Universiteit
Leiden
The Netherlands

Translational molecular pathology of myxoid liposarcoma and leiomyosarcoma of soft tissue

Graaff, M.A. de

Citation
Graaff, M. A. de. (2017, February 7). *Translational molecular pathology of myxoid liposarcoma and leiomyosarcoma of soft tissue*. Retrieved from https://hdl.handle.net/1887/45811

Version:	Not Applicable (or Unknown)
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/45811

Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/45811 holds various files of this Leiden University dissertation

Author: Graaff, M.A. de
Title: Translational molecular pathology of myxoid liposarcoma and leiomyosarcoma of soft tissue
Issue Date: 2017-02-07

Translational molecular pathology of myxoid liposarcoma and leiomyosarcoma of soft tissue

Marieke Anna de Graaff

Translational molecular pathology of myxoid liposarcoma and leiomyosarcoma of soft tissue.

Cover layout by F.K. de Jong and B. Kleverlaan

Top right picture of myxoid liposarcoma and bottom left picture leiomyosarcoma of soft tissue.

The research described in this thesis was supported by the Liddy Shriver Sarcoma Initiative.

Financial support of this thesis was kindly provided by the Department of Pathology, Leiden University Medical Center.

Layout: Ferdinand van Nispen tot Pannerden, *my*-thesis.nl

Printing: GVO drukkers & vormgevers B.V.

Copyright © 2017, M.A. de Graaff, Leiden, The Netherlands.

ISBN: 978-94-6332-130-3

All rights reserved. No part of this publication may be reproduced or transmitted in any form or by any means without permission in writing from the author.

Translational molecular pathology of myxoid liposarcoma and leiomyosarcoma of soft tissue

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 7 februari 2017
klokke 13:45 uur

door

Marieke Anna de Graaff

geboren op 12 februari 1986
te Haarlem

Promotor

Prof. dr. J.V.M.G. Bovée

Copromotor

Dr. K. Szuhai

Leden promotiecommissie

Prof. dr. A.J. Gelderblom

Prof. dr. M. Debiec-Rychter (University of Leuven, Belgium)

Dr. E. Danen

"Nullius in verba"
(Quintus Horatius Flaccus, 20 BC)

Voor mijn ouders

Contents

Chapter 1	General Introduction	9
Chapter 2	Mediator complex subunit 12 exon 2 mutation analysis in different subtypes of smooth muscle tumors confirms genetic heterogeneity. Human Pathology. 2013; 44(8): 1597-1604.	37
Chapter 3	A translocation t(6;14) in two cases of leiomyosarcoma: molecular cytogenetic and array-based comparative genomic hybridization characterization. Cancer Genetics. 2015; 208(11): 537-544.	55
Chapter 4	Inhibition of Bcl-2 family members sensitizes soft tissue leiomyosarcomas to chemotherapy. British Journal of Cancer. 2016; 114(11): 1219-1226.	77
Chapter 5	NY-ESO-1 (CTAG1B) expression in mesenchymal tumors. Modern Pathology. 2015; 28(4): 587-595.	97
Chapter 6	Establishment and characterization of a new human myxoid liposarcoma cell line (DL-221) with the FUS-DDIT3 translocation. Laboratory Investigation. 2016; 96(8): 885-894.	117
Chapter 7	High-throughput screening of myxoid liposarcoma cell lines reveals survivin as novel druggable target. Submitted	143
Chapter 8	Summary and concluding remarks	165
Chapter 9	Nederlandse samenvatting	175
Chapter 10	Curriculum vitae	185
	List of publications	188
	Dankwoord	191
	List of abbreviations	193

Chapter 1

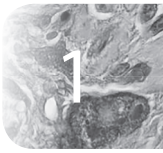
General introduction

Classification of benign and malignant soft tissue tumours

Soft tissue sarcomas are rare malignant tumours originating from the mesenchymal or neuroectodermal tissues, comprising less than 1% of all cancers. The annual incidence of soft tissue sarcomas is around 5 (4.74) per 100,000 with a 5-year survival rate of around 49% (1, 2). Nevertheless, there are more than 50 different histological subtypes described, which differ in biology and are characterized by a unique clinical behaviour and prognosis and therefore require specialized treatment (3). This emphasizes the pivotal importance of a correct diagnosis, which can be challenging for pathologists as these tumours are rare and due to the numerous different histological subtypes of which some have overlapping features. Uniform classification of bone and soft tissue sarcomas is performed on the international consensus guidelines of the World Health Organisation (WHO)(3). The biological potential of tumours varies and four main groups can be discerned: benign, locally aggressive, rarely metastasizing and malignant.

Soft tissue sarcomas can be grouped based on their line of differentiation, thus, based on the normal tissue that the tumour most closely resembles, including for example adipocytes, smooth muscle cells, striated muscle cells, fibroblasts, histiocytes, pericytes, endothelial cells, perineurial-like cells and Schwann cells. Some soft tissue tumours are composed of a combination of two or more cell types and there is also a group of tumours of uncertain differentiation. Mesenchymal tumours can occur anywhere in the body, although the majority is located in the extremities (up to 75%), the trunk wall (around 10%) and the retroperitoneum (10%). Some subtypes even have a strong predilection site, for instance, two-third of the myxoid liposarcomas develop in the thigh. Moreover, the age of patients is important to consider as some sarcomas have an age-related incidence, for example, embryonal rhabdomyosarcoma is encountered almost exclusively in young children while alveolar soft part sarcoma is most frequently detected in young adults and soft tissue leiomyosarcoma and undifferentiated pleomorphic sarcoma occur mainly in elderly patients (3). The overall male to female ratio is 1.1/1 for soft tissue sarcomas. In the subgroup of visceral sarcomas, including gastrointestinal stromal tumour (GIST), a female preponderance is observed (1.4/1) (4).

On the other end of the spectrum of malignant soft tissue tumours, are the benign mesenchymal lesions. This group of neoplasms is also



heterogeneous and consists of multiple different histological subtypes. The incidence of some of these lesions is relatively high, and is estimated to outnumber sarcomas by a factor of minimal 100. A well-known example of a benign soft tissue tumour is a conventional lipoma, responsible for around 30% of all benign soft tissue tumours (3, 5). Conventional lipomas are most frequently located subcutaneously or in the deep soft tissues on the trunk or extremities. Generally they are maximum a few centimetres in diameter, macroscopically well circumscribed and histologically composed of lobules of mature adipocytes without cytonuclear atypia (3). Other common benign neoplasms are for example leiomyomas, which will be discussed in more detail below, nodular fasciitis and schwannoma (3).

Histological classification and grading of soft tissue neoplasia

As mentioned before, soft tissue sarcomas, comprises a rare group of malignancies and accurate diagnosis is challenging for pathologist who are not familiar with the broad range of histological subtypes, therefore the risk of initial diagnostic error ranges from 8-25% (3, 4, 6). This has been investigated by performing a second opinion on diagnosed cases of soft tissue sarcomas by expert pathologists. In the majority of the cases there is either a full agreement on the diagnosis or a partial agreement, situation in which both pathologists diagnosed a sarcoma but with different histopathological grade, between the first and expert pathologist. Other examples of major discrepancies are cases in which benign lesions are mistaken for a sarcoma (10%) or the other way round, a sarcoma is mistaken for a benign neoplasm (4%)(4). Expert pathologists have extensive experience with these rare tumours and knowledge of and access to the latest molecular diagnostic tools like immunohistochemistry, FISH and (Next Generation) Sequencing. In a large series <10% of initial diagnoses were considered major misdiagnoses which were related to histological type or tumour grade (6). The correct diagnosis is crucial for determination of the right treatment, especially for tumours with novel targeted treatments, as this influences patient outcome. Therefore referral to a specialized sarcoma center is often advised for diagnostics and to make appropriate decisions on treatment. These multidisciplinary teams often include surgeons, pathologists,

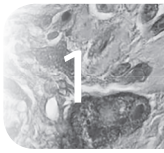
radiologists, medical oncologists, radiation therapists and when applicable organ-based specialists.

The most widely used grading system of sarcomas and also advised by the WHO is the system of the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC)(3, 7). This system is reproducible and the grade is assessed based on three independent histological parameters: tumour differentiation, mitotic activity and necrosis. Tumour differentiation indicates how closely the tumour resembles normal mesenchymal tissue. Poorly differentiated sarcomas, like for example rhabdomyosarcoma, synovial sarcoma and clear cell sarcoma, are scored as undifferentiated per definition (3, 7).

Clinical features and diagnosis

Most soft tissue sarcomas present as slowly growing, painless masses that are detected incidentally and do not, at first instance, interfere with the daily function or general health. Especially at certain locations, as for example the retroperitoneum, tumours can become quite large before they are detected. Due to this seemingly innocent behaviour tumours are often in the beginning characterized as benign lesions. Patients with a superficial lesion of >5 cm in diameter or with a deep soft tissue mass should preferably be directed to a referral center with a multidisciplinary team for diagnosis and treatment (2, 3).

Imaging of soft tissue neoplasia is important as it will provide key information on size of the tumour and relation with other structures, and specialized radiologists might give a good differential diagnosis on the images. Conventional X-ray is often performed in the first place and although it is not detailed it might be helpful in ruling out a primary bone tumour extending in the surrounding soft tissues. Also calcifications in a soft tissue mass, as seen in myositis ossificans, will be detected. Magnetic resonance imaging (MRI) is able to give detailed information on tumour size, tumour components like fat, necrosis, cystic degeneration, haemorrhage and oedema, and relation with surrounding structures, including for example bone and neurovascular bundles. The use of T1-weighted and T2-weighted images might help to discriminate between tumours with a high fat or blood content (high signal on T1W) and fibrous tumours (high signal on T2W). CT-scans are in some instances performed to detect deposition of calcifications



and baseline chest CT might be performed for accurate staging at time of diagnosis (2, 3).

Following adequate imaging, the next step in the work-up is taking of multiple core needle biopsies of the soft tissue mass. In case of a deep seated lesion this might be done with image-guidance. The biopsy location must be chosen in deliberation with the surgeon in such a way that complete resection of the biopsy tract can be achieved during definitive surgery of the tumour (2, 3).

Genetic classification of mesenchymal tumours

Next to the histopathological classification, soft tissue neoplasms can be divided based on the molecular biology of the tumours. From the molecular genetic perspective, tumours are classified in accordance to their genomic profile. Development of cytogenetic and molecular techniques have resulted in the discovery of recurrent genetic aberrations in a large subset of soft tissue tumours. The identification of specific genetic alterations have led to an increased understanding of the biological mechanisms and cellular pathways that have a role in the tumorigenesis.

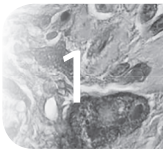
From a genetic point of view soft tissue tumours can be classified in three main categories: tumours with a specific, reciprocal translocation and often a near-diploid karyotype, tumours with specific mutations (activating or inactivating mutation or gene amplification) in a variable genomic background and tumours with an instable genome and as a consequence a complex karyotype (Table 1) (3, 8). Of the 117 soft tissue tumours listed in the WHO Classification of Tumours of Soft Tissue and Bone of 2013, approximately 45% harbour a recurrent cytogenetic or molecular aberration (3, 9). However, it is not inconceivable that this number will increase in the future with the ongoing development of molecular genetic techniques.

The overall fraction of soft tissue tumours with a reciprocal translocation is estimated at ~20% (3, 9, 10). The occurrence of a chromosomal translocation is considered a crucial genomic event for a cell and often plays an important role in tumorigenesis. A chromosome translocation is formed in a complex one-step event and is influenced by several factors, including for example the occurrence of double strand breaks (DSB), the concentration of repair proteins around the broken chromosome ends and the three-dimensional location of

chromosomes with DSB in the interphase nucleus (11, 12). Dependent on the exact location of the breakpoints and the resulting composition of the fusion product, translocations can have several consequences, it can form a chimeric fusion protein in case the coding sequence of two genes are fused, it can disrupt a tumour suppressor gene or it can place a tumour-promoting gene behind the promoter of a transcriptional active gene (promotor swapping). In all these three cases the cell can experience a proliferative advantage and this can subsequently lead to tumorigenesis (11, 13). An example of a soft tissue sarcoma with a chimeric fusion gene is myxoid liposarcoma characterized by an *FUS-DDIT3* or *EWSR1-DDIT3* translocation; which was first described at cytogenetic level in 1986 and the involved genes were identified more than six years later (3, 14, 15). Translocations and point mutations are not exclusively found in malignant tumours as also multiple benign neoplasm harbour recurrent cytogenetic aberrations (16). The translocations found in aneurysmal bone cyst (ABC), a locally aggressive neoplasm, are caused by juxtaposition of the *USP6* gene to any of the highly transcriptional active promotors of the *CDH11*, *COL1A1*, *OMD*, *TRAP150* or *ZNF9* genes (12, 17). A subset of lipomas show rearrangements at 12q14.3, leading to deregulation of the *high-mobility group AT-hook 2* (*HMGA2*) gene and several partner genes of *HMGA2* have been identified (3).

The second category consist of tumours with specific in- or activating mutations or gene amplifications which often lead to alterations in important signalling pathways. A well-known example is gastrointestinal stromal tumour (GIST) characterized by oncogenic, activating mutations in *KIT*, *PDGFRA* or *BRAF*, resulting in a continuous signalling of the KIT pathway (8). These tumours might harbour a variation in secondary genetic changes and in some cases DNA copy number changes seem to be related to the occurrence of metastasis (18).

The last group comprise tumours with an instable genome and as a consequence a complex karyotype, these tumours often contain multiple non-recurrent numerical and structural chromosome alterations (3, 8). These alterations can accumulate gradually over time during cancer development, although complex rearrangements can also develop in a single catastrophic event of reshuffling of chromosomes, known as chromothripsis (19). Leiomyosarcoma, pleomorphic liposarcoma and undifferentiated pleomorphic sarcoma (UPS) are examples of karyotypically complex sarcomas (3).



The discovery of specific, recurrent translocations or mutations in certain soft tissue neoplasms are unique features that discriminates them from other lesions and have contributed significantly to the classification of mesenchymal neoplasms. The presence of a pathognomonic molecular genetic alteration has also proven to be of tremendous value in the diagnostic process (9). Molecular genetic techniques provide a helpful diagnostic tool, especially in discriminating tumours with similar histology, but different biological behaviour or clinical outcome (9, 10). Due to the ongoing development of genetic profiling techniques, for example the broad spectrum of sequencing options, including RNA, whole genome and transcriptome sequencing, tumours that are now classified under the same diagnosis, might in the future be split in different molecular subtypes. The seemingly 'ever increasing' number of molecular subtypes will inevitably lead to a discussion between the 'splitters' and the 'lumpers. It is important to focus on those molecular subtypes that differ in clinical prognosis or that have alterations in molecular pathways which might be candidates for targeted therapies.

Table 1. The molecular genetic subtypes of soft tissue tumours.

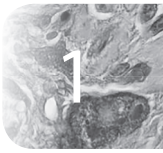
Genetic profile	Example
Specific translocation and relatively simple karyotype	Myxoid liposarcoma: t(12;16)(q13;p11) or t(12;22)(q13;q12)
	Synovial sarcoma: t(X;18)(p11;q11)
Specific mutation in variable genomic background	Gastrointestinal stromal tumour: KIT, PDGFRA, BRAF
	Dedifferentiated liposarcoma: amplification MDM2, CDK4
Complex karyotype without recurrent aberration	Leiomyosarcoma of soft tissue
	Myxofibrosarcoma

Therapeutic strategies in soft tissue sarcomas

The cornerstone of the treatment of soft tissue sarcomas is surgery with a wide tumour resection with negative margins, which implies that the tumour needs to be surrounded by a rim of normal tissue or the tumour may not cross anatomical borders like muscular fasciae or epineurium. Radiation and chemotherapy, neo-adjuvant or adjuvant, are frequently part of the treatment regimen and the decision of these therapies are made in a specialised multidisciplinary team by taking into account several variables, including: specific sarcoma subtype, chemosensitivity of histological subtype, resection margins (if

possible to operate at all), tumour location, tumour size, tumour grade, stage of the disease (especially localized versus advanced and/or metastatic disease), patient's general condition, relevant comorbidities, previous chemotherapy and treatment goal (2, 20-22). The first-line chemotherapy of choice is the anthracycline doxorubicin, often used as monotherapy but individual cases might benefit from the combination with ifosfamide (2, 23). Over the past years several trials have compared these two chemotherapeutic regimens in soft tissue sarcomas and there is no evidence that multi-agent chemotherapy leads to a statistical significant longer overall survival compared to doxorubicin alone, although in some studies a higher regression rate was observed (23, 24).

The specific alterations that have been identified in the genomes of sarcomas over the past years, can be used to develop molecular targeted therapy. In particular tumours with a pathognomonic genetic aberration are good candidates for targeted therapy. The most well-known example is GIST, which can be targeted by the KIT kinase inhibitor imatinib, which gives a partial response or stable disease in around 77% of patients with advanced or metastatic GIST (25). Other targeted (tyrosine kinase) inhibitors are being investigated in several sarcoma types. A thorough understanding of the different mechanisms of pathogenesis in soft tissue tumours is of pivotal importance in the search for novel therapeutic targets. Most translocation-associated sarcomas are characterized by gene-fusions which act as aberrant transcription factors, thereby influencing the expression of a multitude of genes promoting oncogenesis. In this way the chimeric transcription factor dysregulates downstream signalling of several cellular pathways. As a result, specific targeting of only one affected signalling pathway might have a limited effect in individual cases. Secondary changes occurring during tumour progression might serve as a potential target as well. For instance, ~18% of myxoid liposarcomas, characterized by FUS-DDIT3 fusion products, harbour additional *PIK3CA* mutations resulting in altered signalling of the mammalian target of rapamycin (mTOR) pathway. However, targeting the mTOR pathway alone might not be sufficient in myxoid liposarcoma, since the tumour cells are likely to show disturbances in other pathways as well due to altered signalling caused by the aberrant transcription factor (26). Occasionally, a translocation may lead to receptor or ligand activation leading to aberrant receptor tyrosine kinase signalling that more often have specific, inhibitory small molecules. An example of this type of translocation-associated sarcomas is



dermatofibrosarcoma protuberans. This locally aggressive neoplasm harbours a *COL1A1-PDGFB* chimeric fusion gene and the fusion product is processed to the normal PDGFB ligand (3, 27). Tumour cells carry the membrane kinase receptor, resulting in autocrine signalling and stimulating tumorigenesis. This autocrine growth signalling loop can be disrupted by the PDGFR inhibitor imatinib.

Recently, the field of cancer immunology has become more important and several immunotherapeutic approaches, like cytokine-based immunotherapy, immune checkpoint inhibitors, tumour vaccines and adoptive T-cell therapy, are under investigation (8, 28). The family of cancer-testes antigens constitute more than 200 proteins which are by definition expressed in the primitive spermatogonium of the testis, but not in other adult tissues (29). They have immunogenic properties, but as they are separated and protected by the blood-testis barrier, no natural immune response is provoked. There are different subgroups and some are more immunogenic than others. Well-known examples of cancer-testes antigens are MAGE, PRAME, GAGE and SSX2. New York oesophageal squamous cell carcinoma (NY-ESO-1) or CTAG1B belongs to the CTA family and was first detected in an oesophageal carcinoma. It is also highly expressed in the majority of myxoid liposarcomas and synovial sarcomas (30-32). Therefore it is regarded as an attractive target for immunotherapy. NY-ESO-1 can be targeted by dendritic vaccine based strategies resulting in $CD8^+$ and $CD4^+$ T-cell responses. Since the direct cytotoxic effect of $CD8^+$ T-cells against tumour cells is the most important, another option is adoptive cell therapy, involving the transfer of autologous cytotoxic T-cells or natural killer (NK-)cells which are genetically engineered to express T-cell receptors directed against NY-ESO-1 (28).

Smooth muscle tumours

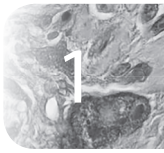
Leiomyomas

Benign smooth muscle tumours are quite frequently encountered in the smooth muscle tissue of the uterus. Uterine fibroids have an estimated incidence of ~70% of women in reproductive age and often more than one lesion is present (33, 34). The tumours are usually well-circumscribed and have a slight varying cell density of a population of uniform long spindle cells with

eosinophilic cytoplasm and elongated blunt-ended nuclei without substantial cytonuclear atypia.

Genetically, uterine leiomyomas can be classified in at least four different groups. The majority, around 70%, of the tumours harbour a mutation in mediator complex subunit 12 (*MED12*), located on chromosome Xq13.1 (33). Germline mutations in this gene were first described in patients with FG syndrome (Opitz-Kaveggia syndrome)(35). MED12 is a member of the mediator complex which is involved in regulation of transcription of RNA polymerase II-dependent genes. The molecular pathogenesis responsible for tumorigenesis in *MED12* mutated fibroids is still not completely elucidated, but it has been postulated that interaction with β-catenin and increased canonical WNT signalling; and loss of repressor element 1 silencing transcription factor (REST) and increased signalling of PI3K/AKT/mTOR might have an important role (34, 36). Nuclear β-catenin is also known to directly interact with MED12, resulting in transcription of target genes of the canonical Wnt-signalling pathway. Markowski et al found high expression of wingless-type MMTV integration site family, member 4 (WNT4), also a member of the Wnt pathway, of which expression is regulated by estrogen in Müllerian mesenchymal tissues (37). Multiple chromosomal alterations have been described in leiomyomas, and the most frequent recurrent translocation is the t(12;14)(q15;q24), fusing high-mobility group AT-hook 2 (*HMGA2*) gene to the *RAD51L1* gene (38). This is the second most common genetic aberration found in uterine leiomyomas. A small percentage of leiomyomas harbour a fumarate hydratase (*FH*) mutation or deletions affecting collagen, type IV, alpha 5 (*COL4A5*) and collagen, type IV, alpha 6 (*COL4A6*) (39). The *HMGA2*, *FH*, *COL4A5-COL4A6* and *MED12* alterations are mutually exclusive, while some other genetic aberrations including *HMGA1* alterations and 7q deletions can co-occur with *MED12* mutations (37, 40, 41). An interesting observation is the occurrence of complex chromosomal rearrangements in some *MED12* mutated and *MED12* wild-type leiomyomas (40). The complex chromosomal rearrangements fulfil to the criteria of chromothripsis, which was first thought to be exclusively associated with malignant tumours, but is also encountered in genomes of some benign lesions and congenital disorders (42, 43).

