to be the optimal strategy to potentiate targeted therapy use and achieve clinical significance. To combine targeted agents two by two or in polytherapy might overcome resistance and the inevitable relapse seen with single agent therapy. Targeted agents may also potentiate standard chemotherapy, allowing it to reduce doses, or prolong its use, which would be relevant regarding doxorubicin dose limiting cardiotoxicity. The eventual question would be: could we spare standard chemotherapy with the use of a targeted polytherapy, translating the HIV model?

Histone deacetylase (HDAC) inhibitors represent a new class of anti-cancer drugs, currently in development. HDAC modifications have been identified in many tumors.¹⁶⁹ HDAC inhibitors induce differentiation, a cell cycle arrest, apoptosis of tumor cells and inhibit tumor growth in preclinical assays.^{170, 171} Among HDAC inhibitors most advanced in clinical studies, Suberoylanilide Hydroxamic Acid or vorinostat has the peculiarity to act on tumors

that express a multi-drug-resistant phenotype (MDR). A synergistic action has been shown with ionizing radiation and inhibitors of kinases. The vorinostat has shown promising results on in vivo models such as prostate and hematological malignancies. It is now approved in cutaneous T cell lymphoma.¹⁷² Several recent arguments suggest an activity of vorinostat on sarcoma cells.^{170, 173, 174}

HSP 90 is a member of heat shock protein family whose expression is increased when cells are exposed to stress such as elevated temperature.^{152, 178} Intracellular heat shock proteins are highly expressed in tumor cells and are essential to their survival within a tumor, because of the hypo oxygenation or post chemotherapy stress. Furthermore, HSP 90 is known to play a critical role in tumor cell survival and growth in several types of cancer. Inhibition of HSP 90 in rhabdomyosarcoma cell lines showed blockade of proliferation and migration and induced apoptosis in vitro, which was confirmed in vivo on mice.¹⁷⁹ The HSP 90 antagonist 17-DMAG is currently in clinical trials and may promise on sarcomas.^{174, 179-181}

Sorafenib is a multikinase inhibitor targeting the recombinant activated factor (RAF) inhibitor, VEGF and PDGFR. The inhibition of these kinases results in anti-tumor activity through the inhibition of cancer cell proliferation and angiogenesis.¹⁶⁵ An action on sarcoma has been showed on synovial sarcoma in vitro.¹⁶⁶ It has been tested in a phase II study as a single agent on metastatic or recurrent sarcomas.¹⁶⁷ An activity was notable against angiosarcoma and was minimal against other sarcomas and concluded that further evaluation of sorafenib in these and possibly other sarcoma subtypes would appear warranted, presumably in combination with cytotoxic or kinase-specific agents.¹⁶⁸

Upregulation of telomerase in most cancer yields to protect tumor cells from aging and death of cancer cells.^{182, 183} An antiretroviral nucleoside analogue called abacavir (Ziagen) has potent antiretroviral activity and a telomerase inhibitory activity in various cellular systems and animal studies. It showed in solid tumors notably prostate cancer cell lines a reduction of proliferation and senescence.¹⁸⁴ It has not been tested on sarcoma cell lines so far.

Many crosstalks are being discovered between the different pattern of inhibition between this four drugs. For instance, on gastrointestinal stromal tumors, it has been observed that HSP 90 was a target for SAHA, which acetylates it then induces apoptosis.¹⁸⁵ Therefore, early phase clinical trials are underway to test targeted therapy combination such as sorafenib and vorinostat in patients with solid tumors.¹⁷⁶

This work aims to study targeted therapy combinations to offer a preclinical background to clinical trials. We sought to identify most active bi and tritherapy combinations of targeted agents and how to timely orchestrate them together and with standard chemotherapy doxorubicin in small cell sarcoma cell lines.