Less frequently leiomyomas appear in the cutis, named cutaneous or piloleiomyomas, and these can occur either sporadic or in association with an inherited disorder. The most well-known is Reed's syndrome or hereditary



leiomyomatosis and renal cell cancer (HLRCC), an autosomal dominant disease, in which patients are predisposed to develop cutaneous and uterine leiomyomas and papillary renal cell cancer. HLRCC is caused by a heterozygotic germline mutation in the gene encoding the Krebs cycle enzyme fumarate hydratase (*FH*), located at 1q43 (44, 45). The accumulation of high levels of fumarate leads to abberant succination of proteins by forming a chemical modification of cysteine residues to S-(2-succino)-cysteine (2SC) (46). In addition to HLRCC cutaneous leiomyomatosis has been associated with several other syndromes and conditions (47).

Leiomyosarcomas

Leiomyosarcomas are malignant tumours with features of smooth muscle differentiation and responsible for around 10 to 20% of all soft tissue sarcomas (3, 48, 49). Tumours are most frequently encountered in the 5th to 6th decade and are slightly more common in women compared to men. Based on the anatomic site the tumours can be classified in five major groups: leiomyosarcomas originating in the deep soft tissues, retroperitoneum, uterus, blood vessels and superficial dermis (3). Tumours confined to the dermis have an indolent clinical course and rarely metastasize, and are therefore better designated "atypical intradermal smooth muscle neoplasm" (50, 51).

Uterine leiomyosarcomas are considered as a separate entity, as they have a Müllerian origin and often express estrogen receptor (ER), progesterone receptor (PR) or Wilms tumour protein (WT1), supporting derivation of the genitourinary tract (52, 53). These tumours are not graded according to the FNCLCC, instead the Federation of Gynaecology and Obstetrics (FIGO) staging system is applied. The prognosis of uterine leiomyosarcoma is often poor and depends on the tumour stage. The 5-year overall survival (OS) rate is 40% (54).

Leiomyosarcomas of the deep soft tissues and the retroperitoneum are often large (>10 cm) at the moment of presentation (55). Histologically lesions are characterized by a population of long spindle cells with eosinophilic, fibrillar cytoplasm, arranged in intersecting bundles (Figure 1A and B). The cells contain cigar-shaped nuclei with often remarkable pleomorphism and hyperchomasia. Bizarre tumour giant cells, a high mitotic rate and necrosis can be present. Tumours are graded according to the FNCLCC system. Immunohistochemically, tumours frequently express smooth muscle actin (SMA), actin (HHF35) and vimentin. In addition, for the diagnosis of leiomyosarcoma focal expression of

at least one of the two smooth muscle markers, desmin and heavy-caldesmon, is more supportive for the diagnosis (3). Tumour depth, tumour size (>5cm), high histological grade, age >60 years and metastasis at presentation are indicators of a poor prognosis (48, 55, 56). The overall disease-specific 5-year survival rate is around 54-64% (48, 55).

As mentioned above, leiomyosarcomas pertain to the sarcomas with an instable genome and complex karyotype (Figure 1C). Although no common cytogenetic aberrations have been identified in these tumours, over the past decades several genes were found to be mutated. Mono-allelic or bi-allelic *TP53* alterations are found in up to 50% of leiomyosarcomas and p53 pathway inactivation is encountered in a minority of leiomyosarcomas by p14 loss (57, 58). Also loss of the tumour suppressor genes *PTEN* and *RB1* is frequently found (59, 60). Myocardin (MYOCD), a transcriptional cofactor regulating smooth muscle differentiation, is amplified in a subset of soft tissue and retroperitoneal tumours (61). *ATRX* mutations, ROR2 overexpression and MYC overexpression are correlated with poor clinical outcome (58, 62, 63). Deregulation of the apoptosis pathway can occur under influence of MYC which affects death-associated protein (DAP) kinase or via high expression of anti-apoptotic proteins of the Bcl family (64).

More recently, molecular profiling including gene expression profiling and array comparative genomic hybridization (array-CGH) identified three molecular subtypes of leiomyosarcoma (49). One subtype demonstrated high expression of multiple muscle-associated genes and is regarded as the 'muscle-enriched' group. This subtype can be identified immunohistochemically with the smooth muscle marker leiomodin 1 (LMOD1) and is associated with an improved disease specific survival (49, 65). High expression of ARL4C, a member of the GTP-binding proteins, can distinguish the leiomyosarcomas of the second molecular genetic subtype, which are associated with a worse disease specific survival (65). The third group had an intermediate outcome and until now no specific marker is discovered to differentiate this group (65).

Leiomyosarcomas are treated with the conventional chemotherapeutics used for most soft tissue sarcomas. Single-agent doxorubicin is the treatment of choice in soft tissue sarcomas and although the combination of doxorubicin with ifosfamide or with dacarbazine has been suggested, there is no randomized trial that proves the effect of these drug combinations in leiomyosarcoma. Docetaxel plus gemcitabine is active in uterine and soft

tissue leiomyosarcomas (66). Trabectedin alone has been used as second-line treatment. The combination of doxorubicin with trabectedin is being investigated, but has not been approved for first-line treatment yet (67, 68). As the driver mechanism responsible for leiomyosarcoma genesis is poorly understood and only a limited number of functional studies have been performed, at present, no targeted therapy is present for leiomyosarcomas.

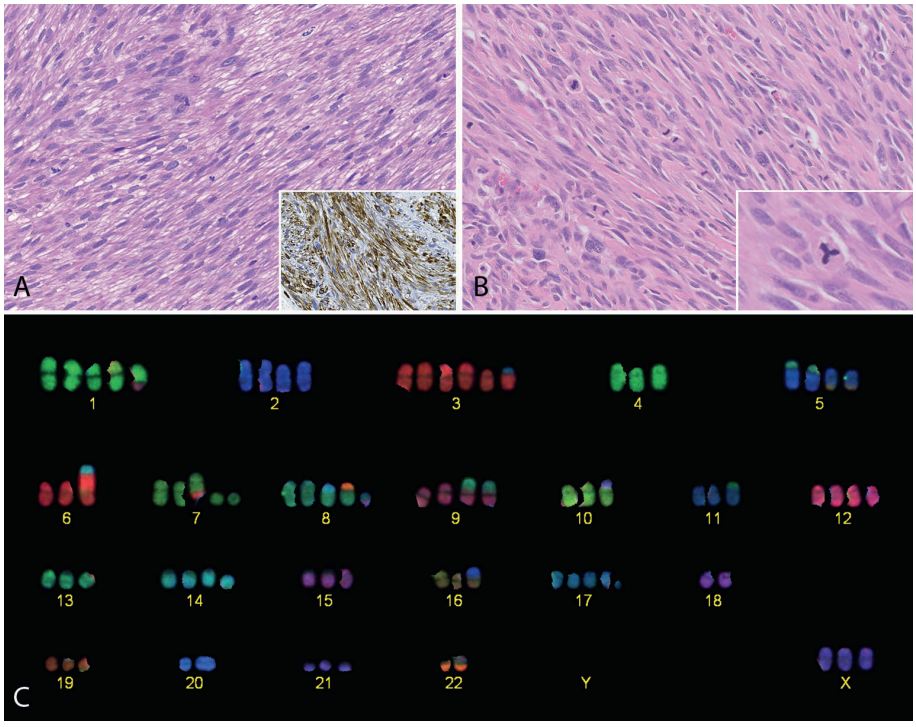


Figure 1. Histology and molecular cytogenetic representation of leiomyosarcoma.

(A) H&E stained section of a soft tissue leiomyosarcoma, grade II, with characteristic elongated spindle cells with fibrillar and eosinophilic cytoplasm arranged in bundles. A moderate variation in size and chromasia is observed and several mitotic figures are present. Inset shows desmin positivity in the majority of the tumour cells. (B) Grade III leiomyosarcoma of soft tissue with increased cytonuclear pleomorphism and atypical mitotic figures (inset). (C) Hypotetraploid karyotype of leiomyosarcoma with multiple translocations.

Liposarcomas

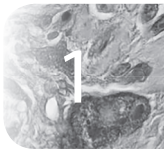
Different subtypes of liposarcomas

Liposarcomas are malignant mesenchymal neoplasms characterized by a varying degree of lipogenic differentiation and can be classified into three subtypes, each with a distinctive histopathology, genetic profile and clinical behaviour (3). The most common subtype is the well-differentiated/dedifferentiated liposarcoma, consisting of areas resembling well-differentiated fat with an often abrupt transition to high-grade, non-lipogenic areas. This subtype is characterized by amplification of various segments of 12q most consistently involving the amplification of *MDM2* and *CDK4* genes, located herein (69). Pleomorphic liposarcoma is rare and resembles histologically an undifferentiated high grade sarcoma with varying amounts of lipoblasts. Genetically this tumour has a complex karyotype without recurrent aberrations (3, 69). Myxoid liposarcoma is responsible for around 20-30% of the liposarcomas and 5% of all soft tissue sarcomas (3).

Myxoid liposarcomas

Myxoid liposarcomas most commonly arise in the deep soft tissues of the thigh (two-third of the cases). The peak age is between the 3rd and 5th decade an no gender predilection is observed (3). Tumours are histologically composed of a variably cellular proliferation of stellate spindle cells with some signet-ring lipoblasts intermingled and embedded in a myxoid matrix with a characteristic plexiform, "crow's feet", vascular network (Figure 2A and B). Areas of undifferentiated small blue round cells with scarce cytoplasm and no matrix might be present and if they compose more than 5% of the total tumour volume, the tumor is considered high grade (round cell variant) and is associated with a higher metastatic potential and a worse prognosis (70-75). The presence of necrosis, male gender, increasing age and multifocal disease are other poor prognostic factors (76-78).

The cytogenetic hallmark of myxoid liposarcoma is the reciprocal translocation t(12;16)(q13;p11) which generates a fusion transcription factor oncogene by fusing *FUS* (fused in sarcoma; also known as *TLS*) on 16p11 with *DDIT3* (DNA-damage-inducible transcript 3; also known as *CHOP*) on 12q13. To date, twelve different fusion transcripts have been described (Figure 2C)(79, 80). In less than 5% of the cases a t(12;22)(q13;q12) is identified resulting in an



EWSR1-DDIT3 fusion, of which 4 different transcripts are known (81). The fusion type in myxoid liposarcoma does not impact clinical outcome, which is similar to the translocation-associated sarcomas Ewing and synovial sarcoma (77, 81-83).

The exact mechanism via which the chimeric fusion product exerts its oncogenic potential remains to be elucidated, however it is postulated that it functions as an aberrant transcription factor by stimulating proliferation while inhibiting adipogenic differentiation (84, 85). The fusion product interferes with heterodimerization of DDIT3 with CCAAT/enhancer-binding protein-β (C/EBPβ). FUS-DDIT3 represses the promotors of C/EBPα and PPARγ, resulting in inhibition of the adipocyte differentiation program in mesenchymal cell progenitors (85). Investigation of C/EBPα and PPARγ at mRNA and protein level revealed under- and overexpression, respectively in myxoid liposarcoma tumour samples, which might be explained by the fact that both factors exert their effect at another stage of the differentiation process. Additionally, the fusion protein stimulates expression of eIF4E, which is able to downregulate the afore mentioned C/EBPα pathway and further inhibits adipogenic differentiation (85). In line with these data, the mRNA expression profile of myxoid liposarcoma is consistent with an immature adipogenic status (86). The fusion product also interacts with NF-kappa-B inhibitor zeta (NFKBIZ), which could contribute to oncogenesis through deregulation of nuclear factor-kB-controlled genes (87, 88). The expression of the *FUS-DDIT3* oncogene is tightly regulated at both the mRNA and protein levels. Also the level of the aberrant transcription factor has shown to differ between individual tumour cells. This once more illustrates that tumorigenesis is a complex process and not all regulatory mechanisms and role of downstream targets are understood yet (89).

MicroRNAs (miRNAs) are small, non-coding RNAs that are able to negatively regulate expression of target genes and they are associated with various cellular processes as differentiation and proliferation. In myxoid liposarcoma the FUS-DDIT3 fusion product is found to suppress miR-486 resulting in increase of cell growth (90). The miR-135b is higher expressed in the round cell component and associated with worse prognosis (91, 92).

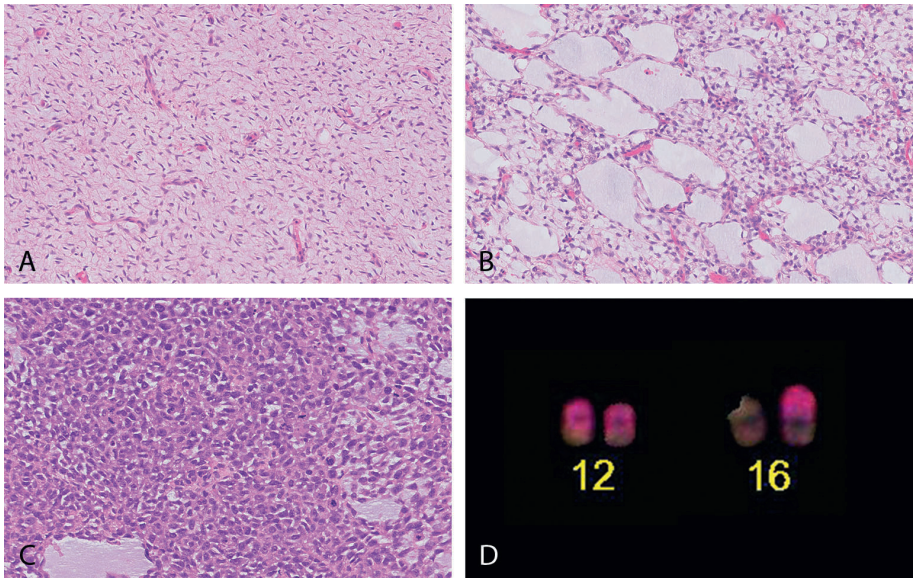
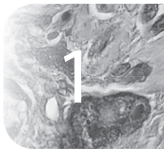


Figure 2. Histology of myxoid liposarcoma. (A) low grade, hypocellular area of stellate spindle cells set in a myxoid background. The fine branching vascular network (crow's feet) is visible. (B) moderate cell rich area with characteristic pools of myxoid matrix. (C) high grade area with closely packed round cells. (D) partial karyotype of myxoid liposarcoma with t(12;16).

Biomarker analysis of myxoid liposarcoma specimens has identified alterations in the IGF/Akt/mTOR axis, involved in crucial cellular processes such as cell survival, proliferation and growth. Overexpression of the receptor tyrosine kinases RET and IGF1R, and the ligand IGF1, are negative prognostic biomarkers known to stimulate the PI3K/Akt pathway (86, 93). *PIK3CA*, the catalytic subunit of phosphatidylinositol 3-kinase (PI3K), harbours activating mutations in 14-18% of the tumours. Loss of PTEN expression, a negative regulator of PI3K, is found in 12% of the cases and is mutually exclusive from *PIK3CA* mutations (94, 95). Increased PI3K/Akt signalling has been demonstrated by high expression of downstream targets like phosphorylated 4EBP1, PRAS40 and S6. The *PIK3CA* mutation rate, IGF1R expression and loss of PTEN were higher in tumors with a round cell component suggesting that this pathway might be involved in round cell transformation and tumor progression (86, 93). Also *TP53* mutations and reduced protein expression of p16INK/p14ARF have been identified in a subset of the tumors, especially in round cell areas (93). Hotspot mutations in the *TERT* (telomerase reverse transcriptase) promoter region were recently reported to be recurrent in myxoid liposarcoma, as well as



in several other solid tumours like for example glioblastomas, melanomas and urothelial carcinomas (96-99). These mutations lead to increased TERT mRNA and protein expression and have been implicated in telomerase dysregulation and the resultant proliferative capability of tumor cells (100). In summary, most of the above listed biomarkers are progression markers rather than myxoid liposarcoma specific markers, again highlighting the need for further research to identify tumour specific, targetable, alterations.

Surgery or a combination of surgery with radiotherapy is an effective treatment for localized disease, however 25-50% of patients develop recurrence or metastases (73, 74, 78, 101). The metastatic pattern is unconventional with spread to sites in soft tissue or bone (retroperitoneum, abdominal wall, thoracic wall, spine) occurring before the tumour metastasizes to the lungs (3, 73, 101). The 5-year disease specific survival rate depends on the histology and is for purely myxoid tumours higher than for tumours with a round cell component (around 91-100% versus 73-79%) (70, 71, 73, 74, 77, 78, 101, 102). Cases with advanced disease at time of diagnosis have a worse disease specific survival of 8% (75).

Compared to other soft tissue sarcomas, myxoid liposarcoma is quite sensitive to radiotherapy and chemotherapy. In the search for new therapeutic agents the pure form of the natural tetrahydroisoquinolone alkaloid was isolated from the Caribbean tunicate *Ecteinascidia turbinata* in 1986. This substance, known as trabectedin or ET-743 was found to be a DNA alkylating agent, it binds the DNA minor groove in GC-rich sequences and the adducts are reversible upon DNA denaturation (103). Trabectedin was shown to block the cell cycle in late S and G2 phases and it is able to disorganize the microtubule network in the cell (104). *In vitro* and *in vivo* studies demonstrated a potent antitumour activity at a relatively low dose in several solid tumours (104, 105). The first, phase 1, clinical trial with advanced, pretreated sarcoma patients revealed an effect of treatment in patients with soft tissue sarcomas (106). Over the past decade several trials were conducted and especially myxoid liposarcoma patients do respond well to treatment with trabectedin (107-109). Therefore, next to doxorubicin also trabectedin has been approved for the treatment of unresectable or metastatic myxoid liposarcoma or leiomyosarcoma (110). In the European Union trabectedin has been approved in 2007 and in the USA the Food and Drug Administration approved the use of trabectedin in 2015 after a large phase III trial trabectedin (67, 111). It was postulated that the drug is able to dislocate the oncogenic FUS-DDIT3

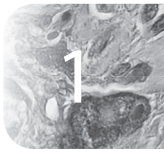
transcription factor from the DNA (112). Interestingly trabectedin has shown to have an effect in other translocation-associated sarcomas as well, such as alveolar rhabdomyosarcoma and mesenchymal chondrosarcoma (113). Worth-mentioning is that trabectedin also demonstrates antitumour activity in leiomyosarcoma and platinum-sensitive ovarium cancer, which are both, as far as we know yet, not characterized by an oncogenic chimeric transcription factor (67, 114). The exact mechanism of trabectedin has been subject of multiple studies over the past years and is currently not entirely elucidated, ongoing research demonstrated that the mechanism of action of trabectedin is complex and involves multiple aspects: binding to DNA-minor groove, interaction with homologous recombination and transcription-coupled nucleotide-excision repair (TC-NER), modulation of transcription regulation and induction of micro-environment changes (115). Recently eribulin has been registered for the treatment of liposarcomas (116).

Table 2. Clinico-pathological characteristics of leiomyosarcoma and myxoid liposarcoma.

Features	Leiomyosarcoma of soft tissue	Myxoid/round cell liposarcoma
Age range	5th - 6th decade	3rd - 5th decade
Frequency	10%-20% of soft tissue sarcomas	~5% of soft tissue sarcomas
Preferential location	Retroperitoneum and other deep soft tissues	Deep soft tissues of extremities (2/3 in thigh)
Survival	5-years survival 54%-64%	5-years survival ~10%-90%
Histomorphology	Spindle cells in intersecting bundles	Stellate cells in myxoid matrix with "crow's feet" vasculature
Histological grading	FNCLCC	Myxoid: low grade; >5% round cell: high grade
Immunohistochemistry	Desmin, h-caldesmon	No specific stain
Normal counterpart	Smooth muscle	Adipose tissue
Genomic profile	Complex karyotype with multiple non-recurrent aberrations	t(12;16)(q13;p11) or t(12;22)(q13;q12)
Cell line models	LMS04, LMS05, IB133, IB140	402-91, 1765-92, DL-221

Aim and outline of the thesis

The aim of this thesis is to gain more insight in the molecular pathology of soft tissue sarcomas, especially leiomyosarcoma and myxoid liposarcoma. As mentioned above, the group of soft tissue sarcomas constitute a large number



of different, mostly rare, neoplasms, each with their own histopathology, genetic profile and clinical features. Here we focus on two different tumours, on one hand the leiomyosarcomas characterized by a complex genetic profile and on the other hand the myxoid liposarcoma with one well-defined recurrent translocation. Over the past decade, we have gained increasing insight into the molecular pathogenesis of leiomyosarcoma and myxoid liposarcoma; however, translating this knowledge into specific therapies has been challenging. Here we focus on the molecular profile of these two soft tissue tumours and performed studies to identify potential targets for specific therapeutic strategies.

Part 1. Leiomyosarcoma

To study leiomyosarcomas we used collected tumour specimens, primary tumour cultures and four well-established cell lines (LMS04, LMS05, IB133 and IB140) that were available through our international collaborators.

In **chapter 2** we investigate the presence of *MED12* exon 2 mutations in both benign and malignant smooth muscle tumours of soft tissues as well as the mutation rate in uterine smooth muscle tumours. Also the possible role of the Wnt-signalling pathway in the pathogenesis of these tumours is investigated by use of β-catenin immunohistochemistry.

In **chapter 3** primary cell cultures of soft tissue leiomyosarcomas are characterized in depth by array-based comparative genomic hybridization and COBRA-FISH karyotyping in order to investigate the genetic alterations in leiomyosarcomas and to search for a common molecular genetic aberration. This might improve the understanding of this malignant tumour and identify candidate targets for future therapeutic regimens.

In **chapter 4** we investigated whether the anti-apoptotic Bcl family proteins play a role in the responsiveness to conventional chemotherapy in leiomyosarcomas. The expression of the Bcl proteins in a large patient series is investigated. Next, we determined whether the Bcl-2 pathway inhibitor ABT-737 sensitises leiomyosarcoma cells to chemotherapy *in vitro*.

Part 2. Myxoid liposarcoma

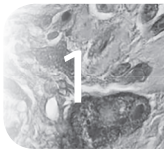
The studies on myxoid liposarcoma were performed within an international consortium, encouraged and partly funded by the Liddy Shriver Sarcoma Initiative, and led by Dr. T. Nielsen, University of British Columbia, Vancouver.

Chapter 5 is a large, multicenter, validation study in which we investigate the immunohistochemical expression of the cancer-testis antigen NY-ESO-1 (CTAG1B) in myxoid liposarcomas as well as multiple other soft tissue and bone neoplasms. The aim is to identify tumour subtypes with a high expression rate, which might benefit from NY-ESO-1 targeted immunotherapy.

Chapter 6 reports the detailed molecular cytogenetic profile, phenotypic properties and xenograft properties of a newly established myxoid liposarcoma cell line (DL-221). Reliable *in vitro* and *in vivo* models are of pivotal importance to investigate novel therapies, and to study the mechanisms of action and causes of resistance. Although myxoid liposarcoma is a relatively common soft tissue sarcoma subtype, only few model systems are available to study the disease. Thus far only two cell lines were available (402-91 and 1765-92), both of which are immortalized by transfection with the SV40 large T-antigen. One of the goals of the consortium was to increase the number of available models. We were able to establish one new cell line (DL-221) with xenograft model, which is so far the only spontaneously immortalized cell line model of myxoid liposarcoma.

In **chapter 7** we use this newly established cell line, in addition to the two cell lines that were already available, to perform a high-throughput drug screen exploring the effectivity of 273 different drugs. Our aim is to identify auspicious candidate targets which might serve as a new therapeutic option for myxoid liposarcoma.

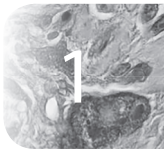
Finally, **chapter 8** summarizes and discusses the results of the studies described in the preceding chapters.



References

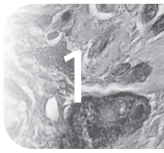
1. Gatta G, van der Zwan JM, Casali PG, et al. Rare cancers are not so rare: the rare cancer burden in Europe. Eur J Cancer. 2011;47(17):2493-511.
2. Group EESNW. Soft tissue and visceral sarcomas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2014;25 Suppl 3:iii102-12.
3. Fletcher CDM, Bridge JA, Hogendoorn PCW, et al. World Health Organisation Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. 4th ed. Lyon: IARC Press; 2013.
4. Honore C, Meeus P, Stoeckle E, et al. Soft tissue sarcoma in France in 2015: Epidemiology, classification and organization of clinical care. J Visc Surg. 2015;152(4):223-30.
5. Myhre-Jensen O. A consecutive 7-year series of 1331 benign soft tissue tumours. Clinicopathologic data. Comparison with sarcomas. Acta Orthop Scand. 1981;52(3):287-93.
6. Ray-Coquard I, Montesco MC, Coindre JM, et al. Sarcoma: concordance between initial diagnosis and centralized expert review in a population-based study within three European regions. Ann Oncol. 2012;23(9):2442-9.
7. Trojani M, Contesso G, Coindre JM, et al. Soft-tissue sarcomas of adults; study of pathological prognostic variables and definition of a histopathological grading system. Int J Cancer. 1984;33(1):37-42.
8. Taylor BS, Barretina J, Maki RG, et al. Advances in sarcoma genomics and new therapeutic targets. Nat Rev Cancer. 2011;11(8):541-57.
9. Bridge JA. The role of cytogenetics and molecular diagnostics in the diagnosis of soft-tissue tumors. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2014;27 Suppl 1:S80-97.
10. Hameed M. Molecular diagnosis of soft tissue neoplasia: clinical applications and recent advances. Expert review of molecular diagnostics. 2014;14(8):961-77.
11. Roukos V, Misteli T. The biogenesis of chromosome translocations. Nature cell biology. 2014;16(4):293-300.
12. Mitelman F, Johansson B, Mertens F. The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer. 2007;7(4):233-45.
13. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
14. Turc-Carel C, Limon J, Dal Cin P, et al. Cytogenetic studies of adipose tissue tumors. II. Recurrent reciprocal translocation t(12;16)(q13;p11) in myxoid liposarcomas. Cancer genetics and cytogenetics. 1986;23(4):291-9.
15. Aman P, Ron D, Mandahl N, et al. Rearrangement of the transcription factor gene CHOP in myxoid liposarcomas with t(12;16)(q13;p11). Genes Chromosomes Cancer. 1992;5(4):278-85.
16. Marino-Enriquez A, Fletcher CD. Shouldn't we care about the biology of benign tumours? Nat Rev Cancer. 2014;14(11):701-2.
17. Oliveira AM, Perez-Atayde AR, Dal CP, et al. Aneurysmal bone cyst variant translocations upregulate USP6 transcription by promoter swapping with the ZNF9, COL1A1, TRAP150, and OMD genes. Oncogene. 2005;24(21):3419-26.
18. Lartigue L, Neuville A, Lagarde P, et al. Genomic index predicts clinical outcome of intermediate-risk gastrointestinal stromal tumours, providing a new inclusion criterion for imatinib adjuvant therapy. Eur J Cancer. 2015;51(1):75-83.
19. Stephens PJ, Greenman CD, Fu B, et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell. 2011;144(1):27-40.
20. Gronchi A, Miceli R, Shurell E, et al. Outcome prediction in primary resected retroperitoneal soft tissue sarcoma: histology-specific overall survival and disease-free survival nomograms built on major sarcoma center data sets. J Clin Oncol. 2013;31(13):1649-55.
21. Cahlon O, Brennan MF, Jia X, et al. A postoperative nomogram for local recurrence risk in extremity soft tissue sarcomas after limb-sparing surgery without adjuvant radiation. Ann Surg. 2012;255(2):343-7.

22. Blay JY, Sleijfer S, Schoffski P, et al. International expert opinion on patient-tailored management of soft tissue sarcomas. Eur J Cancer. 2014;50(4):679-89.
23. Judson I, Verweij J, Gelderblom H, et al. Doxorubicin alone versus intensified doxorubicin plus ifosfamide for first-line treatment of advanced or metastatic soft-tissue sarcoma: a randomised controlled phase 3 trial. Lancet Oncol. 2014;15(4):415-23.
24. Edmonson JH, Ryan LM, Blum RH, et al. Randomized comparison of doxorubicin alone versus ifosfamide plus doxorubicin or mitomycin, doxorubicin, and cisplatin against advanced soft tissue sarcomas. J Clin Oncol. 1993;11(7):1269-75.
25. Heinrich MC, Corless CL, Demetri GD, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol. 2003;21(23):4342-9.
26. Sanfilippo R, Dei Tos AP, Casali PG. Myxoid liposarcoma and the mammalian target of rapamycin pathway. Curr Opin Oncol. 2013;25(4):379-83.
27. Simon MP, Pedeutour F, Sirvent N, et al. Deregulation of the platelet-derived growth factor B-chain gene via fusion with collagen gene COL1A1 in dermatofibrosarcoma protuberans and giant-cell fibroblastoma. Nat Genet. 1997;15(1):95-8.
28. Burgess M, Tawbi H. Immunotherapeutic approaches to sarcoma. Curr Treat Options Oncol. 2015;16(6):26.
29. Gjerstorff MF, Andersen MH, Ditzel HJ. Oncogenic cancer/testis antigens: prime candidates for immunotherapy. Oncotarget. 2015;6(18):15772-87.
30. Chen YT, Boyer AD, Viars CS, et al. Genomic cloning and localization of CTAG, a gene encoding an autoimmunogenic cancer-testis antigen NY-ESO-1, to human chromosome Xq28. Cytogenet Cell Genet. 1997;79(3-4):237-40.
31. Pollack SM, Jungbluth AA, Hoch BL, et al. NY-ESO-1 is a ubiquitous immunotherapeutic target antigen for patients with myxoid/round cell liposarcoma. Cancer. 2012;118(18):4564-70.
32. Lai JP, Robbins PF, Raffeld M, et al. NY-ESO-1 expression in synovial sarcoma and other mesenchymal tumors: significance for NY-ESO-1-based targeted therapy and differential diagnosis. Mod Pathol. 2012;25(6):854-8.
33. Makinen N, Mehine M, Tolvanen J, et al. MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. Science. 2011;334(6053):252-5.
34. Varghese BV, Koohestani F, McWilliams M, et al. Loss of the repressor REST in uterine fibroids promotes aberrant G protein-coupled receptor 10 expression and activates mammalian target of rapamycin pathway. Proc Natl Acad Sci U S A. 2013;110(6):2187-92.
35. Risheg H, Graham JM, Jr., Clark RD, et al. A recurrent mutation in MED12 leading to R961W causes Opitz-Kaveggia syndrome. Nature genetics. 2007;39(4):451-3.
36. Kim S, Xu X, Hecht A, et al. Mediator is a transducer of Wnt/beta-catenin signaling. J Biol Chem. 2006;281(20):14066-75.
37. Markowski DN, Bartnitzke S, Loning T, et al. MED12 mutations in uterine fibroids--their relationship to cytogenetic subgroups. Int J Cancer. 2012;131(7):1528-36.
38. Schoenmakers EF, Huysmans C, Van de Ven WJ. Allelic knockout of novel splice variants of human recombination repair gene RAD51B in t(12;14) uterine leiomyomas. Cancer Res. 1999;59(1):19-23.
39. Mehine M, Kaasinen E, Heinonen HR, et al. Integrated data analysis reveals uterine leiomyoma subtypes with distinct driver pathways and biomarkers. Proc Natl Acad Sci U S A. 2016;113(5):1315-20.
40. Mehine M, Kaasinen E, Makinen N, et al. Characterization of uterine leiomyomas by whole-genome sequencing. N Engl J Med. 2013;369(1):43-53.
41. Kampjarvi K, Makinen N, Mehine M, et al. MED12 mutations and FH inactivation are mutually exclusive in uterine leiomyomas. British journal of cancer. 2016.
42. de Pagter MS, van Roosmalen MJ, Baas AF, et al. Chromothripsis in healthy individuals affects multiple protein-coding genes and can result in severe congenital abnormalities in offspring. American journal of human genetics. 2015;96(4):651-6.
43. Zhang CZ, Leibowitz ML, Pellman D. Chromothripsis and beyond: rapid genome evolution from complex chromosomal rearrangements. Genes & development. 2013;27(23):2513-30.



44. Tomlinson IP, Alam NA, Rowan AJ, et al. Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. Nature genetics. 2002;30(4):406-10.
45. Launonen V, Vierimaa O, Kiuru M, et al. Inherited susceptibility to uterine leiomyomas and renal cell cancer. Proc Natl Acad Sci U S A. 2001;98(6):3387-92.
46. Bardella C, El-Bahrawy M, Frizzell N, et al. Aberrant succination of proteins in fumarate hydratase-deficient mice and HLRCC patients is a robust biomarker of mutation status. The Journal of pathology. 2011;225(1):4-11.
47. Malik K, Patel P, Chen J, et al. Leiomyoma cutis: a focused review on presentation, management, and association with malignancy. American journal of clinical dermatology. 2015;16(1):35-46.
48. Abraham JA, Weaver MJ, Hornick JL, et al. Outcomes and prognostic factors for a consecutive case series of 115 patients with somatic leiomyosarcoma. The Journal of bone and joint surgery American volume. 2012;94(8):736-44.
49. Beck AH, Lee CH, Witten DM, et al. Discovery of molecular subtypes in leiomyosarcoma through integrative molecular profiling. Oncogene. 2010;29(6):845-54.
50. Hornick JL, Fletcher CD. Criteria for malignancy in nonvisceral smooth muscle tumors. Annals of diagnostic pathology. 2003;7(1):60-6.
51. Kraft S, Fletcher CD. Atypical intradermal smooth muscle neoplasms: clinicopathologic analysis of 84 cases and a reappraisal of cutaneous "leiomyosarcoma". The American journal of surgical pathology. 2011;35(4):599-607.
52. Patil DT, Laskin WB, Fetsch JF, et al. Inguinal smooth muscle tumors in women-a dichotomous group consisting of Mullerian-type leiomyomas and soft tissue leiomyosarcomas: an analysis of 55 cases. The American journal of surgical pathology. 2011;35(3):315-24.
53. D'Angelo E, Prat J. Uterine sarcomas: a review. Gynecol Oncol. 2010;116(1):131-9.
54. Burghaus S, Halmen S, Gass P, et al. Outcome and prognosis in uterine sarcoma and malignant mixed Mullerian tumor. Archives of gynecology and obstetrics. 2015.
55. Italiano A, Lagarde P, Brulard C, et al. Genetic profiling identifies two classes of soft-tissue leiomyosarcomas with distinct clinical characteristics. Clin Cancer Res. 2013;19(5):1190-6.
56. Gladdy RA, Qin LX, Moraco N, et al. Predictors of survival and recurrence in primary leiomyosarcoma. Ann Surg Oncol. 2013;20(6):1851-7.
57. Perot G, Chibon F, Montero A, et al. Constant p53 pathway inactivation in a large series of soft tissue sarcomas with complex genetics. Am J Pathol. 2010;177(4):2080-90.
58. Yang CY, Liau JY, Huang WJ, et al. Targeted next-generation sequencing of cancer genes identified frequent TP53 and ATRX mutations in leiomyosarcoma. American journal of translational research. 2015;7(10):2072-81.
59. Gibault L, Ferreira C, Perot G, et al. From PTEN loss of expression to RICTOR role in smooth muscle differentiation: complex involvement of the mTOR pathway in leiomyosarcomas and pleomorphic sarcomas. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2012;25(2):197-211.
60. Dei Tos AP, Maestro R, Doglioni C, et al. Tumor suppressor genes and related molecules in leiomyosarcoma. The American journal of pathology. 1996;148(4):1037-45.
61. Perot G, Derre J, Coindre JM, et al. Strong smooth muscle differentiation is dependent on myocardin gene amplification in most human retroperitoneal leiomyosarcomas. Cancer Res. 2009;69(6):2269-78.
62. Tsiatis AC, Herceg ME, Keedy VL, et al. Prognostic significance of c-Myc expression in soft tissue leiomyosarcoma. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2009;22(11):1432-8.
63. Edris B, Espinosa I, Muhlenberg T, et al. ROR2 is a novel prognostic biomarker and a potential therapeutic target in leiomyosarcoma and gastrointestinal stromal tumour. J Pathol. 2012;227(2):223-33.
64. Bialik S, Kimchi A. The DAP-kinase interactome. Apoptosis : an international journal on programmed cell death. 2014;19(2):316-28.

65. Guo X, Jo VY, Mills AM, et al. Clinically Relevant Molecular Subtypes in Leiomyosarcoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(15):3501-11.
66. Pautier P, Floquet A, Penel N, et al. Randomized multicenter and stratified phase II study of gemcitabine alone versus gemcitabine and docetaxel in patients with metastatic or relapsed leiomyosarcomas: a Federation Nationale des Centres de Lutte Contre le Cancer (FNCLCC) French Sarcoma Group Study (TAXOGEM study). The oncologist. 2012;17(9):1213-20.
67. Demetri GD, von Mehren M, Jones RL, et al. Efficacy and Safety of Trabectedin or Dacarbazine for Metastatic Liposarcoma or Leiomyosarcoma After Failure of Conventional Chemotherapy: Results of a Phase III Randomized Multicenter Clinical Trial. J Clin Oncol. 2016;34(8):786-93.
68. Pautier P, Floquet A, Chevreau C, et al. Trabectedin in combination with doxorubicin for first-line treatment of advanced uterine or soft-tissue leiomyosarcoma (LMS-02): a non-randomised, multicentre, phase 2 trial. Lancet Oncol. 2015;16(4):457-64.
69. Mertens F, Fletcher CD, Dal Cin P, et al. Cytogenetic analysis of 46 pleomorphic soft tissue sarcomas and correlation with morphologic and clinical features: a report of the CHAMP Study Group. Chromosomes and MorPhology. Genes Chromosomes Cancer. 1998;22(1):16-25.
70. Fiore M, Grosso F, Lo VS, et al. Myxoid/round cell and pleomorphic liposarcomas: prognostic factors and survival in a series of patients treated at a single institution. Cancer. 2007;109(12):2522-31.
71. Haniball J, Sumathi VP, Kindblom LG, et al. Prognostic factors and metastatic patterns in primary myxoid/round-cell liposarcoma. Sarcoma. 2011;2011:538085.
72. Kilpatrick SE, Doyon J, Choong PF, et al. The clinicopathologic spectrum of myxoid and round cell liposarcoma. A study of 95 cases. Cancer. 1996;77(8):1450-8.
73. Moreau LC, Turcotte R, Ferguson P, et al. Myxoid\round cell liposarcoma (MRCLS) revisited: an analysis of 418 primarily managed cases. Ann Surg Oncol. 2012;19(4):1081-8.
74. ten Heuvel SE, Hoekstra HJ, van Ginkel RJ, et al. Clinicopathologic prognostic factors in myxoid liposarcoma: a retrospective study of 49 patients with long-term follow-up. Ann Surg Oncol. 2007;14(1):222-9.
75. Hoffman A, Ghadimi MP, Demicco EG, et al. Localized and metastatic myxoid/round cell liposarcoma: clinical and molecular observations. Cancer. 2013;119(10):1868-77.
76. Antonescu CR, Elahi A, Healey JH, et al. Monoclonality of multifocal myxoid liposarcoma: confirmation by analysis of TLS-CHOP or EWS-CHOP rearrangements. Clinical cancer research : an official journal of the American Association for Cancer Research. 2000;6(7):2788-93.
77. Antonescu CR, Tschernyavsky SJ, Decuseara R, et al. Prognostic impact of P53 status, TLS-CHOP fusion transcript structure, and histological grade in myxoid liposarcoma: a molecular and clinicopathologic study of 82 cases. Clin Cancer Res. 2001;7(12):3977-87.
78. Nishida Y, Tsukushi S, Nakashima H, et al. Clinicopathologic prognostic factors of pure myxoid liposarcoma of the extremities and trunk wall. Clin Orthop Relat Res. 2010;468(11):3041-6.
79. Powers MP, Wang WL, Hernandez VS, et al. Detection of myxoid liposarcoma-associated FUS-DDIT3 rearrangement variants including a newly identified breakpoint using an optimized RT-PCR assay. Mod Pathol. 2010;23(10):1307-15.
80. Crozat A, Aman P, Mandahl N, et al. Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. Nature. 1993;363(6430):640-4.
81. Bode-Lesniewska B, Frigerio S, Exner U, et al. Relevance of translocation type in myxoid liposarcoma and identification of a novel EWSR1-DDIT3 fusion. Genes Chromosomes Cancer. 2007;46(11):961-71.
82. ten Heuvel SE, Hoekstra HJ, Bastiaannet E, et al. The classic prognostic factors tumor stage, tumor size, and tumor grade are the strongest predictors of outcome in synovial sarcoma: no role for SSX fusion type or ezrin expression. Appl Immunohistochem Mol Morphol. 2009;17(3):189-95.
83. Le Deley MC, Delattre O, Schaefer KL, et al. Impact of EWS-ETS fusion type on disease progression in Ewing's sarcoma/peripheral primitive neuroectodermal tumor: prospective results from the cooperative Euro-E.W.I.N.G. 99 trial. J Clin Oncol. 2010;28(12):1982-8.
84. Tan AY, Manley JL. The TET family of proteins: functions and roles in disease. J Mol Cell Biol. 2009;1(2):82-92.



85. Perez-Mancera PA, Bermejo-Rodriguez C, Sanchez-Martin M, et al. FUS-DDIT3 prevents the development of adipocytic precursors in liposarcoma by repressing PPARgamma and C/EBPalpha and activating eIF4E. PLoS One. 2008;3(7):e2569.
86. Cheng H, Dodge J, Mehl E, et al. Validation of immature adipogenic status and identification of prognostic biomarkers in myxoid liposarcoma using tissue microarrays. Hum Pathol. 2009;40(9):1244-51.
87. Goransson M, Andersson MK, Forni C, et al. The myxoid liposarcoma FUS-DDIT3 fusion oncoprotein deregulates NF-kappaB target genes by interaction with NFKBIZ. Oncogene. 2009;28(2):270-8.
88. Willems SM, Schrage YM, Bruijn IH, et al. Kinome profiling of myxoid liposarcoma reveals NF-kappaB-pathway kinase activity and casein kinase II inhibition as a potential treatment option. Mol Cancer. 2010;9:257.
89. Aman P, Dolatabadi S, Svec D, et al. Regulatory mechanisms, expression levels and proliferation effects of the FUS-DDIT3 fusion oncogene in liposarcoma. The Journal of pathology. 2016;238(5):689-99.
90. Borjigin N, Ohno S, Wu W, et al. TLS-CHOP represses miR-486 expression, inducing upregulation of a metastasis regulator PAI-1 in human myxoid liposarcoma. Biochem Biophys Res Commun. 2012;427(2):355-60.
91. Nezu Y, Hagiwara K, Yamamoto Y, et al. miR-135b, a key regulator of malignancy, is linked to poor prognosis in human myxoid liposarcoma. Oncogene. 2016.
92. Yu PY, Balkhi MY, Ladner KJ, et al. A selective screening platform reveals unique global expression patterns of microRNAs in a cohort of human soft-tissue sarcomas. Lab Invest. 2016;96(4):481-91.
93. Negri T, Virdis E, Brich S, et al. Functional mapping of receptor tyrosine kinases in myxoid liposarcoma. Clin Cancer Res. 2010;16(14):3581-93.
94. Barretina J, Taylor BS, Banerji S, et al. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. Nat Genet. 2010;42(8):715-21.
95. Demicco EG, Torres KE, Ghadimi MP, et al. Involvement of the PI3K/Akt pathway in myxoid/round cell liposarcoma. Mod Pathol. 2012;25(2):212-21.
96. Koelsche C, Renner M, Hartmann W, et al. TERT promoter hotspot mutations are recurrent in myxoid liposarcomas but rare in other soft tissue sarcoma entities. J Exp Clin Cancer Res. 2014;33(1):33.
97. Saito T, Akaike K, Kurisaki-Arakawa A, et al. promoter mutations are rare in bone and soft tissue sarcomas of Japanese patients. Molecular and clinical oncology. 2016;4(1):61-4.
98. Fan X, Wang Y, Liu Y, et al. Brain regions associated with telomerase reverse transcriptase promoter mutations in primary glioblastomas. Journal of neuro-oncology. 2016;128(3):455-62.
99. Bell RJ, Rube HT, Xavier-Magalhaes A, et al. Understanding TERT Promoter Mutations: A Common Path to Immortality. Molecular cancer research : MCR. 2016;14(4):315-23.
100. Vinagre J, Almeida A, Populo H, et al. Frequency of TERT promoter mutations in human cancers. Nat Commun. 2013;4:2185.
101. Fuglo HM, Maretty-Nielsen K, Hovgaard D, et al. Metastatic pattern, local relapse, and survival of patients with myxoid liposarcoma: a retrospective study of 45 patients. Sarcoma. 2013;2013:548628.
102. Baxter KJ, Govsyeyev N, Namm JP, et al. Is multimodality therapy necessary for the management of pure myxoid liposarcomas? A multi-institutional series of pure myxoid liposarcomas of the extremities and torso. Journal of surgical oncology. 2015;111(2):146-51.
103. Pommier Y, Kohlhagen G, Bailly C, et al. DNA sequence- and structure-selective alkylation of guanine N2 in the DNA minor groove by ecteinascidin 743, a potent antitumor compound from the Caribbean tunicate Ecteinascidia turbinata. Biochemistry. 1996;35(41):13303-9.
104. Izbicka E, Lawrence R, Raymond E, et al. In vitro antitumor activity of the novel marine agent, ecteinascidin-743 (ET-743, NSC-648766) against human tumors explanted from patients. Ann Oncol. 1998;9(9):981-7.
105. Hendriks HR, Fiebig HH, Giavazzi R, et al. High antitumour activity of ET743 against human tumour xenografts from melanoma, non-small-cell lung and ovarian cancer. Ann Oncol. 1999;10(10):1233-40.
106. Delaloge S, Yovine A, Taamma A, et al. Ecteinascidin-743: a marine-derived compound in advanced, pretreated sarcoma patients--preliminary evidence of activity. J Clin Oncol. 2001;19(5):1248-55.