Material and Method

Cell culture

TC71 human Ewing's sarcoma cells were cultured in RPMI1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine. A204 human undifferentiated rhabdomyosarcoma cell line was cultured in Mc Coy Media with 10% fetal bovine serum, 2 mmol/L L-glutamine. RD18 human rhabdomyosarcoma cell line was cultured in modified Dulbecco's Modified Eagle's Medium (DMEM) 12 media, supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. All cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). They were negative for Mycoplasma, as determined by UT-MD Anderson CCSG Characterized Cell Line Core and were maintained at 37 °C in a humidified incubator, with 5% CO₂. Cell lines are described in Table 1.

Chemicals

Vorinostat or suberoylanilide hydroxamic acid (SAHA) was provided by Merck & Co. Inc., (Whitehouse Station, NJ, USA), doxorubicin HCl was obtained from Ben Venue lab. (Benford, OH) and 17 DMAG was purchased from Invivogen (San Diego, CA). Sorafenib and Abacavir were purchased from the University of Texas—MD Anderson Cancer Center Pharmacy. The drugs were dissolved in dimethyl sulfoxide (DMSO) (Fisher Bioreagents) at 10 mmol/l and filtered through 0.22 micron filters, and aliquots were stored in -20°C, protected from light.

Viability assays

Cells were cultured in 100 mm dishes (Corning Life Sciences, Corning, NY) then seeded in 96-well plates at a concentration of 5 x10 cells per well and incubated for 24h, 48h, 72 h with increasing concentrations of SAHA, 17-DMAG, abacavir and sorafenib, initially as monotherapy, then in combination. Based on those first results, each agent and the succeeding combinations was associated with doxorubicin. At the end of incubation, the reduction in cell viability compared with untreated cells was determined by MTS assay through the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega

Corporation, Madison, WI) according to the manufacturer's instructions. Absorbance at 490 nm reflecting the number of living cells in culture was measured using KC Junior software and microplate reader (Bio-Tek Instruments, Winooski, VT). Relative cell viability (%) was calculated as the mean absorbance of replicate treatment-wells minus the mean absorbance of replicate background wells, divided by the mean absorbance of replicate DMSO-treated wells minus the mean absorbance of replicate background wells, multiplied by 100. Direct cell counting were done by counting nuclei (5–25 μm) using an automated Vi-Cell Analyzer (Beckman Coulter) as described previously or Mozi Z cell counter (Orflo)

Cell cycles, apoptosis and caspase 3/7 activity assay

Cell cycle assay was performed by propidium iodide (PI) staining (Roche, Indianapolis, IN, USA) and analysed by a FACS Canto II flow cytometer and FACS Diva 6.1 software (BD Biosciences, San Jose, CA) as previously described¹⁸⁶ Cells was stained by alexa fluor 488 annexin V and PI (Invitrogen, Carlsbad, CA USA) to assess apoptosis by flow cytometry and caspase 3 and 7 activities were measured using the Apo-One homogeneous Capase-3/7 kit (Promega Inc., Madison, WI, USA) according to manufacturer's instructions.

Synergy analysis

The combination index (CI) method of Chou and Talalay was used to determine whether the combinations were synergistic (<1), additive ($=1$), or antagonistic (>1). Briefly, combination index (CI) was calculated based on the fraction of the cells affected (Fa) by the dose measured cell viability and apoptosis assays, and CIs were generated using CalcuSyn software (Biosoft, Cambridge, UK). The combinations were considered synergistic if the CI was inferior to 1, additive for a CI equal to 1, or antagonistic for a CI superior to 1.

Results

Bi targeted therapies with vorinostat, sorafenib and 17-DMAG result in a synergistic decrease in viability in small cell sarcoma cell lines.

We first determined the IC₅₀ of each targeted therapy drug as a single agent in each cell line. The abacavir IC₅₀ was very high and not even reached in RD18 cell line. We based or

combination data on the IC₂₅ of each drug and then determined an IC₅₀ of each targeted agent. We estimated the CI using Chou and Talalay method as reported in Table 2. Abacavir was found antagonistic with each drug. However, vorinostat, sorafenib and 17-DMAG had a synergistic activity, which was consistent across the different cell lines except in RD18 for the 17 DMAG plus vorinostat combination. Vorinostat plus 17 DMAG achieved the best synergy. Abacavir was not tested further.