107. Le Cesne A, Cresta S, Maki RG, et al. A retrospective analysis of antitumour activity with trabectedin in translocation-related sarcomas. Eur J Cancer. 2012;48(16):3036-44.

108. Blay JY, Italiano A, Ray-Coquard I, et al. Long-term outcome and effect of maintenance therapy in patients with advanced sarcoma treated with trabectedin: an analysis of 181 patients of the French ATU compassionate use program. BMC Cancer. 2013;13:64.

109. Gronchi A, Bui BN, Bonvalot S, et al. Phase II clinical trial of neoadjuvant trabectedin in patients with advanced localized myxoid liposarcoma. Ann Oncol. 2012;23(3):771-6.

110. Gordon EM, Sankhala KK, Chawla N, et al. Trabectedin for Soft Tissue Sarcoma: Current Status and Future Perspectives. Advances in therapy. 2016;33(7):1055-71.

111. Katz D, Boonsirikamchai P, Choi H, et al. Efficacy of first-line doxorubicin and ifosfamide in myxoid liposarcoma. Clin Sarcoma Res. 2012;2(1):2.

112. Forni C, Minuzzo M, Virdis E, et al. Trabectedin (ET-743) promotes differentiation in myxoid liposarcoma tumors. Mol Cancer Ther. 2009;8(2):449-57.

113. Kawai A, Araki N, Sugiura H, et al. Trabectedin monotherapy after standard chemotherapy versus best supportive care in patients with advanced, translocation-related sarcoma: a randomised, open-label, phase 2 study. Lancet Oncol. 2015;16(4):406-16.

114. Poveda A, Vergote I, Tjulandin S, et al. Trabectedin plus pegylated liposomal doxorubicin in relapsed ovarian cancer: outcomes in the partially platinum-sensitive (platinum-free interval 6-12 months) subpopulation of OVA-301 phase III randomized trial. Ann Oncol. 2011;22(1):39-48.

115. D'Incalci M, Galmarini CM. A review of trabectedin (ET-743): a unique mechanism of action. Mol Cancer Ther. 2010;9(8):2157-63.

116. Schoffski P, Chawla S, Maki RG, et al. Eribulin versus dacarbazine in previously treated patients with advanced liposarcoma or leiomyosarcoma: a randomised, open-label, multicentre, phase 3 trial. Lancet. 2016;387(10028):1629-37.

Chapter 2

MED12 exon 2 mutation analysis in different subtypes of smooth muscle tumors confirms genetic heterogeneity

Marieke A. de Graaff, Anne-Marie Cleton-Jansen, Károly Szuhai, Judith V.M.G. Bovée

Human Pathology. 2013; 44(8): 1597-1604

Abstract

Recently heterozygous mutations in exon 2 of the *mediator complex subunit 12* (*MED12*) gene have been described in 50-70% of uterine leiomyomas; the recurrent nature of these mutations suggests an important role in their pathogenesis. MED12 is involved in regulation of transcription and Wnt-signaling. So far, little is known about the pathogenesis of the different subtypes of extra-uterine leiomyomas and leiomyosarcomas. We performed mutation analysis of *MED12* and immunohistochemistry for β-catenin, using 69 tumors of 64 patients including 19 uterine-, 6 abdominal-, 9 angio-, 5 piloleiomyomas, 7 uterine- and 23 soft tissue leiomyosarcomas. In line with previous observations, 58% of uterine leiomyomas carried a *MED12* mutation. However, all other extra-uterine leiomyomas were negative with the exception of one abdominal leiomyoma with a likely primary uterine origin. Of the 30 leiomyosarcomas, only one uterine tumor harbored a mutation. A new observation is the identification of three tumors with a homozygous mutation; a monosomy X or interstitial deletion was excluded. β-catenin immunohistochemistry showed nuclear positivity in only 55% of the *MED12* mutated uterine leiomyomas suggesting the involvement of pathways other than canonical Wnt-signaling in tumorigenesis. Interestingly, 80% of *MED12* wild type sporadic piloleiomyomas displayed nuclear β-catenin positivity indicating its involvement in this leiomyoma subtype. The lack of *MED12* mutations in extra-uterine leiomyomas and leiomyosarcomas indicate that these tumors arise through a different pathway, emphasizing the genetic heterogeneity of smooth muscle tumors.

Keywords:
β-catenin, leiomyoma, leiomyosarcoma, *MED12* mutation, Wnt-pathway.

Introduction

Leiomyomas are benign tumors (fibroids) consisting of a proliferation of smooth muscle cells. The majority of these tumors arise in the uterus and by age 45 60% of women has at least one leiomyoma [1]. The tumors can reach a substantial size and multiple tumors are often present; leading to a considerable morbidity. Surgery is the mainstay of treatment to relieve symptoms [1,2]. At histological examination the lesions are composed of interlacing fascicles of spindle shaped cells with eosinophilic cytoplasm and blunt-ended cigar shaped nuclei [3].

An identical histology can be seen in smooth muscle tumors in the abdomen and in the dermis (piloleiomyoma), although these leiomyomas are far less frequent compared to the uterine lesions. In angioleiomyomas smooth muscle cells spin off from vessel walls, presenting as a small and painful mass located in the subcutis of the lower extremities. Simple surgical excision is an adequate treatment for symptomatic cases [4-6].

Uterine leiomyomas are genetically heterogeneous since approximately 30-50% of the tumors display chromosomal aberrations involving (balanced) translocations of chromosomes t(6;14) or t(12;14) and deletions of chromosome 7 [7-9]. The *HMGA1* and *HMGA2* genes mapping to chromosome 6p21.31 and 12q14.3 respectively are frequently involved in the breakpoints; and as no partner gene on chromosome 14 has been identified so far, the deregulation of *HMGA1/2* is believed to be important for tumor development [10-12]. Mäkinen et al. recently identified *MED12* mutations in 70% (159 of 225) of uterine leiomyomas of 80 different patients in a study using next generation sequencing [13]. All mutations were heterozygous and resided in exon 2 and each tumor only contains one mutation, suggesting that these alterations contribute to tumorigenesis. *MED12* changes and 12q14 rearrangements turned out to be mutually exclusive [14]. In five consecutive studies mutation rates of 52.2% (35/67), 58.8% (47/80), 66.6% (6/9), 67% (100/148) and 50% (14/28) were identified, including a study with African patients [14-18]. The occurrence of *MED12* mutations in other leiomyoma subtypes has not been investigated.

MED12 consists of 45 exons and is located at chromosome Xq13.1. It is a subunit and activator of the CDK8-module, which includes in addition to MED12 also MED13, CCNC and CDK8. The CDK8-module associates with 25 other complexes in the highly conserved Mediator complex, which is responsible

for the transcription of all RNA polymerase II dependent genes and functions as a transcription factor [19-21]. The CDK8-module controls global and gene specific transcription and regulates specific transcription of members of the Wnt-pathway. MED12 is involved in the binding of transcription factors to the Mediator complex and it is crucial for activation of the CDK8 histone kinase [20-22]. Moreover, MED12 interacts directly with β-catenin, a member of the Wnt-pathway, and mediates β-catenin-activated transcription [23].

Leiomyosarcomas are malignant tumors originating from smooth muscle cells. They account for 10-15% of the soft tissue sarcomas and predilection sites are the retroperitoneum, the large blood vessels and the soft tissues of the lower extremities [6,24]. They can also arise in the uterus with an estimated incidence of 0.40 per 100,000 women per year [25]. Although leiomyomas of the uterus are quite common, uterine leiomyosarcoma is very rare. There is not much known about the pathogenesis of this group of highly malignant tumors. Cytogenetic analysis reveals often complex karyotypes with multiple non-recurrent genetic aberrations, probably resulting from genetic instability [6,26,27]. A tumor progression model has been postulated for leiomyoma and leiomyosarcoma as both originate from smooth muscle cells; with an estimated frequency of <0.1%, however such a relation is still controversial as no convincing evidence is present [26]. In a recent study, mutations in *MED12* have been found in 2 of 10 uterine leiomyosarcomas [17], while the occurrence in leiomyosarcoma of soft tissue has, so far, not been investigated.

To determine a possible role for *MED12* mutations both in benign and malignant extra-uterine smooth muscle tumors, we evaluated the presence of mutations in different smooth muscle tumor subtypes occurring in the soft tissues. As MED12 has both direct and indirect interactions with β-catenin we performed immunohistochemistry for β-catenin to study activity of canonical Wnt-signaling and correlate expression to mutation status.

Materials and Methods

Tissue samples

A total of 69 formalin fixed paraffin embedded (FFPE) tissue samples of smooth muscle tumors from 64 different patients were selected for DNA extraction and mutation analysis and were retrieved from the archives of the department

of Pathology at Leiden University Medical Center (Leiden, The Netherlands); including 6 abdominal leiomyomas, 9 angioleiomyomas, 5 piloleiomyomas, 19 uterine leiomyomas, 7 leiomyosarcomas of the uterus and 23 leiomyosarcomas of soft tissue (Table 1). Of one patient, three different uterine leiomyomas were included as well as a leiomyosarcoma of the femoral artery; another patient underwent surgical removal of an abdominal leiomyoma and shortly thereafter leiomyoma-related hysterectomy, both tumors were included.

Tissue microarrays (TMAs) were constructed from a panel of FFPE tumors including 7 uterine leiomyomas, 53 leiomyosarcomas (30 of which were also used for mutation analysis), 14 myxofibrosarcomas, 4 pleomorphic liposarcomas, 2 rhabdomyosarcomas, 6 undifferentiated spindle cell sarcomas and 24 undifferentiated pleomorphic sarcomas. The TMAs were constructed using a semi-automated TMA apparatus (TMA Master, 3DHistech, Budapest, Hungary) to transfer tumor punches to the recipient block. A biopsy needle of 1.5 mm was used resulting in a surface area of 1,767 mm^2 per core; all tumor samples are present in triplicates. Cores from colon, liver, placenta, prostate, skin and tonsil were included for control and orientation purposes. Using a tape-transfer system (Instrumedics, Hackensack, NJ, USA), 4-µm sections were transferred to coated glass slides. For 12 of the uterine leiomyomas and 20 extra-uterine leiomyomas regular tissue sections were obtained from paraffin blocks, as the amount of tissue was too low to punch three tissue cores.

The histological diagnosis of all samples was confirmed by reviewing the hematoxylin and eosin (HE) stained slides by a bone and soft tissue tumor specialist (JVMGB). At least one of the smooth muscle markers h-caldesmon or desmin was positive in the smooth muscle tumors. Malignant tumors were graded according to the FNCLCC grading system [6]. All samples were handled in a coded fashion, and all procedures were performed according to the ethical guidelines, "Code for Proper Secondary Use of Human Tissue in the Netherlands" (Dutch Federation of Medical Scientific Societies).

Table 1. Patient characteristics per group, including results of *MED12* mutation analysis and ß-catenin immunohistochemistry.

Tumor type	Included tumors	Gender M : F	Median age (range)	Median tumor size in cm (range)	MED12 exon 2 mutation	β-catenin nuclear staining (%)	β-catenin cytoplasmic staining (%)
Abdominal leiomyoma	6	2 : 4	62.3 (42-80)	5.8 (0.5-17.0)	1/6	0/6 (0%)	6/6 (100%)
Angioleiomyoma	9	5 : 4	52.8 (36-70)	1.6 (0.2-5.2)	0/9	0/9 (0%)	9/9 (100%)
Piloleiomyoma	5	3 : 2	44.6 (19-62)	0.8 (0.4-1.1)	0/5	4/5 (80%)	5/5 (100%)
Uterine leiomyoma	19	0 : 19	48.7 (34-71)	6.5 (1.5-20.0)	11/19	6/19 (31.6%)	18/19 (94.7%)
LMS soft tissue	23	9 : 14	58.6 (30-86)	7.3 (1.1-23.5)	0/23	3/23 (13.0%)	22/23 (95.7%)
Uterine LMS	7	0 : 7	58.0 (39-76)	12.1 (5.0-23.0)	1/7	0/7 (0%)	7/7 (100%)
LMS TMA	23	9 : 14	58.7 (23-80)	8.0 (1.0-23.0)	NA	7/23 (30.4%)	22/23 (95.7%)
PI LPS TMA	4	2 : 2	67.5 (53-82)	10.5 (4.5-26.5)	NA	1/4 (25%)	4/4 (100%)
MFS TMA	14	6 : 8	67.9 (47-91)	9.8 (2.2-20.0)	NA	3/14 (21.4%)	12/14 (85.7%)
RMS TMA	2	2 : 0	56.5 (55-58)	5.2 (5.0-5.3)	NA	1/2 (50%)	2/2 (100%)
USCS TMA	6	4 : 2	72.3 (46-89)	5.7 (2.5-8.3)	NA	6/6 (100%)	6/6 (100%)
UPS TMA	24	16 : 8	64.9 (36-94)	8.4 (2.4-17.0)	NA	9/24 (37.5%)	23/24 (95.8%)

LMS: leiomyosarcoma; PI LPS: pleomorphic liposarcoma; MFS: myxofibrosarcoma; RMS: rhabdomyosarcoma; USCS: undifferentiated spindle cell sarcoma; UPS: undifferentiated pleomorphic sarcoma; TMA: tissue microarray; NA: not available.

***MED12* mutation analysis**

Representative tumor areas from FFPE samples were selected to punch 3 cores of 0.6 mm in diameter for DNA isolation, whereas microdissection was performed on small tumors; using a total of five 10-µm thick hematoxylin stained sections for each case. A tumor percentage of >90% was achieved for all tumors. Genomic DNA was isolated using NucleoSpin tissue kit from Macherey-Nagel (Düren, Germany). DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Breda, The Netherlands). Oligonucleotide primers were designed to amplify exon 2 of *MED12* (NCBI gene ID: 9968; ChrX:70,338,406-70,357,793 [GRCh37/hg19]) with the use of Primer3 program (http://frodo.wi.mit.edu/). Primers included forward: AACAACTAAACGCCGCTTTC and reverse: TGTCCCTATAAGTCTTCCCAACC with the addition of universal M13-tails (M13 forward: TGTAAAACGACGGCCAGT and M13 reverse: CAGGAAACAGCTATGACC) to facilitate standardization of PCR and subsequent sequencing. PCR was performed on 10 ng of genomic DNA in combination with iQ SYBRgreen supermix (Bio-Rad, Veenendaal, The

Netherlands) using a CFX96 Real-Time PCR Detection System (Bio-Rad). All PCR products were purified with the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). Purified PCR products were electrophoresed using a 0.8% agarose gel in order to separate amplified fragments as a quality control. Direct sequencing of both the forward and reverse PCR products was performed by Macrogen (Macrogen Corporation, Amsterdam, The Netherlands) and analysis of the sequence graphs was performed both manually and with the computer program Mutation Surveyor V4.0.4 (Softgenetics, State College, PA, USA).

Immunohistochemistry

Immunohistochemistry (IHC) was performed using the β-catenin antibody (clone 14, Becton Dickinson Transduction Laboratories, San Diego, CA, USA) in a 1:4000 dilution, following antigen retrieval by heating in citrate buffer. Signal detection was performed with Envision+ (DAKO Cytomation, Carpinteria, CA, USA) and the chromogen 3,3'-diaminobenzidine according to manufacturer's instructions. Nuclear and cytoplasmatic or membranous staining were separately scored on a 0-7 scale based on the staining intensity (1=weak, 2=moderate, 3=strong) added to the percentage of positive cells (1=0-24%, 2=25-49%, 3=50-74% and 4=75-100%) by two independent observers and a score of >0 was considered positive [28]. IHC pictures were taken using the Pannoramic MIDI scanner (3DHistech).

Fluorescence *in situ* hybridization (FISH)

In order to determine the copy number status of *MED12* loci on the X chromosome, a two color interphase FISH was performed using a probe mix containing bacterial artificial chromosome (BAC) RP11-523P2 clone labeled with Biotin-dUTP and detected with streptavidin conjugated to Cy3 (Roche diagnostics GmbH, Mannheim, Germany); a direct labeled centromere X probe (FITC-dUTP) (Roche) was used as a reference. Probes were hybridized on a 4 µm thick FFPE section from cases with a detected homozygous *MED12* mutation. Probe labeling and hybridization reaction were performed using standard protocols published earlier [29].

Statistical analysis

The PASW 18.0 for Windows software package (SPSS Inc., Chicago, IL, USA) was used to analyze the results. The statistical significance of differences between

two groups was assessed by the independent-samples *t*-test and the one-way ANOVA-test was used for comparisons of more than two groups, a p-value of <0.05 was considered statistically significant.

Results

MED12 mutation analysis

Mutations were absent in all extra-uterine leiomyomas with the exception of abdominal leiomyoma L4041 in the pelvic region of a 42-year-old female harboring a homozygous c.[130G>A];[130G>A] base substitution. The tumor cells expressed the estrogen receptor alpha, suggesting an uterine origin of the tumor. The patient underwent hysterectomy due to a large uterine leiomyoma a few months earlier and this tumor L3981 showed a heterozygous c.131G>C mutation (Figure 1a-c; Supplementary Table 1). Interphase FISH showed the presence of two copies of both the centromere X signal (green) and the RP11-523P2 probe signal (red) in the majority of the cells (Figure 1e) excluding the possibility of a hemizygous deletion of one allele leading to the detected homozygous mutation.

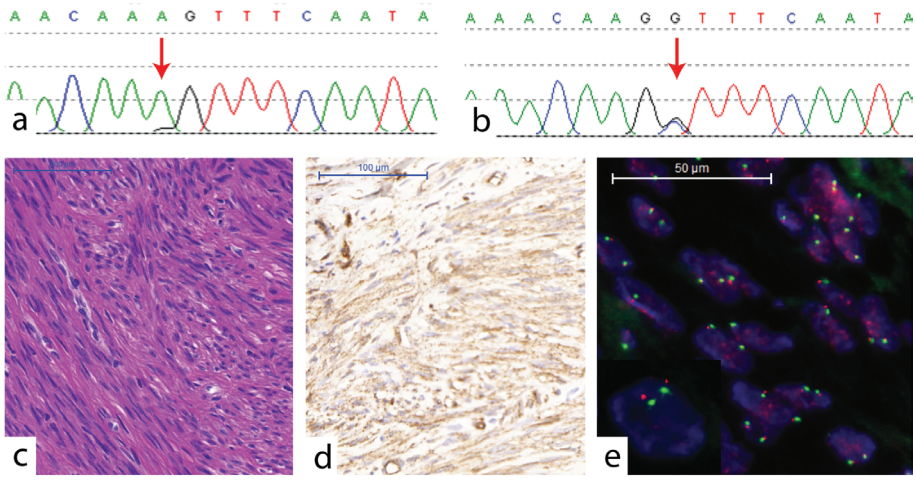


Figure 1. Histological and molecular cytogenetic results in leiomyomas.

Sequence chromatogram of homozygous c.130G>A mutation in codon 44 of the *MED12* gene in abdominal leiomyoma L4041; the altered base is indicated by a red arrow (a). Sequence graph showing a heterozygous c.131G>C alteration in the uterine leiomyoma L3981 of the same patient (b). HE stained slide of L4041 (c) and immunohistochemical staining of β-catenin showing staining of the cytoplasm

but no nuclear localization of the protein (d). FISH performed on a tissue section of mutated abdominal leiomyoma with centromere X probe (green) and BAC-probe RP11-523P2 (red) close to the *MED12* locus showing two signals in the majority of the nuclei, thereby excluding a deletion of chromosome X or the region around the *MED12* gene (e, inset).

Of the 19 uterine leiomyomas, 11 (57.9%) revealed a mutation in exon 2 of *MED12*, among which one substitution was homozygous as is confirmed with FISH. All detected base changes were missense mutations occurring at the hot spot positions of base pairs 130 and 131. Four different mutations were observed of which c.130G>A was the most frequent with an incidence of 36.4% (4/11) followed by c.131G>A and c.131G>C which were both responsible for 27.3% (3/11) of the *MED12* alterations. The c.130G>C alteration was only found once (9.1%) (Supplementary Table 1). From sample L3984 having multiple uterine leiomyomas three tumors were included, two of three leiomyomas showed a c.131G>A substitution, while c.131G>C was found in the third leiomyoma. A mutation was absent in the vascular leiomyosarcoma of the same patient.

Of the 30 leiomyosarcomas, only 1 of 7 (14.3%) uterine tumors harbored a homozygous c.[133_144del];[133_144del] deletion leading to a deletion of four amino acids p.F45_Q48del. The homozygous change was not caused by an allelic loss as interphase FISH revealed retained copies of both X chromosomes and *MED12* loci. This tumor was graded as high grade based on the nuclear pleomorphism, the mitotic index of 32 mitoses per 10 high power fields and necrotic fields [6,30]. All 23 soft tissue leiomyosarcomas were *MED12* exon 2 wild type (Table 1).

Correlation of *MED12* mutation status to clinicopathological data

Uterine leiomyomas harboring a mutation in exon 2 of *MED12* were significantly smaller compared to *MED12* wild type leiomyomas (p=0.013) with a mean size of 4.1 cm for the mutated tumors versus 9.9 cm in the *MED12* wild type lesions. We found no association between the c.130G>A and c.131G>A transitions and larger size (p = 0.708), which was reported previously by others [14]. No correlation was observed between the age at hysterectomy and the mutation status (p = 0.364) (Table 2) [13,14].

Table 2. Clinicopathological data of uterine leiomyomas in *MED12* mutated and wild type cases.

	***MED12* wild type**	***MED12* mutation**	**Statistical significance**
Median age (range)	51.5 (36-71)	46.7 (34-66)	p = 0.364
Median size (range)	9.9 (1.5-20.0)	4.1 (1.5-7.0)	p = 0.013[1]
β-catenin nuclear score (range)	0.0 (0.0)	1.7 (0.7-6.0)	p = 0.042[1]
β-catenin cytoplasmic score (range)	2.8 (0.0-5.0)	4.2 (2.7-7.0)	p = 0.050[1]

P-values marked with a [1] are statistically significant.