Targeted tritherapy does not reach synergism compared to a bitherapy

Drugs that were synergistic 2 by 2 were combined together. Cells were exposed to vorinostat, 17 DMAG and sorafenib at the doses of 0.25, 0.5 and 1 uM for homogeneity. Cell viability revealed by MTS assay showed that the lowest viability was achieved by the triple therapy (Figure 1A and B). However, the action of the 3 drugs together was either additive or antagonistic compared to targeted bitherapies in all 3 cell lines (mean of CIs = 1.2).

Doxorubicin plus a targeted bitherapy

In order to identify an optimal combination, we first tested doxorubicin with each 3 targeted agent: vorinostat, 17 DMAG and sorafenib. (Fig 2). We obtained a reduction of cell viability by the combination of doxorubicin that was moderate for 17 DMAG (Fig. 2A), with a mild synergism (CI: 0.8), almost none observed with sorafenib (Fig. 2B) and more substantial with vorinostat (Fig. 2C) with a synergistic CI of 0.7. We thus tested the combination therapy consisting of doxorubicin with either vorinostat plus 17 DMAG (Fig 3A) or sorafenib plus 17 DMAG (Fig 3B). A modest reduction in cell viability was observed with the combination of doxorubicin plus a bitargeted therapy vorinostat plus 17 DMAG while no effect was observed for doxorubicin added to sorafenib plus 17 DMAG (Fig 3 A and B). Both CIs were above 1 for these triple combinations.

Timing of combination

To try to understand the mechanism of action and the interactions between targeted therapies and standard chemotherapy agents like doxorubicin, we studied further the most successful triple combination consisting of doxorubicin, vorinostat and 17 DMAG. Using different treatment sequences, we tested cell viability to assess the optimal timing to use each

therapeutic class. Our results show first that to treat first cells with the bitargeted therapy seems more active than a sequence starting by chemotherapy. Furthermore, a sequencing treatment was not superior to a concomitant one. (Fig 3C)

Apoptosis of Doxorubicin plus vorinostat-17 DMAG bitargeted therapy

The triple therapy vorinostat, 17 DMAG and doxorubicin did not show synergism (CI:1.2) but transiently increased the subG1 population at 24H, 70% compared to about 30% in monotherapy (Fig 4 A and B). Mechanism of action was further investigated and showed an increase in caspase 3 dependent apoptosis with the combination (Fig 5) and an early caspase-independent apoptosis (11% to 36%). (Fig 6)

Discussion

While standard treatment of small cell sarcoma still involves chemotherapy agents, targeted therapies represent a new field to explore to enrich the therapeutic palette of these poor prognosis diseases. This work provides preclinical evidence of synergism of dual targeted therapy combinations. Indeed, a synergism is achieved with biotherapies including vorinostat, 17 DMAG and sorafenib in small cell sarcoma cell lines. Targeted tritherapy not including doxorubicin does not achieve synergism and suggests more side effects for a modest cell viability reduction. In adjunction to doxorubicin, the targeted biotherapy combinations enhance doxorubicin cytotoxicity at therapeutically relevant concentrations,

allowing to reduce its dose or prolong its administration. The most efficient combination revealed by our screening was doxorubicin associated to the targeted biotherapy vorinostat and 17 DMAAG who achieved a synergistic induction of caspase-mediated and -independent apoptosis. According to our data, the most promising combination had also to include the HDAC inhibitor vorinostat. Our results do not support the use of targeted-only polytherapy as the triple combination of agents synergistic 2 by 2 did not achieve synergism.

The concept of tumor heterogeneity discussed in numerous recent articles stress the need of combination of therapies.^{153, 201, 202} Indeed, failure of targeted therapy in clinical trials may be due to the classic monotherapy scheme used to develop classic drugs. The phase I process has the objective to reach the maximal tolerated dose (MTD) and might not be appropriate for targeted therapy. So far, no new design has been developed such as whether the drug reaches its target or whether the pathway is inhibited, even if it is often reported as secondary objective. Moreover, the introduction of a second or third targeted agent might overcome resistance and therapeutic failure due to clone emerged by Darwinian selection but the whole drug development should be remodelled to adapt to targeted polytherapy clinical investigation.²⁰²

In the new era of personalized medicine, targeted therapies may represent hope for a more accurate and relevant approach but a better understanding of the biologic mechanism of the tumor itsek=lf is also needed. Many sarcoma have a specific chromosomal translocation such as small cell sarcoma: PAX3 or PAX7-FOXO1 in alveolar RMS⁴⁵, loss of heterozygosity in chromosome 11⁴⁸, FLT1-EWS1 in Ewing's sarcoma.^{48, 192-194} These translocations might sensitize cells to DNA disrupting agents such as trabectedin.²¹¹ The key tumor suppressor gene p53 might also be of interest in tumor response to targeted agents. The majority of Ewing sarcomas are wild type for p53^{195, 196}, and studies have demonstrated that the chimeric protein EWS-FLI1 silences p53 activity^{197, 198}. Recent data suggest that the molecular mechanism about the abrogation of p53 by EWS-FLI1 involves a decrease of acetylation of p53 and increase of mdm2-mediated p53 degradation¹⁹⁹. In this perspective the use of HDAC inhibitors may re-acetylate p53 and results in an increase of p53 stability and activity. In our study, TC71 cell line is mutated for *P53* but was sensitive to vorinostat and

was the cell line that reached the best viability reductions in combination therapies. In opposition to Ewing's sarcoma, *P53* is mutated in the majority of RMS²⁰⁰. *In vitro*, the wild-type *P53* A204 cells was sensitive to different agents in monotherapy but the combinations failed to achieve synergism compared to other cell lines. Finally, RD18 was mutated for *P53* and was sensitive to both monotherapies and combinations.

In vitro studies present inherent limitations. Indeed, the cell lines may not represent the real spectrum of small cell sarcoma, exemplified by their p53 status but due to the so many disappointing early phase clinical trials, we cannot spare such a model to increase our knowledge to push further combination of targeted agents in clinic. Conversely, a drug such as abacavir, regarding its mechanism of action as anti-telomerase would have made a good candidate for early phase clinical trial but was proved not to have effect in monotherapy. However, an activity might have been seen in combination as no decrease in viability may not absolutely mean no synergism with other agents.¹⁹¹ Therefore abacavir was also tested in combination but failed to potentiate its partner agents which led us to abandon the drug for further testing and not to retain it for clinical trial in small cell sarcoma.

Targeted therapies represent a new paradigm with regards to drug developpement and how to orchestrate them together and to optimally use them with standard chemotherapy. Preclinical studies such as ours offer some background to test targeted biotherapy in association with doxorubicin in small cell sarcoma.

Figures

Table 1. Cell line descriptions and P53 status.

Table 2. IC50 of monotherapy and combinations and their CI - additive (CI=1), synergistic (CI<1) or antagonistic effect (CI>1).

Figure 1. Cell viability of cell lines with triple therapy (A) TC71 and (B) RD18. S=sorafenib, D=17 DMAG, V=vorinostat

Figure 2. Cell viability of doxorubicin plus either (A) 17 DMAG (RD18), (B) sorafenib (RD18) or (C) vorinostat (TC71)

Figure 3. Cell viability of doxorubicin plus 17 DMAG and (A) vorinostat (TC71) or (B) sorafenib (RD18) and (C) the sequential or concomitant administration of doxorubicin plus vorinostat-17 DMAG (RD18).