β-catenin immunohistochemistry

Nuclear β-catenin staining was present in 80% (4/5) of the piloleiomyomas (p=0.0001) and semi-quantitative analysis revealed a mean immunohistochemical score of 4.0, while the nuclei of all other extra-uterine leiomyomas were negative (score 0) (Figure 2a-c). All extra-uterine leiomyomas expressed β-catenin in the cytoplasm, however the cytoplasmic staining was most intense in the piloleiomyomas but not statistically significant compared to the other two subtypes (p=0.087). Nuclear staining of β-catenin was found in 54.5% (6/11) of the uterine leiomyomas carrying a *MED12* mutation with a mean immunohistochemical score of 1.7, while the nuclei of all wild type uterine leiomyomas were negative (score 0) (p=0.042) (Figure 2a). Cytoplasmic staining of β-catenin was found in 94.7% (18/19) of the uterine leiomyomas with a considerably higher expression in the mutated tumors (p=0.050), membranous staining was absent. Nuclear staining was found in 18.9% (10/53) of all leiomyosarcomas present on the TMAs. Three of 23 (13%) soft tissue leiomyosarcomas expressed β-catenin in the nucleus while the nuclei of all uterine leiomyosarcomas were negative. Cytoplasmic (96%; 51/53) as well as membranous (22.6%; 12/53) staining was however frequently observed in leiomyosarcomas. Variable nuclear expression of β-catenin was observed in 25% (1/4) of pleomorphic liposarcomas, 21.4% (3/14) of myxofibrosarcomas, 100% (6/6) of undifferentiated spindle cell sarcomas, 50% (1/2) of rhabdomyosarcomas and 37.5% (9/24) of undifferentiated pleomorphic sarcomas (Table 1).

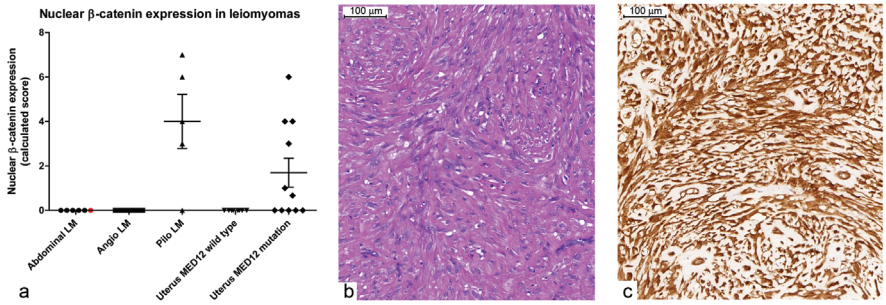


Figure 2. Nuclear ß-catenin expression in different leiomyoma subtypes.

Results for ß-catenin immunohistochemistry. Dot plot presenting results of immunohistochemical nuclear ß-catenin expression in abdominal leiomyomas, angioleiomyomas, piloleiomyomas and *MED12* wild type and mutated uterine leiomyomas, demonstrating a high expression in 80% of the piloleiomyomas and a high expression in the majority of the uterine leiomyomas with a *MED12* mutation. The abdominal leiomyoma with a mutation is marked with a red dot (a). HE stained slide (b) and positive nuclear ß-catenin labeling in one piloleiomyoma (c).

Discussion

Heterozygous mutations in exon 2 of the *mediator complex subunit 12* (*MED12*) gene have been described in the majority of uterine leiomyomas [13-18], and their recurrent nature suggests an important role in the pathogenesis of these lesions. Since the role of *MED12* mutations in extra-uterine leiomyomas, and in leiomyosarcomas is currently unclear, we investigated the occurrence of mutations in these lesions. Moreover, since MED12 is involved in regulating Wnt-signaling, we evaluated the expression pattern of β-catenin, as a read-out for canonical Wnt-signaling.

We demonstrated that *MED12* mutations were absent in all angioleiomyomas, piloleiomyomas and in 5 of 6 investigated abdominal leiomyomas. One patient presented with both an abdominal and uterine leiomyoma, and we identified a homozygous mutation in the abdominal tumor (Figure 1). Monosomy X or an interstitial deletion of the *MED12* locus was excluded using interphase FISH. The uterine leiomyoma of the same patient revealed a different mutation, confirming that both tumors developed from a different cellular clone, however, a primary uterine origin of the abdominal tumor is likely considering the expression of estrogen receptor [31].

None of the 23 soft tissue leiomyosarcomas harbored a *MED12* exon 2 alteration. Interestingly, we identified a homozygous

c.[133_144del];[133_144del] deletion in one of seven (14.3%) uterine leiomyosarcomas. This mutation has been described previously in an uterine leiomyoma [16]. Including our cases, in total 22 uterine leiomyosarcomas have been studied [15,17]. Two other uterine leiomyosarcomas were identified by Pérot et al. to harbor a heterozygous missense mutation at the hot spot region of codon 44 [17]. Thus, while *MED12* mutations are found in 50-70% of uterine leiomyomas, only 13.6% (3/22) of uterine leiomyosarcomas carry *MED12* mutations. This may suggest a decreased propensity of *MED12* mutated leiomyomas to progress to malignancy, however, it is still under debate whether uterine leiomyosarcomas are primary or arise secondarily within a preexisting benign leiomyoma, as only in sporadic cases progression from a leiomyoma towards leiomyosarcoma is confirmed [26]. Probably, only a small subset of leiomyosarcomas arise in a preexisting leiomyoma, while the majority arise primary. Alternatively, the benign tumor may create an environment which facilitates wild type smooth muscle cells to acquire more genetic aberrations and become invasive, analogous to what has been described for *EXT* mutated osteochondroma, in which wild type cells progress to secondary peripheral chondrosarcoma [32].

The detected mutation rate of 57.9% in the group of uterine leiomyomas is consistent with previous reports, in which the percentage of mutation positive tumors ranges between 50 and 70% [13-18]. The c.130G>A, c.131G>A and c.131G>C base substitutions are responsible for 84.6% (11/13) of all identified alterations in our series, consistent with those of Markowski et al. who also detected these three alterations in the majority of the cases [14]. Of note, different mutations were found in multiple leiomyomas of one patient, thereby supporting the existing evidence for the independent clonal origin of uterine leiomyomas [13,14]. None of the tumors harbors a second mutation as expected in case of pathogenic mutations. Mutated uterine leiomyomas were significantly smaller compared to wild type lesions, no other statistical significant differences were found between the groups (Table 2) [13].

Interestingly, while so far all reported mutations are heterozygous, we report for the first time homozygous mutations in three cases: in an abdominal leiomyoma, an uterine leiomyoma and in an uterine leiomyosarcoma. MED12 has a transcriptional repressive and chromatin modifying function and McGuire et al. hypothesized that the gene acts as a tumor suppressor gene [16,21]. However the absence of nonsense mutations, the presence of in-frame

deletions and the existence of hot spot mutations might be regarded as data supporting the hypothesis of activating mutations [13,17]. As the *MED12* locus is localized on the X chromosome, the predominant inactivation of the wild type allele [13] leads to the same effect as a homozygous mutation. Others have confirmed that *MED12* mutated leiomyomas predominantly express the mutated allele [13,14,16,17]. The biological relevance of the observed homozygous mutation remains to be elucidated.

Mäkinen et al. observed significant alterations of three pathways in *MED12* mutated leiomyomas, namely extra-cellular matrix receptor interaction, focal adhesion and Wnt-signaling [13]. Under normal conditions β-catenin is tightly regulated by the tumor suppressor gene APC and the membrane bound E-cadherin, which mediates contact inhibition and suppresses tumor invasion, disruption of this equilibrium can result in uncontrolled growth [33]. After stabilization β-catenin travels to the nucleus and interacts with MED12 of the Mediator complex stimulating transcription of target genes of the canonical Wnt-signaling pathway [23]. We investigated the β-catenin expression pattern by immunohistochemistry to see if activation of this pathway can be confirmed. As 80% of the piloleiomyomas displayed nuclear β-catenin, this strongly suggests that activation of the Wnt-pathway is an important mechanism in the development of these cutaneous smooth muscle tumors. However, since we showed *MED12* mutations to be absent, other mechanisms are probably involved for which alterations in β-catenin, APC or E-cadherin but also members of the CDK8 subcomplex are possible candidates. Our data suggest that angioleiomyomas develop through a different, *MED12* and Wnt-signaling independent mechanism, as no nuclear β-catenin staining or *MED12* mutation was found. Thus, our data confirm that despite morphological similarities, smooth muscle tumors at different locations arise through different genetic mechanisms.

Pérot et al. found membranous β-catenin staining of *MED12* mutated uterine leiomyomas in 66.7% (4/6) suggesting that the canonical Wnt-pathway is not activated and subsequently not responsible for uterine leiomyoma development [17]. However, a mouse model that expresses constitutively activated β-catenin in the mesenchyme of the uterus results in the development of leiomyoma-like tumors in 100% of the mice [34]. We identified in 54.5% of the mutated leiomyomas concurrent nuclear β-catenin positivity, indicating that activation of the Wnt-pathway might be important in a subset of these tumors.

Moreover, Markowski et al. identified high expression of wingless-type MMTV integration site family member (*WNT4*) in *MED12* mutated uterine leiomyomas which is a member of the Wnt-pathway. Expression of WNT4 is stimulated by estrogen in tissues originating from mesenchyme of the Müllerian duct [35]. They hypothesized that *MED12* and estrogen cooperate in the pathogenesis of uterine leiomyomas, which is consistent with our results as *MED12* mutations are exclusively found in uterine tumors and are absent in extra-uterine smooth muscle tumors. Since these extra-uterine leiomyomas are quite rare in contrast to the high incidence of uterine leiomyomas, and also occur in males being insensitive to female steroid hormones, we postulate that they might develop through a different mechanism.

In conclusion, we investigated the presence of *MED12* mutations in benign and malignant extra-uterine smooth muscle tumors to obtain insight in their pathogenesis. We demonstrate the absence of *MED12* mutations in angioleiomyomas, piloleiomyomas and in 5 of 6 abdominal leiomyomas, and conclude that *MED12* is not involved in the development of benign smooth muscle tumors outside the uterus. Nuclear ß-catenin staining indicative of active canonical Wnt-signaling was found in only half of the *MED12* mutated uterine leiomyomas. Intriguingly, a high proportion of ß-catenin activation was observed in piloleiomyomas, indicating an important role for *MED12* independent activation of Wnt-signaling in this subtype. Our results confirm the hypothesis that smooth muscle tumors are a heterogeneous group of tumors which arise through different mechanisms.

Acknowledgements

We would like to thank Daniëlle de Jong, Maayke van Ruler, Inge H. Briaire-de Bruijn and Pauline Wijers-Koster for their excellent technical assistance.

Duality of interest

The authors declare that they have no conflicts of interest.

Reference List

1. Okolo S. Incidence, aetiology and epidemiology of uterine fibroids. Best Pract Res Clin Obstet Gynaecol 2008;22:571-88.
2. Stewart EA. Uterine fibroids. Lancet 2001;357:293-8.
3. Flake GP, Andersen J, Dixon D. Etiology and pathogenesis of uterine leiomyomas: a review. Environ Health Perspect 2003;111:1037-54.
4. Weedon D, Williamson RM, Patterson JW. Smooth and skeletal muscle tumours. In: LeBoit PE, et al, editors. World Health Organization Classification of Tumours. Pathology and Genetics of Skin Tumours. Lyon: IARC Press;2006. p. 250-3.
5. Ramesh P, Annapureddy SR, Khan F, Sutaria PD. Angioleiomyoma: a clinical, pathological and radiological review. Int J Clin Pract 2004;58:587-91.
6. Fletcher CDM, Unni KK, Mertens F. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. IARC Press, Lyon edn. Lyon: IARC Press; 2002.
7. Hennig Y, Deichert U, Stern C, et al. Structural aberrations of chromosome 6 in three uterine smooth muscle tumors. Cancer Genet Cytogenet 1996;87:148-51.
8. Ishwad CS, Ferrell RE, Davare J, et al. Molecular and cytogenetic analysis of chromosome 7 in uterine leiomyomas. Genes Chromosomes Cancer 1995;14:51-5.
9. Pandis N, Heim S, Bardi G, et al. Chromosome analysis of 96 uterine leiomyomas. Cancer Genet Cytogenet 1991;55:11-8.
10. Van de Ven WJ. Genetic basis of uterine leiomyoma: involvement of high mobility group protein genes. Eur J Obstet Gynecol Reprod Biol 1998;81:289-93.
11. Schoenberg-Fejzo M, Ashar HR, Krauter KS, et al. Translocation breakpoints upstream of the HMGIC gene in uterine leiomyomata suggest dysregulation of this gene by a mechanism different from that in lipomas. Genes Chromosomes Cancer 1996;17:1-6.
12. Williams AJ, Powell WL, Collins T, Morton CC. HMGI(Y) expression in human uterine leiomyomata. Involvement of another high-mobility group architectural factor in a benign neoplasm. Am J Pathol 1997;150:911-8.
13. Makinen N, Mehine M, Tolvanen J, et al. MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. Science 2011;334:252-5.
14. Markowski DN, Bartnitzke S, Loning T, et al. MED12 mutations in uterine fibroids-their relationship to cytogenetic subgroups. Int J Cancer 2012.
15. Je EM, Kim MR, Min KO, Yoo NJ, Lee SH. Mutational analysis of MED12 exon 2 in uterine leiomyoma and other common tumors. Int J Cancer 2012.
16. McGuire MM, Yatsenko A, Hoffner L, et al. Whole exome sequencing in a random sample of North American women with leiomyomas identifies MED12 mutations in majority of uterine leiomyomas. PLoS One 2012;7:e33251.
17. Perot G, Croce S, Ribeiro A, et al. MED12 alterations in both human benign and malignant uterine soft tissue tumors. PLoS One 2012;7:e40015.
18. Makinen N, Heinonen HR, Moore S, et al. MED12 exon 2 mutations are common in uterine leiomyomas from South African patients. Oncotarget 2011;2:966-9.
19. Takagi Y, Kornberg RD. Mediator as a general transcription factor. J Biol Chem 2006;281:80-9.
20. Akoulitchev S, Chuikov S, Reinberg D. TFIIH is negatively regulated by cdk8-containing mediator complexes. Nature 2000;407:102-6.
21. Taatjes DJ. The human Mediator complex: a versatile, genome-wide regulator of transcription. Trends Biochem Sci 2010;35:315-22.
22. Knuesel MT, Meyer KD, Donner AJ, Espinosa JM, Taatjes DJ. The human CDK8 subcomplex is a histone kinase that requires Med12 for activity and can function independently of mediator. Mol Cell Biol 2009;29:650-61.
23. Kim S, Xu X, Hecht A, Boyer TG. Mediator is a transducer of Wnt/beta-catenin signaling. J Biol Chem 2006;281:14066-75.
24. Farshid G, Pradhan M, Goldblum J, Weiss SW. Leiomyosarcoma of somatic soft tissues: a tumor of vascular origin with multivariate analysis of outcome in 42 cases. Am J Surg Pathol 2002;26:14-24.

25. Koivisto-Korander R, Martinsen JI, Weiderpass E, Leminen A, Pukkala E. Incidence of uterine leiomyosarcoma and endometrial stromal sarcoma in Nordic countries: results from NORDCAN and NOCCA databases. Maturitas 2012;72:56-60.
26. Hodge JC, Morton CC. Genetic heterogeneity among uterine leiomyomata: insights into malignant progression. Hum Mol Genet 2007;16 Spec No 1:R7-13.
27. Sreekantaiah C, Davis JR, Sandberg AA. Chromosomal abnormalities in leiomyosarcomas. Am J Pathol 1993;142:293-305.
28. Bovee JVMG, Cleton-Jansen AM, Kuipers-Dijkshoorn NJ, et al. Loss of heterozygosity and DNA ploidy point to a diverging genetic mechanism in the origin of peripheral and central chondrosarcoma. Genes Chromosomes Cancer 1999;26:237-46.
29. Rossi S, Szuhai K, Ijszenga M, et al. EWSR1-CREB1 and EWSR1-ATF1 fusion genes in angiomatoid fibrous histiocytoma. Clin Cancer Res 2007;13:7322-8.
30. Trope CG, Abeler VM, Kristensen GB. Diagnosis and treatment of sarcoma of the uterus. A review. Acta Oncol 2012;51:694-705.
31. Bodner K, Bodner-Adler B, Kimberger O, Czerwenka K, Mayerhofer K. Estrogen and progesterone receptor expression in patients with uterine smooth muscle tumors. Fertil Steril 2004;81:1062-6.
32. de Andrea CE, Reijnders CM, Kroon HM, et al. Secondary peripheral chondrosarcoma evolving from osteochondroma as a result of outgrowth of cells with functional EXT. Oncogene 2012;31:1095-104.
33. Ilyas M, Tomlinson IP. The interactions of APC, E-cadherin and beta-catenin in tumour development and progression. J Pathol 1997;182:128-37.
34. Tanwar PS, Lee HJ, Zhang L, et al. Constitutive activation of Beta-catenin in uterine stroma and smooth muscle leads to the development of mesenchymal tumors in mice. Biol Reprod 2009;81:545-52.
35. Deutscher E, Hung-Chang YH. Essential roles of mesenchyme-derived beta-catenin in mouse Mullerian duct morphogenesis. Dev Biol 2007;307:227-36.

Supplementary Information

Supplementary Table 1. Patient data and somatic *MED12* mutations observed in the series of 69 smooth muscle tumors.

Sample ID	Age at diagnosis	Tumor type	Tumor size (cm)	Mutation type	Location	Nucleotide change	Predicted protein change
L3980	41	LM uterus	7.0	missense	exon 2	c.130G>A	p.G44S
L3871	46	LM uterus	4.0	missense	exon 2	c.131G>C	p.G44A
L3872	49	LM uterus	5.9	missense	exon 2	c.130G>C	p.G44R
L3875	40	LM uterus	7.0	missense	exon 2	c.130G>A	p.G44S
L3983	46	LM uterus	2.3	missense	exon 2	c.131G>A	p.G44D
L3985	66	LM uterus	1.5	missense	exon 2	c.130G>A	p.G44S
L3974	34	LM uterus	2.0	missense	exon 2	c.[130G>A]; [130G>A]	p.G44S
L3984-G[1]	50	LM uterus	1.7	missense	exon 2	c.131G>A	p.G44D
L3984-I[1]	50	LM uterus	2.5	missense	exon 2	c.131G>C	p.G44A
L3984-J[1]	50	LM uterus	5.5	missense	exon 2	c.131G>A	p.G44D
L3981[2]	42	LM uterus	5.2	missense	exon 2	c.131G>C	p.G44A
L4041[2]	42	LM abdomen	17.0	missense	exon 2	c.[130G>A]; [130G>A]	p.G44S
L3976	50	LMS uterus	17.0	deletion	exon 2	c.[133_144del]; [133_144del]	p.F45_Q48del

The three and two samples marked with [1] and [2] respectively belong to the same patients.

Chapter 3

A translocation t(6;14) in two cases of leiomyosarcoma: Molecular cytogenetic and array based comparative genomic hybridization characterization.

Marieke A. de Graaff, Daniëlle de Jong, Inge H. Briaire-de Bruijn, Pancras C.W. Hogendoorn, Judith V.M.G. Bovée, Károly Szuhai

Cancer Genetics. 2015; 208(11): 537-544.

Abstract

Leiomyosarcomas are malignant mesenchymal tumors recapitulating smooth muscle cell differentiation. Tumors are characterized by a genetic heterogeneity with complex karyotypes without a tumor-specific genetic aberration. Their pathobiology is still poorly understood and no specific targeted treatment is currently available for these aggressive tumors. For six leiomyosarcomas cells were cultured and analyzed by COBRA-FISH karyotyping. A t(6;14)(p;q) was identified in two cases. FISH breakpoint mapping of case L1339 reveals a breakpoint at chromosome 6p21.31 close to *HMGA1* and a small deletion was observed on the distal side of the gene. A small homozygous deletion was also found in the breakpoint region of chromosome 14q24.1 involving *ACTN1*. The second case revealed a der(6) t(6;14)(p21.1;q21.3), with a duplication adjacent to the breakpoint at chromosome 6. Confirmatory FISH revealed a second leiomyosarcoma with an aberration at 14q24.1. Alterations at this locus are found in 5% (2/39) of the leiomyosarcomas in this study. The other identified breakpoints appear to be non-recurrent, as they were not detected in other leiomyosarcomas, uterine leiomyomas, undifferentiated spindle cell sarcomas or undifferentiated pleomorphic sarcomas.

Keywords:
Leiomyosarcoma, soft tissue sarcoma, translocation, FISH, array-CGH, HMGA1.

Introduction

Leiomyosarcomas are malignant tumors originating from smooth muscle cells and responsible for 10-15% of adult soft tissue sarcomas. Tumors show a varying degree of smooth muscle differentiation which is inversely proportional to tumor grade. Histologically low grade tumors are composed of sharply demarcated bundles of spindle cells intersecting at right angles, whereas high grade lesions might contain poorly differentiated regions with nuclear pleomorphism and atypical mitoses. Areas of myxoid change, epithelioid morphology, fibrosis, hemorrhage or necrosis can be present. Smooth muscle differentiation can be detected with desmin and h-caldesmon immunohistochemistry. The retroperitoneum is the most common location but the large blood vessels, with a predilection for the vena cava inferior, the uterus and other soft tissue sites as the lower extremities are also frequently involved [1]. Lesions of the retroperitoneum and blood vessels are encountered more frequently in women; no gender predilection is present for other soft tissue locations. Leiomyosarcomas are responsible for a substantial morbidity and mortality since they are characterized by aggressive behavior, with a 5-year overall survival (OS) of 64% [2;3]. A high tumor grade, deep tumor location and large tumor size (>5 cm) were independent adverse prognostic factors for metastatic disease and overall survival [2-4]. Wide surgical excision remains the mainstay of treatment often in combination with radiation or conventional chemotherapy. No specific targeted treatment against leiomyosarcomas is at the moment available as the pathophysiology remains still poorly understood [5].

Genetically leiomyosarcomas are characterized by complex karyotypes with numerous reported non-recurrent and non-specific aberrations which result from a high degree of genetic instability [1;6;7]. Although gains and losses of many different chromosomes have been reported and the inter-tumor heterogeneity is extensive, also some similarities are described. Both cytogenetic and array-based comparative genomic hybridization (array-CGH) analyses have revealed recurrent involvement of losses of 1p, 2p, 10q, 11q, 13q, 16q and 17p and gains of 17q and 17p [2;8]. The main objective of this study is to investigate the genetic alterations in leiomyosarcomas and to search for a common molecular genetic aberration in order to improve the understanding of this malignant tumor and to identify prognostic factors or candidate targets for future therapeutic regimens. We identified two cases with t(6;14) by using

state-of-the-art molecular karyotyping tools and fine mapped breakpoint regions were tested on a tissue microarray (TMA) consisting of a large panel of leiomyosarcomas, uterine leiomyomas, undifferentiated spindle cell sarcomas and undifferentiated pleomorphic sarcomas using interphase fluorescence *in situ* hybridization (FISH).