Figure 4. Cell cycle distribution of TC71 cells treated with vorinostat, doxorubicin and 17 DMAG as (A) monotherapy and (B) in combination.

Figure 5. Measure of apoptosis by Caspase 3/7 activity of doxorubicin, vorinostat and 17 DMAG combination in TC71 cell line

Figure 6. Apoptosis measured with Alexa Fluor 488 Annexin V and PI staining of doxorubicin, vorinostat and 17 DMAG combination in TC71 cell line

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Table 1

Cell line	Type	p53	Fusion	References
A204	undifferentiated RMS	wilde type	None	1
RD18	ERMS	wilde type	None	2
TC71	Ewing	mutant (637 C>T)	EWS-FLI1	3 http://www-p53.iarc.fr

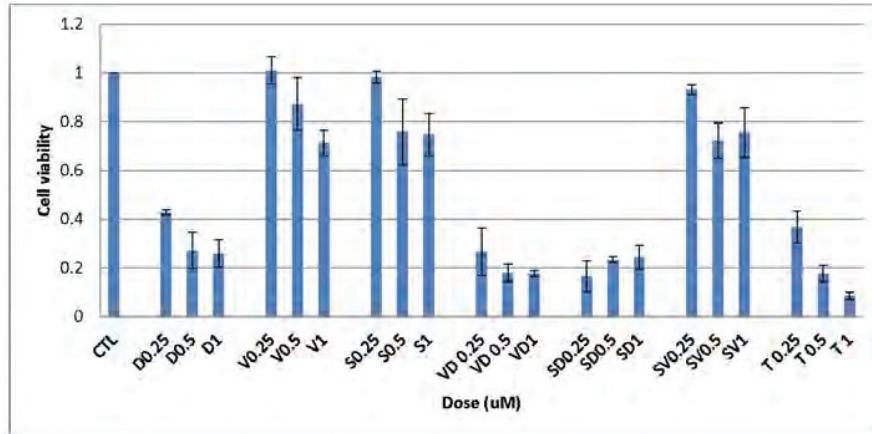
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Table 2

	RD 18		A204		TC71	
Monotherapy	IC50 (uM)					
sorafenib	5		5		5	
17-DMAG	10		10		3	
abacavir	Not reached		750		375	
vorinostat	3		1		2	
Combination	IC50 (uM)	CI	IC50 (uM)	CI	IC50 (uM)	CI
sorafenib	2.5	0.8	2.5	0.7	2.5	0.5
17-DMAG	5		10		5	
sorafenib	2.5	1.1	5	1.4	2.5	1.1
abacavir	375		188		188	
sorafenib	5	0.7	5	0.9	2.5	0.8
vorinostat	1.5		3		3	
17-DMAG	10	1.1	5	1.2	5	1
abacavir	188		750		188	
17-DMAG	1.5	3	1.5	0.7	1.5	0.6
vorinostat	0.75		3		0.75	
abacavir	750	1.9	750	3.7	188	1.4
vorinostat	3		3		3	