Materials and Methods

Cell culture and COBRA-FISH

Tumor specimens were collected directly after surgery and manually dissected in small fragments. After the addition of IMDM, supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco, Life Technologies, Bleiswijk, The Netherlands), they were placed in a humidified 5% CO_2 incubator at 37°C. Cells were cultured for several passages and harvested after 25 min incubation with Calyculin A (Sigma-Aldrich, Zwijndrecht, The Netherlands). Subsequently cells were placed in a hypotonic 0.075 M KCl solution followed by three fixation steps of methanol/glacial acetic acid (4:1). Slides with metaphase spreads were made in a conditioned chamber (25°C, 50% humidity; Thermotron, Holland, MI, USA).

Combined binary ratio labeling fluorescence *in situ* hybridization (COBRA-FISH) karyotyping was performed on slides with metaphase spreads of six leiomyosarcomas, no conventional G-banding studies were performed. A detailed protocol on the pre- and post-hybridization treatments has been published earlier [9]. A DMRA (Leica Microsystems, Rijswijk, The Netherlands) fluorescence microscope was used to capture images, which were analyzed with the in house developed software tool ColorProc (LUMC, Leiden, The Netherlands). Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN, 2013)[10]. In three leiomyosarcomas no detectable cytogenetic abnormalities were found in the analyzed cells. Tumor cells and fibroblasts show a similar, spindle cell morphology *in vitro,* thereby making it difficult to discriminate them morphologically. The leiomyosarcomas with a seemingly normal karyotype might have contained a mix in which fibroblast have overgrown the tumor cells, these three cases are therefore excluded for further analysis.

FISH breakpoint mapping

Two-color FISH was performed to investigate the exact breakpoints of chromosomes 6p and 14q of two selected leiomyosarcoma cases. To detect possible rearrangements of the region of *HMGA1* on chromosome 6p21 bacterial artificial chromosomes (BAC) clones, RP3-349A12, proximal to the breakpoint, and RP11-936A3, distal to the breakpoint, were selected. BAC clones used in the fine mapping of the deletion breakpoints on chromosome 6 are RP11-175A, RP5-1077I5, RP3-468B3 and RP11-974K1. On region 14q24, around the *ACTN1* gene, RP11-204K16 was selected proximal and BAC clone RP11-486O13 was located distal from the breakpoint. Other BAC clones used in the fine mapping of the breakpoint on chromosome 14 are RP11-226F19 and RP11-565I3. All BAC clones were obtained from Wellcome Trust, Sanger Institute. Probes were directly labeled with FITC or Cy3 using nick translation labeling reaction. Probes were hybridized overnight on metaphase slides using a protocol otherwise identical to the COBRA-FISH protocol as described above.

Table 1: Clinicopathological and COBRA-FISH karyotyping results of three leiomyosarcoma cases.

Case	Age/ Sex	Tumor grade	Tumor size (cm)	Location	Histological features	Karyotype
L1091	70/M	3	8x7x7	Gastrocnemius muscle	Myxoid	46,XY,t(6;7)(p;p) [3]/46,XY[15]*
L1339	39/F	N/A	9x7	Uterus	Regular/ Spindle cell	45,XX,der(1;13)t(1;13) (q10;q10), t(2;10) (q;q),t(6;14)(p21.31;q24.1-24.2),der(13)t(6;13) (q?;q10),-14[6]
L1814	56/F	3	10.5x5x4.3	Hamstring muscle	Epithelioid	81,XXXX,del(X)(q),der(1;10) (q10;q10),der(1)t(1;9) (p10;p?),der(3;6) (q10;p10),+der(3)t(3;17) (q10;p10),-4,der(5)t(5;8) (q10;p10)x3, t(5;16) (q;p),+der(5)t(5;16)(q;p),-6,der(6)t(6;14)(p21.1;q21.3) hsr(6)(p?), der(7) t(6;7) (q?;q?) ,i(8)(q10)x2,der(8) t(8;22)(q10;q10), der(8),der(9)t(8;9)(q;p) x2,-10,-10,-11, der(11) t(1;11) (p;p),-13,+der(14) t(8;14)(q10;q10),-15,der(15)t(15;18)(p10 ;p?),-16,-18,-18,-19,-20,-22, der(22)t (8;9;22)(q;p;q)[3]

Abbreviation: N/A (not applicable). Uterine leiomyosarcomas are not FNCLCC-graded according to the WHO.

Array-Comparative Genomic Hybridization Analysis

Genomic DNA was isolated from frozen tumor sections of the two selected cases with a Nucleospin DNA extraction protocol (Macherey-Nagel, Düren, Germany). Tumor content was confirmed to exceed 80-90% by histologic evaluation. DNA labeling and purification were done according to the BioPrime Total Genomic Labeling System (Invitrogen); labeling efficiency was calculated with a Nanodrop ND-1000 spectrophotometer measuring A_{260} (DNA), A_{550} (Cy3) and A_{649} (Cy5). Array-based comparative genomic hybridization was performed using oligonucleotide-based human genome CGH microarray 4x44 k (Agilent Technologies, Santa Clara, CA, USA) at 65°C for 40 hr. Slides were washed according to the manufacturer's protocols and scanned using a Agilent microarray scanner with a 5 µm scan resolution. Images were imported to Feature Extraction Software and Genomic Workbench (Agilent) with human reference genome and standard settings for analysis. Commercially available healthy male or female DNA (Promega, Madison, WI, USA) was used as reference DNA. Test and reference samples were hybridized as a gender mismatch to illustrate the dynamic range of hybridization at the X and Y chromosomes.

Tissue microarray

A TMA with a broad selection of soft tissue tumors diagnosed at the Department of Pathology of the Leiden University Medical Center (LUMC), was used for confirmatory FISH analysis [11]. In this study the evaluable cases included 37 leiomyosarcomas, 17 undifferentiated pleomorphic sarcomas, 4 undifferentiated spindle cell sarcomas and 4 uterine leiomyomas. The histological diagnosis of all samples was confirmed by reviewing the hematoxylin and eosin (H&E) stained slides by a specialized bone and soft tissue tumor pathologist (JVMGB, PCWH). At least one of the smooth muscle markers h-caldesmon or desmin was positive in the leiomyosarcomas. The included leiomyosarcomas were negative for HMB45, which is a marker positive in PEComas. Soft tissue sarcomas were graded according to the FNCLCC grading system as accepted by the WHO classification [1]. Exclusion criteria were pretreatment with neoadjuvant chemotherapy or radiotherapy and radiation-induced sarcomas. All samples were handled in a coded fashion and all procedures were performed according to the ethical guidelines, "Code for Proper Secondary Use of Human Tissue in the Netherlands" (Dutch Federation of Medical Scientific Societies).

Confirmatory TMA FISH and scoring

Four different sets of BAC clones were used to screen the samples on the tissue microarray for a recurrent breakpoint as that of the two index cases (Supplementary Table 1). For the interphase FISH on TMA sections probes were labeled with biotin-dUTP or digoxigenin-dUTP using a nick translation labeling reaction. Biotin and digoxigenin labeled probes were visualized by indirect detection with Cy3-labeled streptavidin (1:500; Sigma), FITC-labeled mouse antidigoxigenin antibody (1:250; Sigma) and FITC-labeled rabbit anti-mouse antibody (1:1000; Sigma). A detailed protocol of two-color FISH on formalin-fixed, paraffin-embedded (FFPE) tissue sections has been published previously [12]. For digital archiving and annotation, all slides used for various staining procedures were scanned with the Pannoramic MIDI scanner (3DHistech, Budapest, Hungary) using brightfield or fluorescent settings for H&E sections or interphase FISH staining, respectively. Scanned images were visualized using the Pannoramic Viewer (v15.0), scoring was performed visually. For the TMA slides, at least 50 nuclei were evaluated per core with retained tissue and good signals.

Results

Clinicopathological data and COBRA-FISH karyotyping

In this study cultured tumor cells from three leiomyosarcoma patients were suitable for cytogenetic analysis. Two patients were diagnosed with a high grade, grade 3 according to the FNCLCC grading system, leiomyosarcoma of soft tissue located in the muscles of the lower limb. One patient presented with a uterine leiomyosarcoma, which was staged according to the FIGO criteria [13;14]. All tumors expressed desmin using immunohistochemistry (Table 1).

The uterine leiomyosarcoma L1339 harbored a diploid karyotype with translocations involving several chromosomes: 45,XX,der(1;13)t(1;13)(q10;q10),t(2;10),t(6;14)(p21.31;q24.1-24.2),der(13)t(6;13)(q?;q10),-14[6]. Soft tissue leiomyosarcoma L1814 displayed a complex, hypotetraploid karyotype with multiple chromosomal rearrangements and whole chromosome gains and losses: 81,XXXX,del(X)(q),der(1;10)(q10;q10),der(1)t(1;9)(p10;p?),der(3;6)(q10;p10),+der(3)t(3;17)(q10;p10),-4,der(5)t(5;8)(q10;p10)x3,t(5;16)(q;p),+der(5)t(5;16)(q;p),-6,der(6)t(6;14)(p21.1;q21.3)hsr(6)(p?),der(7)t(6;7)

(q?;q?),i(8)(q10)x2,der(8)t(8;22)(q10;q10),der(8),der(9)t(8;9)(q;p)x2,-10,-10,-11,der(11)t(1;11)(p;p),-13,+der(14)t(8;14)(q10;q10),-15,der(15)t(15;18)(p10;p?),-16,-18,-18,-19,-20,-22,der(22)t(8;9;22)(q;p;q)[3] (Figure 1).

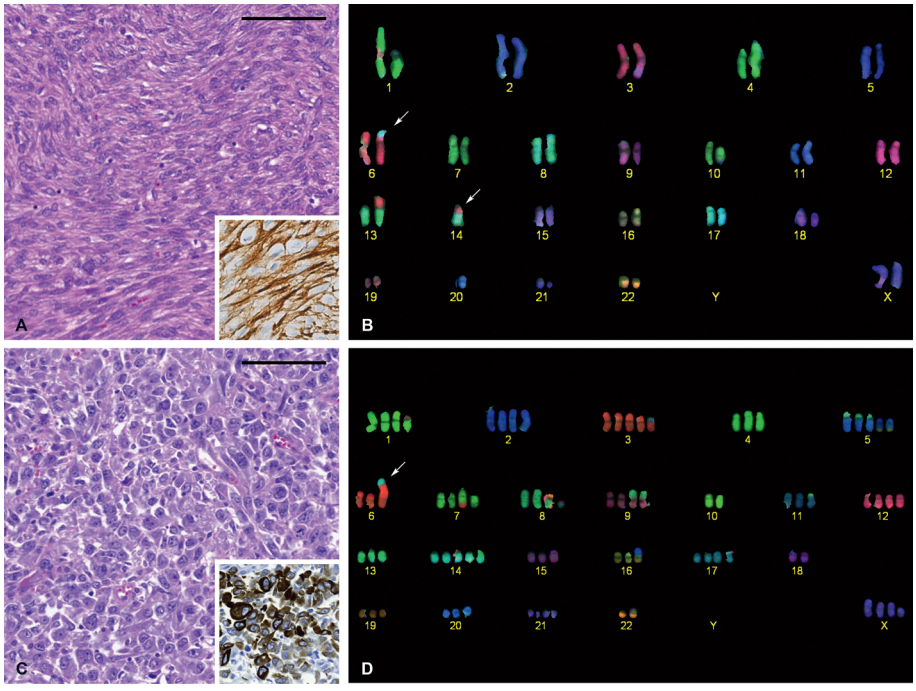


Figure 1. Histology and molecular cytogenetic results of two leiomyosarcoma cases.

H&E stained section of uterine leiomyosarcoma L1339 showing intersecting bundles of spindle cells, inset shows immunoreactivity for h-caldesmon, scale bar represents 100 µm (A). COBRA-FISH karyotype of L1339, the balanced t(6;14)(p21.31;q24.1-24.2) is indicated with white arrows (B). H&E stained section of grade 3 leiomyosarcoma L1814 with nuclear pleomorphism and multiple mitotic figures. Tumor cells exhibit epithelioid morphology with eosinophilic cytoplasm, inset shows a focal strong positive desmin staining (C). Hypotetraploid karyotype of L1814 with several translocations, including the der(6)t(6;14)(p21.1;q21.3) indicated by an arrow (D).

FISH breakpoint mapping and array-CGH profile

A seemingly balanced translocation was observed in case L1339. The breakpoint regions on chromosomal arms 6p and 14q were compatible with translocation breakpoint regions described in uterine leiomyoma involving the *HMGA1* and the *RAD51L1* genes on chromosome 6 and chromosome 14, respectively [15]. FISH mapping of the translocation regions was performed using probe sets depicted in Figure 2. Clones RP3-349A12 and RP11-936A3, proximal of and partly overlapping with the *HMGA1* locus respectively, demonstrated a split

apart in which the RP3-349A12 signal was retained on chromosome 6 while the signal of RP11-936A3 was translocated to the derivative chromosome 14. Fine mapping of this region revealed a deletion of clones RP11-175A4 and RP3-468B3 and the retention of RP5-1077I5 and RP11-974K1 bracketing this region on chromosome 6p. Breakpoint mapping on chromosome 14 using BAC clones located proximal (RP11-204K16) and distal (RP11-486O13) to *ACTN1* on chromosome 14q24, showed split signal and the distal clone was located on the derivative chromosome 6. The mapping also revealed a homozygous deletion of clones RP11-226F19 and RP11-565I3 (Figure 2B), covering the *ACTN1* gene. As secondary rearrangements at or around translocation breakpoint regions have been observed more often, we further characterized these regions by using a high resolution array-CGH approach. The array-CGH profile of L1339 detected a 603 kb deletion on 6p21.31-21.32, located 333 kb distal of *HMGA1* without the involvement of the *HMGA1* locus itself (Figure 2A). *TAPBP* was overlapping with the last distal deleted probe and might be involved in the deletion breakpoint. No copy number alteration was identified at the translocation breakpoint proximal to *HMGA1*. On chromosome 14q24.1 a 449 kb deletion was observed involving the *ACTN1* and *WDR22* genes (Supplementary Table 2). A second, 77 kb deletion was detected inside the distal part of *RAD51L1*, close to the locus of the BAC clone RP11-204K16 which is retained to chromosome 14 in the translocation. These deletions on chromosome 14 resulted in a homozygous loss of the involved chromosomal regions as monosomy 14 was observed in all tumor cells. A 7.3 Mb region at 14q21.1-21.3 was retained based on the array-CGH and likely was inserted into one of the derivative chromosomes containing chromosome 14 as no other regions were stained by whole chromosome specific paint (wcp) probes in a confirmatory FISH test using wcp 6 and wcp 14 (data not shown). Taking this together with the FISH mapping results, a balanced translocation with complex rearrangements at translocation breakpoint regions involving the *HMGA1* and *RAD51L* loci can be concluded (Figure 2).

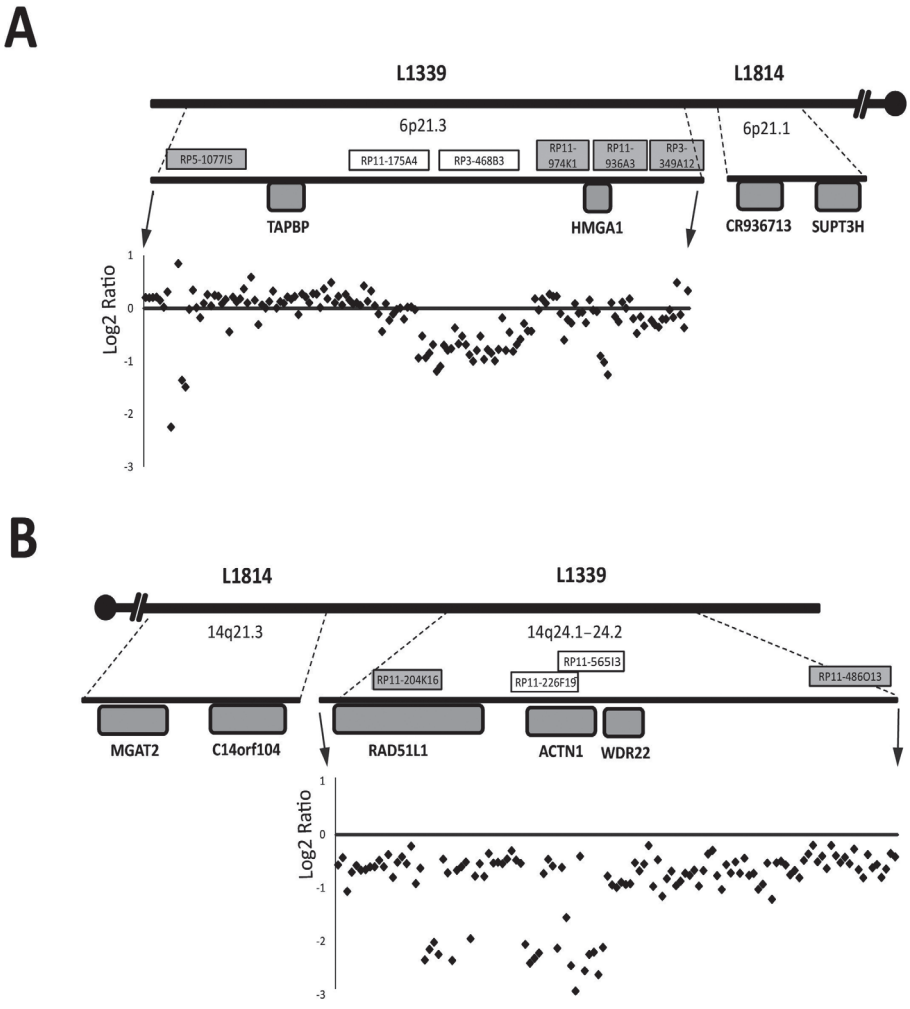
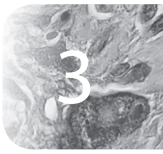


Figure 2. Schematic representation of breakpoints of leiomyosarcoma L1339 and L1814.

Schematic picture of 6p21 region with location of genes and BAC clones used for fine mapping of the translocation and deletion breakpoints of L1339; and the relation of this breakpoint compared to the breakpoint of L1814 (A). Schematic diagram of region 14q21-q24 including the genes and BAC clones involved in the translocation and deletion breakpoints (B). BAC clones in white boxes represent clones deleted in L1339, not to scale.

The hypotetraploid karyotype of L1814 resulted in a complex array-CGH profile in which the copy number varied from two to five or more of certain genomic regions. A derivative chromosome 6 involving translocations between chromosome 6 and chromosome 14 was detected. Array-CGH profiling showed

that the der(6)t(6;14) translocation was complicated with an amplification of 3.8 Mb in size at locus 6p21.1. The probes spanning the proximal breakpoint of the amplicon were 40 kb separated and *SUPT3H* was located in this region. The distance between the two probes spanning the distal amplicon breakpoint was 52 kb and was overlapping with *CR936713*. The amplification was located 6.7 Mb proximal to the *HMGA1* locus. On chromosome 14 a gain of 5.3 Mb in size was found at 14q24.2-24.3, located 3.7 Mb distal to *ACTN1*. The proximal and distal breakpoints of this gained region were spanning the *DPF3* and *ADCK1* loci, respectively. Based on the array-CGH data the translocation breakpoint was located at 14q21.3, as a slight change in copy number variation was noticed at that location. The proximal probe of the translocation breakpoint was corresponding to the *MGAT2* locus and the distal probe was overlapping with *C14orf104*. More detailed FISH-based fine mapping of the der(14)t(6;14)(p21.1;q21.3) could not be performed due to a lack of cultured tumor cells.



The unbalanced cytogenetic aberrations identified with COBRA-FISH were also confirmed by the array-CGH profiles of both leiomyosarcoma cases. Furthermore, the higher resolution of array-CGH revealed several other small deletions and gains in both cases.

Validation of breakpoints in other soft tissue tumors

A TMA with several soft tissue tumors was used to investigate the recurrence of the aberrations by using interphase FISH of four BAC clone sets corresponding to the regions of *HMGA1* at 6p21.31, *TAPBP* at 6p21.32, the amplicon at 6p21.1 and *ACTN1* at 14q24 (Supplementary Table 1). The two index cases were also present on the TMA and a split apart at the *HMGA1* and *ACTN1* locus and a deletion around *TAPBP* was confirmed in L1339. L1814 harbored numerous copies of BAC clone RP11-501I18 at 6p21.1, 2 Mb distal from *SUPT3H*, consistent with an amplification of that particular region; the tumor cells only contain 2 to 3 copies of centromere 6, which was included as a control. The majority of the cells of one leiomyosarcoma (L1566) revealed two times co-localization of the signals of the BAC clone set bracketing *ACTN1* and two additional signals of clone RP11-486O13 distal to *ACTN1* were present indicating an unbalanced translocation of this region. In this series, alterations involving a split apart of the signals in the 1.6 Mb region at 14q24.1-24.2 were found with FISH in 5% (2/39) of all investigated leiomyosarcomas. Copy number alterations with an equal number of the red and green signals were observed in

several tumors. None of the investigated loci showed any structural alteration in any of the additional evaluable cases including 37 leiomyosarcomas, 17 undifferentiated pleomorphic sarcomas, 4 undifferentiated spindle cell sarcomas and 4 uterine leiomyomas.

Discussion

Leiomyosarcomas belong to a group of sarcomas with a complex genetic profile without a yet identified specific, known genetic aberration. The complex karyotypes of most leiomyosarcomas might merely represent tumor progression associated changes; however, a causative role of a hidden, recurrent chromosomal aberration cannot be excluded in the development of these malignancies. In this study COBRA-FISH karyotyping of three tumors revealed a translocation t(6;14)(p;q) in two unrelated cases.

In case L1339 *HMGA1* might be involved in the breakpoint. HMGA proteins are evolutionary conserved members of the heterogeneous high-mobility group family of non-histone DNA binding proteins, they bind in the minor groove to AT-rich DNA sequences and regulate gene transcription by affecting the promotor activity and influencing the accessibility of transcription factors to target genes. They are involved in the differentiation and proliferation of mesenchymal tissues during embryonic development [16]. Some lipomas, angiomyxomas and uterine leiomyomas harbor a translocation involving *HMGA2,* at 12q15, leading to a chimeric fusion product with different partners [15;17-19]. Translocations involving *HMGA1* are identified in a subset of lipomas and uterine leiomyomas including benign uterine metastasizing leiomyoma, resulting in an upregulated expression of the chimeric protein [16;20-22]. Elevated expression has been encountered in synovial sarcoma and malignant peripheral nerve sheath tumor and is correlated with increased tumor grade and poor prognosis [23;24]. The molecular mechanism is considerably complex, as overexpression of wild type HMGA leads to deregulation of the cell cycle machinery and apoptosis in normal cells [25], while overexpression in immortalized cells stimulates tumor development [26]. The leiomyosarcomas in this study showed no intragenic *HMGA1* involvement, but a transregulatory effect due to the complex rearrangements cannot be excluded. Previously published benign mesenchymal tumors with *HMGA1* translocations mostly

revealed a break at the 3'-end, instead of an intragenic break; the breakpoint in L1339 is located less than 428 kb from the 3'-end. It has been postulated that transcriptional upregulation due to the genomic rearrangement is able to induce tumor formation [20]. Similarly complex rearrangements, involving regulatory sequences upstream of the coding sequence, have been described recently in chondromyxoid fibromas [27].

FISH mapping showed that the breakpoint at 14q24.1 in case L1339 was located at the *ACTN1* (α-actinin-1) locus, which is, like other cytoskeletal proteins, a member of the spectrin superfamily. This isoform is mainly expressed in non-muscular cells and regulates the role of focal adhesions and stress fibers and the association with cell motility [28]. High resolution array-CGH, showed a homozygous deletion of the *ACTN1* gene, which might be relevant for tumorigenesis. *RAD51L1*, located less than 200 kb to the 14q24 breakpoint, has been identified as a fusion partner of *HMGA2* in leiomyomas [15;29]. It is part of the homologous recombination repair machinery of double-strand DNA breaks. Although FISH mapping showed that the probe overlapping with *RAD51L1* was still retained to chromosome 14, array-CGH revealed a small intragenic deletion indicating a more complex rearrangement at the translocation breakpoint region with involvement of *RAD51L1*. In this way both fusion and a truncated protein expression might be possible, like mono-allelic knockout of the uterus-specific RAD51L1-11c splice variant as described in leiomyomas [30]. A uterine leiomyoma from a pseudo-Meigs' syndrome patient harbored a fast growing subclone with a deletion of the second *RAD51L1* allele next to the *RAD51L1/HMGA2* fusion [31]. Due to the monosomy 14, L1339 also has no normal copies of the gene left; the importance for tumor progression remains uncertain. Recently *RAD51L1* gene fusions were found in three uterine PEComas, it is interesting that these different mesenchymal tumors share involvement of the same gene [32]. Further research needs to be done to elucidate whether there is a common pathogenetic mechanism in the early development of these tumors. However, in our cases PEComa was excluded using immune-histochemical markers.

In L1814 a duplication at 6p21.1 was complicating the translocation breakpoint, forming the der(6)t(6;14)(p21.1;q21.3). These breakpoints differ from those in L1339. Confirmatory FISH on a TMA revealed a second leiomyosarcoma with an aberration at 14q24.1, however without involvement of the investigated regions on chromosome 6. In this study, alterations at

genomic region 14q24.1 were identified in 5% of the leiomyosarcomas. None of the other alterations appeared to be recurrent in the investigated soft tissue tumors. Nevertheless, an influence of cryptic rearrangements around *HMGA1, ACTN1* or *RAD51L1* cannot be excluded completely as they are expressed in smooth muscle cells and play a role in the development of these tumors.

Both cases harbored several other structural chromosomal aberrations, but as no other recurrent translocation was identified, these were not further investigated. In one case (L1091) we detected a balanced t(6;7)(p;p), the breakpoint region on chromosome 6 might be compatible with the *HMGA* locus. The breakpoint on chromosome 7p is different from the regions described in earlier studies [21]. However, as we had no access to more tumor cells, FISH mapping experiments could not be performed on this case. The quite simple karyotype is remarkable for a high grade leiomyosarcoma, but in the Mitelman Database of Cancer 9 out of the 129 reported cases showed a similarly simple translocation indicating a genetic subtype of leiomyosarcoma. None of the reported cases had translocation breakpoints similar to the one observed by us [33]. The case was included on the TMA, but unfortunately few cells were left with good signals so this case was not eligible for scoring.

With the currently available techniques it is challenging to identify common aberrations in these genetically heterogeneous and instable tumors. Improvements in the field of next generation sequencing have led to an increased possibility to detect aberrations. Whole-genome sequencing of osteosarcomas, a genomically complex and instable bone sarcoma, has recently shown that tumors undergo chromothripsis, leading to numerous genomic rearrangements due to reshuffling of small chromosome segments probably during a crisis-phase [34]. Chromothripsis is also found in 42% of uterine leiomyomas and it has been postulated that similar events occur during the development of leiomyosarcomas [35]. Modern sequencing techniques might be helpful to identify pathogenetic mechanisms responsible for the development of these tumors. Amplification of myocardin (*MYOCD*) at 17p is frequently encountered in leiomyosarcomas [36]. Some genetic alterations are found in only a subset of tumors indicating that they might be secondary rather than the causative, tumor initiating event. Recently mutations in dystrophin (*DMD*) have been identified in high grade mesenchymal tumors with certain level of muscle differentiation, including leiomyosarcomas, rhabdomyosarcomas and gastrointestinal stromal tumors [37]. In that study a

high percentage of the tumors has aberrations in *DMD*, suggesting that these different tumors might develop via a similar way. It will be interesting to study the role of *DMD* as deregulation of this gene might play a role in the early tumorigenesis of myogenic sarcomas.

This study reports the identification of a t(6;14)(p;q) in two leiomyosarcoma cases. As the translocation breakpoints differ, a common driver alteration for leiomyosarcomas was not found which is consistent with the genetic heterogeneity of these tumors. However, we identified genes that might be involved in the pathogenesis of these particular leiomyosarcoma cases. In the future, next generation sequencing can be a helpful tool in the search for a common driver alteration of these malignant tumors.

Acknowledgements
The authors would like to thank Marja van der Burg for the excellent technical assistance.

References

1. Lazar AJ, Evans HL, Shipley J. Smooth-muscle tumours. In: Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F, editors. WHO Classification of Tumors of Soft Tissue and Bone, Lyon: IARC, 2013; pp. 109-113.
2. Italiano A, Lagarde P, Brulard C, Terrier P, Lae M, Marques B, Ranchere-Vince D, Michels JJ, Trassard M, Cioffi A, Piperno-Neumann S, Chevreau C, Blay JY, Delcambre C, Isambert N, Penel N, Bay JO, Bonvalot S, Le CA, Coindre JM, Chibon F. Genetic profiling identifies two classes of soft-tissue leiomyosarcomas with distinct clinical characteristics. Clin Cancer Res 2013;19:1190-1196.
3. Svarvar C, Bohling T, Berlin O, Gustafson P, Folleras G, Bjerkehagen B, Domanski HA, Sundby HK, Tukiainen E, Blomqvist C. Clinical course of nonvisceral soft tissue leiomyosarcoma in 225 patients from the Scandinavian Sarcoma Group. Cancer 2007;109:282-291.
4. Gladdy RA, Qin LX, Moraco N, Agaram NP, Brennan MF, Singer S. Predictors of survival and recurrence in primary leiomyosarcoma. Ann Surg Oncol 2013;20:1851-1857.
5. Radaelli S, Stacchiotti S, Casali PG, Gronchi A. Emerging therapies for adult soft tissue sarcoma. Expert Rev Anticancer Ther 2014.
6. Corless CL, Barnett CM, Heinrich MC. Gastrointestinal stromal tumours: origin and molecular oncology. Nat Rev Cancer 2011;11:865-878.
7. Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad TG, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science 1998;279:577-580.
8. Sandberg AA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: leiomyosarcoma. Cancer Genet Cytogenet 2005;161:1-19.
9. Szuhai K, Tanke HJ. COBRA: combined binary ratio labeling of nucleic-acid probes for multi-color fluorescence in situ hybridization karyotyping. Nat Protoc 2006;1:264-275.
10. ISCN (2013): An International System for Human Cytogenetic Nomenclature. Basel: S. Karger, 2013.
11. de Graaff MA, Cleton-Jansen AM, Szuhai K, Bovee JV. Mediator complex subunit 12 exon 2 mutation analysis in different subtypes of smooth muscle tumors confirms genetic heterogeneity. Hum Pathol 2013;44:1597-1604.
12. Rossi S, Szuhai K, Ijszenga M, Tanke HJ, Zanatta L, Sciot R, Fletcher CD, Dei Tos AP, Hogendoorn PC. EWSR1-CREB1 and EWSR1-ATF1 fusion genes in angiomatoid fibrous histiocytoma. Clin Cancer Res 2007;13:7322-7328.
13. Giuntoli RL, Lessard-Anderson CR, Gerardi MA, Kushnir CL, Cliby WA, Metzinger DS, Gostout BS. Comparison of current staging systems and a novel staging system for uterine leiomyosarcoma. Int J Gynecol Cancer 2013;23:869-876.
14. D'Angelo E, Prat J. Uterine sarcomas: a review. Gynecol Oncol 2010;116:131-139.
15. Dal Cin P, Quade BJ, Weremowicz S, Morton CC, Pauwels P, Van den Berghe H. Primary parauterine leiomyoma with a t(6;14). Genes Chromosomes Cancer 1999;26:385-386.
16. Reeves R. Molecular biology of HMGA proteins: hubs of nuclear function. Gene 2001;277:63-81.
17. Kazmierczak B, Pohnke Y, Bullerdiek J. Fusion transcripts between the HMGIC gene and RTVL-H-related sequences in mesenchymal tumors without cytogenetic aberrations. Genomics 1996;38:223-226.
18. Rawlinson NJ, West WW, Nelson M, Bridge JA. Aggressive angiomyxoma with t(12;21) and HMGA2 rearrangement: report of a case and review of the literature. Cancer Genet Cytogenet 2008;181:119-124.
19. Petit MM, Schoenmakers EF, Huysmans C, Geurts JM, Mandahl N, Van de Ven WJ. LHFP, a novel translocation partner gene of HMGIC in a lipoma, is a member of a new family of LHFP-like genes. Genomics 1999;57:438-441.
20. Kazmierczak B, Dal CP, Wanschura S, Borrmann L, Fusco A, Van den Berghe H, Bullerdiek J. HMGIY is the target of 6p21.3 rearrangements in various benign mesenchymal tumors. Genes Chromosomes Cancer 1998;23:279-285.
21. Nucci MR, Drapkin R, Dal CP, Fletcher CD, Fletcher JA. Distinctive cytogenetic profile in benign metastasizing leiomyoma: pathogenetic implications. Am J Surg Pathol 2007;31:737-743.

22. Bowen JM, Cates JM, Kash S, Itani D, Gonzalez A, Huang D, Oliveira A, Bridge JA. Genomic imbalances in benign metastasizing leiomyoma: characterization by conventional karyotypic, fluorescence in situ hybridization, and whole genome SNP array analysis. Cancer Genet 2012;205:249-254.
23. Hui P, Li N, Johnson C, De W, I, Sciot R, Manfioletti G, Tallini G. HMGA proteins in malignant peripheral nerve sheath tumor and synovial sarcoma: preferential expression of HMGA2 in malignant peripheral nerve sheath tumor. Mod Pathol 2005;18:1519-1526.
24. Reeves R. Nuclear functions of the HMG proteins. Biochim Biophys Acta 2010;1799:3-14.
25. Fedele M, Pierantoni GM, Berlingieri MT, Battista S, Baldassarre G, Munshi N, Dentice M, Thanos D, Santoro M, Viglietto G, Fusco A. Overexpression of proteins HMGA1 induces cell cycle deregulation and apoptosis in normal rat thyroid cells. Cancer Res 2001;61:4583-4590.
26. Wood LJ, Mukherjee M, Dolde CE, Xu Y, Maher JF, Bunton TE, Williams JB, Resar LM. HMG-I/Y, a new c-Myc target gene and potential oncogene. Mol Cell Biol 2000;20:5490-5502.
27. Nord KH, Lilljebjorn H, Vezzi F, Nilsson J, Magnusson L, Tayebwa J, de JD, Bovee JV, Hogendoorn PC, Szuhai K. GRM1 is upregulated through gene fusion and promoter swapping in chondromyxoid fibroma. Nat Genet 2014;46:474-477.
28. Sjoblom B, Salmazo A, Djinovic-Carugo K. Alpha-actinin structure and regulation. Cell Mol Life Sci 2008;65:2688-2701.
29. Schoenmakers EF, Huysmans C, Van de Ven WJ. Allelic knockout of novel splice variants of human recombination repair gene RAD51B in t(12;14) uterine leiomyomas. Cancer Res 1999;59:19-23.
30. Schoenmakers EF, Bunt J, Hermers L, Schepens M, Merkx G, Janssen B, Kersten M, Huys E, Pauwels P, Debiec-Rychter M, van Kessel AG. Identification of CUX1 as the recurrent chromosomal band 7q22 target gene in human uterine leiomyoma. Genes Chromosomes Cancer 2013;52:11-23.
31. Amant F, Debiec-Rychter M, Schoenmakers EF, Hagemeijer-Hausman A, Vergote I. Cumulative dosage effect of a RAD51L1/HMGA2 fusion and RAD51L1 loss in a case of pseudo-Meigs' syndrome. Genes Chromosomes Cancer 2001;32:324-329.
32. Agaram NP, Sung YS, Zhang L, Chen CL, Chen HW, Singer S, Dickson MA, Berger MF, Antonescu CR. Dichotomy of Genetic Abnormalities in PEComas With Therapeutic Implications. Am J Surg Pathol 2015;39:813-825.
33. Mitelman, F., johansson, B., and Mertens, F. Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (2015). http://cgap.nci.nih.gov/Chromosomes/Mitelman .
34. Stephens PJ, Greenman CD, Fu B, Yang F, Bignell GR, Mudie LJ, Pleasance ED, Lau KW, Beare D, Stebbings LA, McLaren S, Lin ML, McBride DJ, Varela I, Nik-Zainal S, Leroy C, Jia M, Menzies A, Butler AP, Teague JW, Quail MA, Burton J, Swerdlow H, Carter NP, Morsberger LA, Iacobuzio-Donahue C, Follows GA, Green AR, Flanagan AM, Stratton MR, Futreal PA, Campbell PJ. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell 2011;144:27-40.
35. Mehine M, Kaasinen E, Makinen N, Katainen R, Kampjarvi K, Pitkanen E, Heinonen HR, Butzow R, Kilpivaara O, Kuosmanen A, Ristolainen H, Gentile M, Sjoberg J, Vahteristo P, Aaltonen LA. Characterization of uterine leiomyomas by whole-genome sequencing. N Engl J Med 2013;369:43-53.
36. Perot G, Derre J, Coindre JM, Tirode F, Lucchesi C, Mariani O, Gibault L, Guillou L, Terrier P, Aurias A. Strong smooth muscle differentiation is dependent on myocardin gene amplification in most human retroperitoneal leiomyosarcomas. Cancer Res 2009;69:2269-2278.
37. Wang Y, Marino-Enriquez A, Bennett RR, Zhu M, Shen Y, Eilers G, Lee JC, Henze J, Fletcher BS, Gu Z, Fox EA, Antonescu CR, Fletcher CD, Guo X, Raut CP, Demetri GD, van de Rijn M, Ordog T, Kunkel LM, Fletcher JA. Dystrophin is a tumor suppressor in human cancers with myogenic programs. Nat Genet 2014;46:601-606.

Supplementary Information

Supplementary Table 1: Combinations of BAC clones used on tissue microarrays.

BAC clone	Start position	End position	Size (kb)	Locus	Labeling	Probe region	Prox-Dist probe distance
RP3-349A12 #	34810549	34943495	132.9	6p21.31	DIG	*HMGA1* (prox)	428.1 kb
RP11-936A3 #	34207886	34382491	174.6	6p21.31	BIO	*HMGA1* (dist)	
RP11-204K16 *	68753875	68924811	170.9	14q24.1	DIG	*ACTN1* (prox)	1600.5 kb
RP11-486O13 *	70525284	70729505	204.2	14q24.2	BIO	*ACTN1* (dist)	
RP11-175A4 *	33360610	33507021	146,4	6p21.31~21.32	BIO	*TAPBP* (prox)	971.1 kb
RP5-1077I5 *	32297092	32389527	92.4	6p21.2~21.32	DIG	*TAPBP* (dist)	
RP11-501I18 *	42780788	42846928	66.1	6p21.1	BIO	2 Mb distal from *SUPT3H*	18.3 Mb
Centromere 6	Centromere 6	Centromere 6		Centromere 6	DIG	Centromere 6	

#UCSC Genome Browser Feb 2009 GRCh37/hg19, *Ensembl Genome Browser GRCh37.

Supplementary Table 2: Agilent probes describing the breakpoints.

Case	Description	Details	Agilent probe	Chromosome	Genomic location#	Genes overlapping with probe location	Distance between single probes	Distance between 2 probe sets
L1339	Deletion at 6p21.3	Last normal probe	A_16_P17503364	6p21.31	33,963,393-33,963,452	none	86.9 kb	0.603 Mb
	Proximal breakpoint	First deleted probe	A_14_P113996	6p21.31	33,876,537-33,876,596	none		(size of deletion)
	Deletion at 6p21.3	Last deleted probe	A_14_P124550	6p21.32	33,273,955-33,274,014	*TABPB*	14.4 kb	
	Distal breakpoint	Next normal probe	A_14_P125596	6p21.32	33,259,603-33,259,651	*RGL2*		
	HMGA1 locus	*HMGA1* locus	A_14_P109294	6p21.31	34,209,880-34,209,939	*HMGA1*		0.333 Mb (deletion to *HMGA1*)
L1339	Retained region 14q21	Last deleted probe	A_14_P130400	14q21.1	43,137,003-43,137,062	none	113.3 kb	7.3 Mb

Supplementary Table 2: continued

Case	Description	Details	Agilent probe	Chromosome	Genomic location#	Genes overlapping with probe location	Distance between single probes	Distance between 2 probe sets
	Proximal breakpoint	First retained probe	A_14_P114243	14q21.1	43,250,347-43,250,406	none		(size of retained region)
	Retained region 14q21	Last retained probe	A_16_P02910911	14q21.3	50,530,528-50,530,587	none	21.4 kb	
	Distal breakpoint	Next deleted probe	A_16_P02910932	14q21.3	50,551,904-50,551,963	*LOC196913*		
L1339	Deletion around *ACTN1*	Last normal probe	A_16_P02937174	14q24.1	69,111,391-69,111,449	none	24.3 kb	449.1 kb
	Proximal breakpoint	First deleted probe	A_16_P20083284	14q24.1	69,135,726-69,135,785	none		(size of deletion)
	Deletion around *ACTN1*	Last deleted probe	A_16_P20084485	14q24.1	69,584,790-69,584,847	*WDR22*	39.1 kb	
	Distal breakpoint	Next normal probe	A_16_P20084596	14q24.1	69,623,872-69,623,931	none		
L1339	Deletion in *RAD51L1*	First proximal deleted probe	A_16_P20081907	14q24.1	68,655,255-68,655,314	*RAD51L1*	76.5 kb	76.5 kb
	Deletion in *RAD51L1*	Last distal deleted probe	A_16_P40226246	14q24.1	68,731,769-68,731,828	*RAD51L1*		(size of deletion)
L1814	Amplification at 6p21.1	Last normal probe	A_16_p17530102	6p21.1	44837840-44837899	*SUPT3H*	40.4 kb	3.8 Mb
	Proximal breakpoint	First amplified probe	A_14_P103947	6p21.1	44797474-44797533	*SUPT3H*		(size of amplification)
	Amplification at 6p21.1	Last amplified probe	A_16_P17520532	6p21.1	40987901-40987960	*CR936713*	51.6 kb	
	Distal breakpoint	Next normal probe	A_14_P130391	6p21.1	40936271-40936330	*CR936713*		
L1814	Translocation breakpoint	Proximal	A_14_P104481	14q21.3	50,088,698-50,088,757	*MGAT2*	6.0 kb	

Supplementary Table 2: continued

Case	Description	Details	Agilent probe	Chromosome	Genomic location#	Genes overlapping with probe location	Distance between single probes	Distance between 2 probe sets
	Translocation breakpoint	Distal	A_14_P115913	14q21.3	50,094,743-50,094,802	*C14orf104*		
L1814	Duplication at 14q24.2	Last normal probe	A_16_P02942905	14q24.2	73145087-73145146	*DPF3*	31.0 kb	5.3 Mb
	Proximal breakpoint	First duplicated probe	A_16_P02942958	14q24.2	73176127-73176186	*DPF3*		(size of duplication)
	Duplication at 14q24.2	Last duplicated probe	A_14_P132604	14q24.3	78389188-78389247	*ADCK1*	38.8 kb	
	Distal breakpoint	Next normal probe	A_16_P20105656	14q24.3	78428021-78428080	none		

#UCSC Genome Browser Feb 2009 GRCh37/hg19

Chapter 4

Inhibition of Bcl-2 family members sensitizes soft tissue leiomyosarcomas to chemotherapy

Marieke A. de Graaff, Marije A.J. de Rooij, Brendy E.W.M. van den Akker, Hans Gelderblom, Fréderic Chibon, Jean-Michel Coindre, Adrian Marino-Enriquez, Jonathan A. Fletcher, Anne-Marie Cleton-Jansen, Judith V.M.G. Bovée

British Journal of Cancer. 2016; 114(11): 1219-1226.

Abstract

BACKGROUND: Leiomyosarcoma is an aggressive soft tissue sarcoma with a 5-year survival rate of 15 to 60%. Treatment options for inoperable or metastatic patients are limited due to frequent resistance of tumours to chemotherapy and radiation. In this study, we hypothesized that anti-apoptotic Bcl-2 family proteins might contribute to leiomyosarcoma chemoresistance and therefore inhibition of Bcl-2 family proteins might sensitize leiomyosarcomas to conventional chemotherapy.

METHODS: Expression of the Bcl-2 family proteins Bcl-xL, Bcl-w and Bcl-2 was investigated using immunohistochemistry on a tissue microarray containing 43 leiomyosarcomas. Furthermore, we investigated whether ABT-737, a potent BH3 mimetic, sensitises leiomyosarcoma cells to doxorubicin treatment *in vitro*.

RESULTS: Seventy-seven percent, 84% and 42% of leiomyosarcomas demonstrated high expression of Bcl-2, Bcl-xL and Bcl-w, respectively. Single-agent treatment with ABT-737 resulted in a minor reduction of cell viability. However, combination treatment of ABT-737 and doxorubicin revealed synergism in all four cell lines, by inducing apoptosis.

CONCLUSION: In conclusion, Bcl-2 family proteins contribute to soft tissue leiomyosarcoma chemoresistance. Anti-apoptotic proteins are highly expressed in leiomyosarcoma of soft tissue, and inhibition of these proteins using a BH3 mimetic increases leiomyosarcoma sensitivity to doxorubicin.