Figure 1

A



B

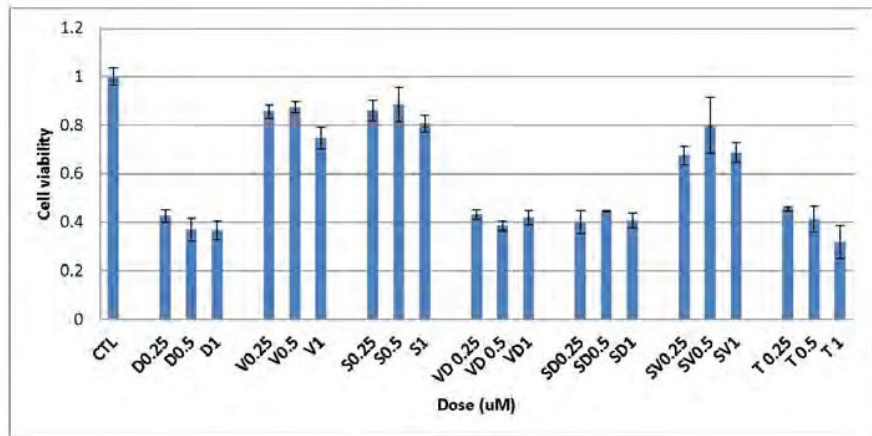


Figure 2

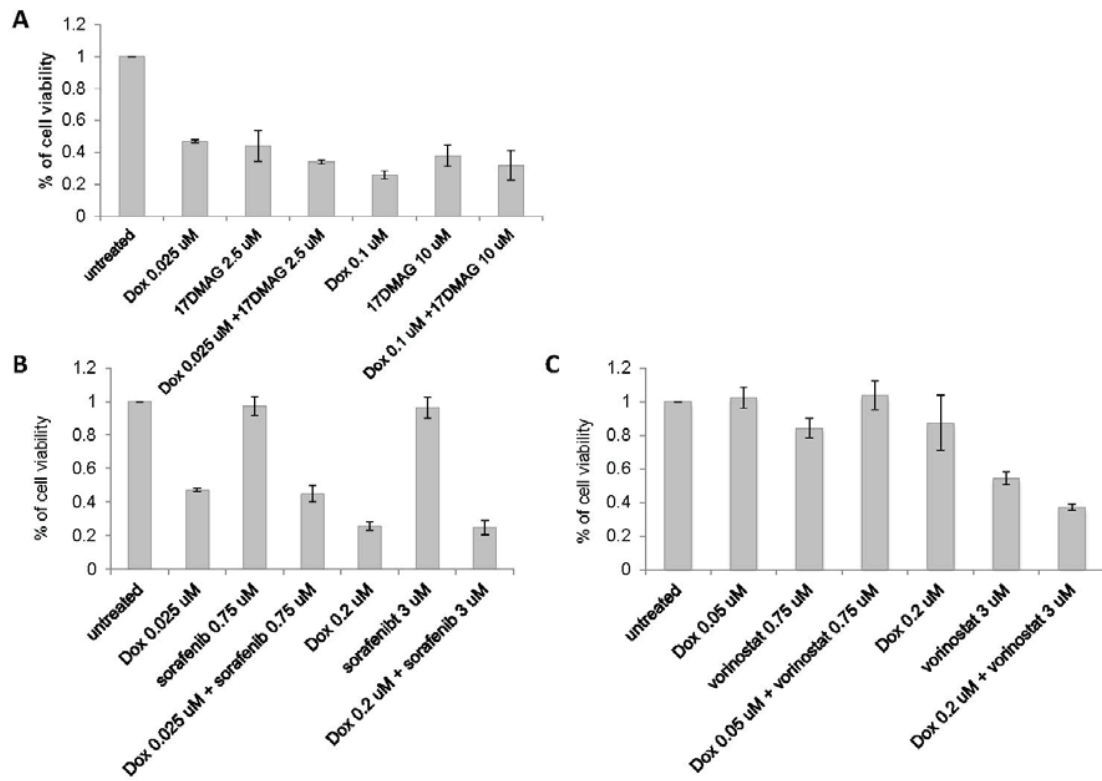


Figure 3

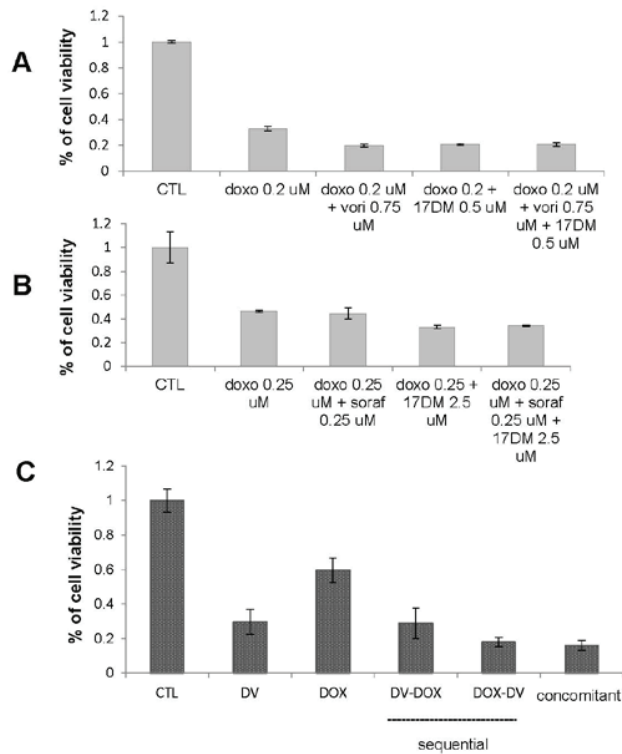
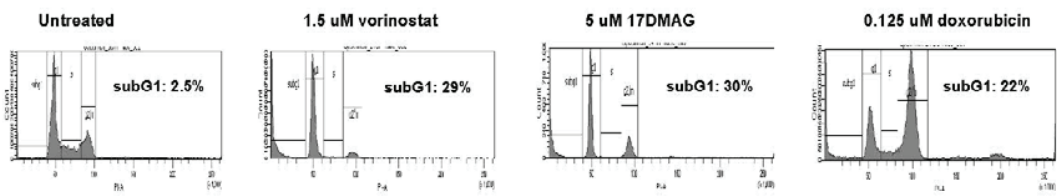


Figure 4

A



B

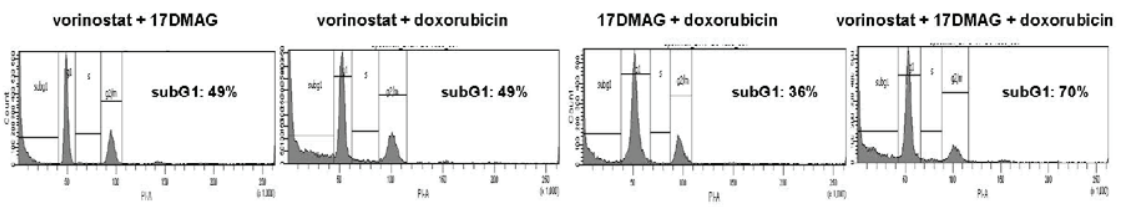


Figure 5

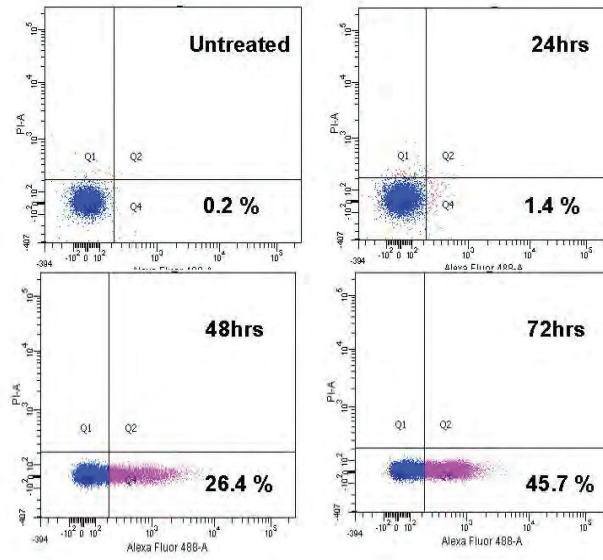
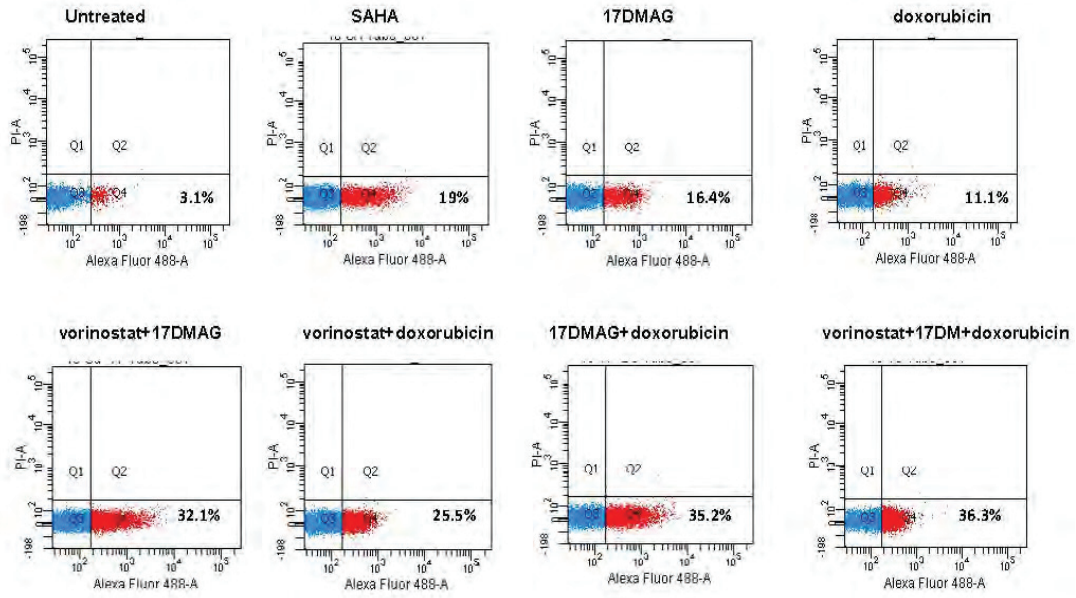


Figure 6



Other works

Publications

Dumont AG, Dumont SN, Trent JC. The favorable impact of PIK3CA mutations on survival: an analysis of 2387 patients with breast cancer. *Chin J Cancer*. 2012 Jul; 31(7):327-34

Reynoso D, Nolden LK, Yang D, Dumont SN, Conley AP, Dumont AG, Zhou K, Duensing A, Trent JC. Synergistic induction of apoptosis by the Bcl-2 inhibitor ABT-737 and imatinib mesylate in gastrointestinal stromal tumor cells. *Mol Oncol*. 2011 Feb;5(1):93-104

Posters and oral communications

Dumont SN, Trent JC, Patel SR, Araujo DS, Dumont AG, Benjamin RS
A phase II study of low-dose protracted irinotecan in patients with advanced sarcomas.
poster at ASCO 2011 and Oral communication at CTOS 2011

Dumont SN, Araujo DS, Dumont AG, Conley AP, Reynoso DR, Munsell MF, Raymond K, Patel SR, Benjamin, Trent JC. Adult Rhabdomyosarcoma: 239 Patients Followed at MD Anderson Cancer Center, poster at CTOS 2010

Dumont AG, Trent JC, Yang D, Katz D, Dumont SN, Reynoso DR, Hughes DP. Histone deacetylase inhibitor SAHA upregulates Notch signaling by a growth arrest in gastrointestinal tumor cells, poster at CTOS 2010

Dumont SN, Lazar AJ, Bridge JA, Benjamin RS, Trent JC, *PAX3/7-FOXO1* translocation by fluorescent in situ hybridization (FISH) using an adult rhabdomyosarcoma tissue microarray, *J Clin Oncol* 28:15s, Poster ASCO 2010

Dumont SN, Aslam MI, McAuliffe JC, Yang D, Nolden LK, Oyedeji CO, Trent JC; M. D. Anderson Cancer Center, Houston, TX CXCR4/CXCL12 axis: a pivotal role as a metastasis mediator *J Clin Oncol* 27:15s, Poster at ASCO 2009 (suppl; abstr 10569)