Key words:
sarcoma, leiomyosarcoma, soft tissue tumour, apoptosis, Bcl-2, Bcl-xL, Bcl-w, ABT-737, chemoresistance.

Introduction

Leiomyosarcomas are malignant mesenchymal neoplasms displaying smooth muscle differentiation (1). They occur in patients over a wide age range with the highest incidence in the 5th and 6th decade of life. The 5-year survival rates range from 15 to 60% depending on the location, size and stage of disease at time of diagnosis (1). These low survival rates are in part attributable to low response rates to chemo- and radiotherapy (2).

Overexpression of multidrug resistance (MDR) proteins, like P-glycoprotein, only partly explains leiomyosarcoma chemoresistance. In addition, overexpression of Bcl-2 family proteins could contribute to chemo- and radiotherapy resistance, since chemo- and radiotherapy are DNA damaging therapies that provoke tumour regression by the activation of the intrinsic apoptotic pathway (3-5). Interestingly, immunohistochemistry shows high expression of the anti-apoptotic protein Bcl-2 in soft tissue sarcomas, including leiomyosarcoma (6, 7). Bcl family members, like Bcl-2, Bcl-xL (BCL2L1) and Bcl-w (BCL2L2) inhibit activation of the intrinsic apoptotic pathway by regulating the mitochondrial outer membrane permeabilization. Specifically, mitochondrial outer membrane pores are formed by dimerization of Bax and Bak proteins. These pores release cytochrome c from mitochondria which leads to activation of the intrinsic apoptotic cascade (5). By binding of the anti-apoptotic proteins Bcl-2/Bcl-xL to the pro-apoptotic family members Bak and Bax, the dimerisation of Bak and Bax is prevented and activation of the apoptotic pathway is inhibited.

In this study we hypothesized that the Bcl-2 family members play an important role in leiomyosarcoma chemoresistance and their inhibition might therefore render leiomyosarcoma cells sensitive to treatment with conventional chemotherapeutic agents. To explore the role of Bcl-2 family members in leiomyosarcoma chemoresistance we first evaluated the protein expression of the anti-apoptotic proteins Bcl-2, Bcl-xL and Bcl-w in a large series of soft tissue leiomyosarcomas by immunohistochemistry, using clinically-annotated tissue microarrays. Subsequently, we determined whether the Bcl-2 pathway inhibitor ABT-737 sensitizes leiomyosarcoma cells to chemotherapy. ABT-737 is a potent BH3 mimetic that inhibits Bcl-2, Bcl-xL and Bcl-w at the mitochondrial membrane by binding to their BH3 domains (8). As chemotherapeutic agent we used doxorubicin which is widely used in the treatment of leiomyosarcoma (9, 10).

Material and methods

Tissue samples

Tissue microarrays were previously constructed containing leiomyosarcomas (soft tissue n=40, uterine n=3), leiomyomas of the uterus (n=7), myxofibrosarcomas (n=16), and undifferentiated pleomorphic/spindle cell sarcomas (n=28). These sarcomas all belong to the genetic subcategory of soft tissue sarcomas with complex karyotypes. Tissue microarrays (TMAs) were constructed using a semi-automated TMA apparatus (TMA Master; 3D Histech, Budapest, Hungary) (11). For each case, 1.767 mm^2 per core was present in triplicate. All samples were handled according to the Dutch code of proper secondary use of human material as accorded by the Dutch society of pathology (Federa). The samples were handled in a coded (pseudo-anonymised) fashion according to the procedures as accorded by the LUMC ethical board. In total, 40 soft tissue leiomyosarcomas were included from 39 patients (23 females and 16 males); of one patient both the primary tumor and a metastatic lesion were analyzed. In addition, three uterine leiomyosarcomas were included. The average age at diagnosis was 60 years. Clinicopathological information is shown in Suppl table 1. Patients were diagnosed between 1989 and 2011 in the LUMC (Leiden, the Netherlands). None of the patients received pre-operative chemotherapy or radiation therapy.

Compounds

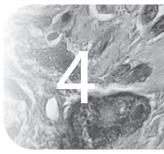
Doxorubicin was obtained from the in-house hospital pharmacy in a 0.9% NaCl solution. ABT-737 (Selleckchem, Houston, USA) was dissolved in dimethyl sulfoxide (DMSO).

Cell culture

In this study we included four leiomyosarcoma cell lines (LMS04, LMS05, IB133 and IB140). These cell lines were established from primary tumours and for the experiments described herein were used between passages 20-50. LMS04 was derived from a high grade retroperitoneal metastasis from the uterus and LMS05 was derived from a grade 2 soft tissue leiomyosarcoma (Suppl table 2). IB140 was derived from a FNCLCC grade 2 leiomyosarcoma from the upper leg and IB133 was derived from a FNCLCC grade 3 retroperitoneal leiomyosarcoma. *TP53* mutation analysis was determined by Sanger sequencing. In addition, the

cell lines were screened for hotspot mutations using the Ion AmpliSeq™ Cancer Hotspot panel v2 (Thermo Fisher Scientific, Bleiswijk, The Netherlands). Fifty reads were considered enough to draw reliable conclusions. The coverage of identified SNPs was ranging from 50 up to 1995 reads.

The leiomyosarcoma cell lines LMS04 and LMS05 were cultured in RPMI1640 medium with 15% FBS and 1% penicillin/streptomycin and the cell lines IB140 and IB133 were cultured in RPMI1640 with 10% FBS, 1% glutamax and 1% pen/strep. The HeLa cell line was included as a reference and cultured in RPMI1640 with 10% FBS and 1% pen/strep. All cell lines were repeatedly checked for mycoplasm and authentication of the cell lines was determined by short tandem repeat (STR) typing (GenePrint 10 system, Promega, Leiden, The Netherlands).



Immunohistochemistry

To detect the expression of anti-apoptotic proteins, immunohistochemistry on TMA was performed according to standard laboratory procedures (11). Further details of the Bcl-2, Bcl-xL and Bcl-w antibodies are shown in Suppl table 3. Scoring was performed by two observers (MAdG and JVMGB) independently and discrepant cases were discussed to reach consensus. Both intensity (0=absent, 1=weak, 2=moderate and 3=strong expression) and percentage of positive tumour cells (0=0%, 1=1-24%, 2=25-49%, 3=50-74%, 4=75-100%) were assessed and scores were added up (12).

Cell viability assays

To analyse the effect of treatment with doxorubicin and ABT-737, cell viability assays were performed with Alamar blue (Life Technologies, Bleiswijk, The Netherlands) according to the manufacturer's instructions. Absorbance was measured with a light spectrometer (Victor V, 1420 Multilabel counter; Perkin Elmer, Groningen, The Netherlands).

The dose-response curves of single treatments with ABT-737 and doxorubicin were determined for each cell line in a range from 0.001 to 12.8μM. For doxorubicin, it is accepted that an *in vivo* range of 5 to 100 μM corresponds to an *in vitro* range from 1 to 10 μM (13). All cell lines were treated with ABT-737 for 24 hours and subsequently doxorubicin for 48 hours. Because in only one of the four cell lines (LMS05) large effects could be observed, the other three cell lines were treated with doxorubicin and ABT-737 combined for 72

hours. The concentrations of doxorubicin used for the combination treatments were selected per cell line, based on the individual dose-response curves after 48 hour (not shown) and 72 hour doxorubicin treatment. Cell viability was calculated relative to untreated cells, all experiments were repeated three times in triplicate.

To evaluate whether the combination treatments of doxorubicin and ABT-737 were synergistic drug interactions, a simplified version of the Bliss independence model was applied (14). When two drugs exert their effects independent to one another, the resulting relative viability after drug treatment is expected to equal the product of the relative viability after treatment with the individual drugs. When the observed viability after combination treatment is lower than the expected viability, this is an indication of a synergistic drug interaction. When the observed viability is higher than the expected based on the individual drug effects, the compounds inhibit each other's effects (antagonism).

Western blot

To evaluate the expression of Bcl-2, Bcl-xL, Bcl-w, Mcl-1, PARP and α-tubulin before and after treatment we performed western blots according to the Cell Signaling Technology protocol enhanced chemiluminescence. Cell lysates were made from cultured cell lines LMS04, LMS05, IB133 and IB140 treated with doxorubicin (respectively 0.2, 0.6, 0.7 and 0.5μM) and with ABT-737 (5μM) for 24 hours. In order to obtain cell lysates, hot SDS was added to the cells and the solution was heated up to 100^{o}C. Gels were loaded with 20μg lysate. Antibodies, described in detail in Suppl table 4, were dissolved in TPS Tween 0.05% in which the blots were also washed. As a positive control for all antibodies except for PARP, the HeLa cell line was included. For PARP, Jurkat apoptosis cell lysate (Cell Signaling Technology) was included as a positive control.

Caspase assay

To confirm that the decrease in cell viability was caused by apoptosis, caspase assays were performed according to the caspase-Glo 3/7 assay protocol of Promega. LMS04, LMS05, IB133 and IB140 cell lines were treated for 24 hours with ABT-737 (5μM), doxorubicin (0.2, 0.6, 0.7 and 0.5μM respectively) or a combination. A 1:1 ratio of Caspase-Glo ® 3/7 reagent volume per sample was used. As negative controls, untreated cells were included. For each condition,

Z-VAD-FMK, an irreversible pan-caspase inhibitor (25μM, Promega) was used. The luciferase units of each sample were measured by the VICTOR3 Label counter with emission filter D572 after 1 hour.

Statistics

GraphPad Prism for Windows version 6.02 (GraphPad Software, La Jolla California, USA) was used to analyze the results. The statistical difference between 2 groups was assessed by an unpaired t-test, *P-values* were considered statistically different if $P<0.05$). Statistical differences in overall survival were calculated with the log-rank (Mantel-Cox) test. Also GraphPad was used for dose-response curves and calculation of IC_{50} values.

Results

High expression of Bcl family proteins in leiomyosarcomas

Immunohistochemistry for anti-apoptotic proteins using tissue microarrays revealed that Bcl-xL and Bcl-w were higher expressed in leiomyosarcomas compared to uterine leiomyomas (Figure 1). Thirty three out of 43 (77%) leiomyosarcomas showed high Bcl-2 expression (IHC score >3). For Bcl-xL and Bcl-w this was 36 out of 43 (84%) and 18 out of 43 (42%), respectively (Table 1). Interestingly, Bcl-xL and Bcl-w were expressed at significantly lower levels in uterine leiomyomas ($p=<0.001$), while Bcl-2 expression was significantly higher in leiomyomas, compared to leiomyosarcomas ($p=0.042$). The three uterine leiomyosarcomas showed a slightly higher Bcl-2 expression compared to the leiomyomas, although this is not statistically significant ($p=0.224$) (Suppl figure 1A). The anti-apoptotic proteins were also expressed in other high grade sarcomas (myxofibrosarcoma and undifferentiated pleomorphic sarcoma) (Table 1).

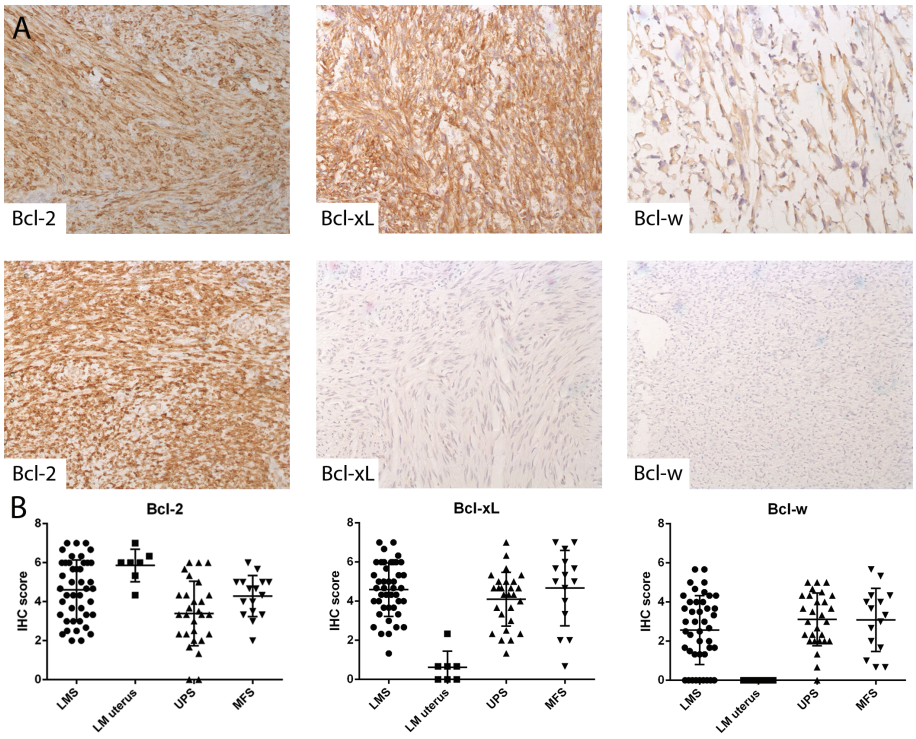


Figure 1. Protein expression of Bcl-2, Bcl-xL and Bcl-w determined by immunohistochemistry. (**A**) In the top panel Bcl-2, Bcl-xL and Bcl-w expression of a representative leiomyosarcoma is displayed were the bottom panel shows expression of a representative uterine leiomyoma (20x magnification). Leiomyosarcomas showed overall higher expression of Bcl-xL and Bcl-w, whereas both leiomyosarcomas and uterine leiomyomas revealed a high expression of Bcl-2. (**B**) The difference in Bcl-2, Bcl-xL and Bcl-w protein expression of leiomyosarcoma (LMS), uterine leiomyoma (LM uterus), myxofibrosarcoma (MFS) and undifferentiated pleomorphic sarcoma (UPS) illustrated by dot plots confirmed an overall higher expression of Bcl-xL and Bcl-w in leiomyosarcomas as compared to uterine leiomyomas.

There was no association between expression and histological grade (Suppl figure 1A). The Kaplan-Meier survival curve showed a trend for high expression of Bcl-2, Bcl-xL and Bcl-w to be associated with shorter overall survival time (Suppl figure 1B). However, the differences in overall survival between the groups of patients with an immunohistochemical score ≤3 or >3 were not significant (p=0.5347 (Bcl-2), p=0.5490 (Bcl-xL) and p=0.2495 (Bcl-w)). Sixteen patients were diagnosed with a metastasis after a period of treatment. In the Kaplan-Meier survival curves no correction was added for metastasis or treatment.

Table 1. Expression of Bcl-2, Bcl-xL and Bcl-w in leiomyosarcomas, leiomyomas and sarcomas.

Expression	LM	LMS	P-value LM vs LMS	MFS	UPS/SCS
Bcl-2					
Low	0/7 (0%)	10/43 (23%)	0.0419	2/16 (13%)	11/28 (39%)
High	7/7 (100%)	33/43 (77%)		14/16 (87%)	17/28 (61%)
Bcl-xL					
Low	7/7 (100%)	7/43 (16%)	<0.0001	3/15 (20%)	7/28 (25%)
high	0/7 (0%)	36/43 (84%)		12/15 (80%)	21/28 (75%)
Bcl-w					
Low	7/7 (100%)	25/43 (58%)	0.0004	7/15 (47%)	14/28 (50%)
High	0/7 (0%)	18/43 (42%)		8/15 (53%)	14/28 (50%)

Leiomyosarcoma (LMS), uterine leiomyoma (LM uterus), myxofibrosarcoma (MFS) and undifferentiated pleomorphic/spindle cell sarcoma (UPS/SCS). Low = IHC score ≤ 3; high = IHC score > 3.

Doxorubicin and ABT-737 act synergistically in leiomyosarcoma cell lines

Since Bcl-2, Bcl-xL and Bcl-w were highly expressed in the majority of leiomyosarcomas, four leiomyosarcomas cell lines (LMS04, LMS05, IB133 and IB140) were selected for further functional evaluation. Clinicopathological as well as genetic characteristics of these cell lines are shown in Suppl table 2. *TP53* mutations were found in LMS04 and IB133, while IB140 demonstrated a *CTNNB1* variation (Suppl table 2 and suppl figure 2).

The response to ABT-737 and doxorubicin were determined separately after 24, 48 and 72 hours of treatment. All cell lines showed maximal response at 72 hours of ABT-737 treatment. IB140 and IB133 demonstrated a slight decrease in cell viability compared to LMS04 and LMS05, however due to the limited sensitivity of single treatment with this drug, no reliable absolute IC_{50} could be calculated (Figure 2A). The cell lines showed a maximal reduction in cell viability of 60-95% after single doxorubicin treatment (Figure 2B). The calculated absolute IC_{50} values of doxorubicin treatment for the four cell lines LMS04, LMS05, IB140 and IB133 were respectively: 0.198; 0.265; 1.314 and 0.580 μM.

To determine possible synergy of combination treatment, ABT-737 (1, 5 and 10μM) was administered for the first 24 hours, followed by the addition

of doxorubicin for 48 hours. A synergistic effect of ABT-737 was found, most pronounced in LMS05 (Figure 2C) and only a moderate effect was observed in IB140, IB133 and LMS04 (not shown). Therefore, these last three cell lines were treated simultaneously with ABT-737 and doxorubicin for 72 hours. The expected cell viability of combination treatment was calculated and compared with the observed viability. For five out of six tested sequential drug combinations in LMS05 a statistical significant difference ($P<0.005$) was found between the expected and observed cell viability, indicating synergism (Figure 2C). IB133 and IB140 both showed synergistic effects with pronounced reduction of cell viability in the three drug combinations with the higher doxorubicin concentration ($p<0.005$) after synchronous treatment for 72 hour (Figure 2E and 2F). In LMS04, on the other hand, the difference between the observed and expected effect of the synchronous combination treatment was most clear in the two combinations with the highest (10μM) ABT-737 concentration ($p=0.07$ and $p<0.005$) (Figure 2D).

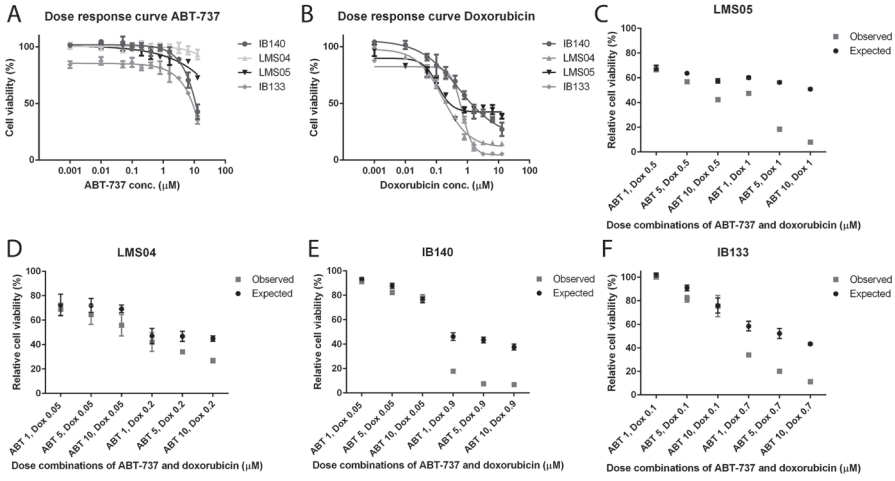


Figure 2. Dose response curves of the four cell lines treated with ABT-737 and doxorubicin. (A) Dose response curves of ABT-737 ranging from 0.001-12.8μM after 72 hours of treatment showed limited effect of this single drug treatment. (**B**) Dose response curves of doxorubicin ranging from 0.001-12.8μM after 72 hours of treatment showed reductions till 5% cell viability. (**C**) Comparison of observed and expected relative cell viability of LMS05 after sequential treatment with ABT-737 for 24 hours followed by 48 hours treatment with doxorubicin. Strong synergism was seen in LMS05. (**D-F**) Comparison of observed and expected relative cell viability of the cell lines LMS04, IB140 and IB133 after synchronous combination treatment with ABT-737 and doxorubicin for 72 hours. A moderate synergistic effect was observed in IB140 and IB133, and LMS04 revealed a mild synergistic effect at ABT-737 10μM.

Western blot performed on untreated cells confirmed expression of all three anti-apoptotic proteins in the four cell lines. Among these, Bcl-xL was most abundantly expressed. Expression of Bcl-2 was highest in LMS05, followed by LMS04, while expression was almost absent in IB133 and IB140 (Figure 3). Mcl-1 was expressed in all cell lines, with a comparable intensity as the HeLa control. LMS04 even showed a stronger Mcl-1 expression. Treatment with ABT-737 and/or doxorubicin did not significantly alter the expression levels of Mcl-1, Bcl-w, Bcl-xL or Bcl-2.

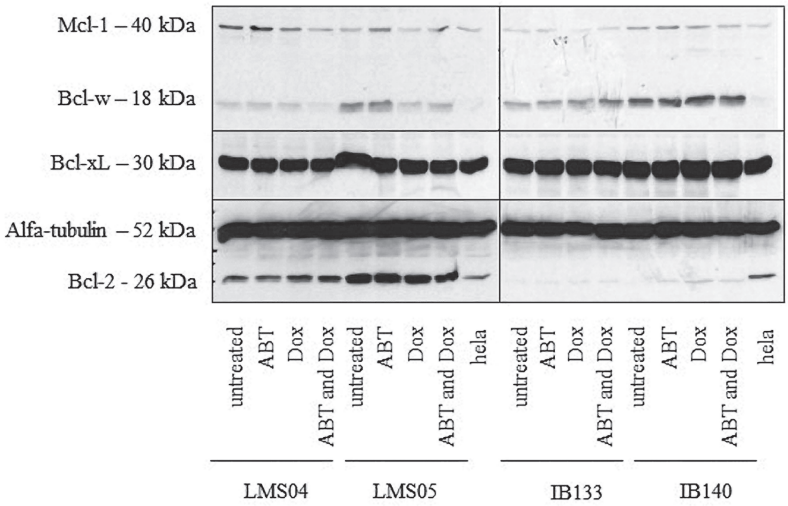


Figure 3. Western blots performed on untreated, single agent treated or combination-agent treated cells. The expression of Mcl-1, Bcl-w, Bcl-xL, α-tubulin and Bcl-2 in four leiomyosarcoma cell lines showed that Bcl-2 was expressed in LMS05 and LMS04, while it was almost absent in the IB133 and IB140 cell lines. Bcl-xL was most abundantly expressed in all four cell lines. Mcl-1 was expressed in all cell lines.

Combination treatment induces apoptosis

Using caspase Glo3/7 assays we demonstrated that reductions in cell viability were caused by apoptosis. All four cell lines showed a dramatic increase in caspase activity after combination treatment compared to single treatment (Figure 4A). Addition of the pan-caspase inhibitor Z-VAD-FMK blocked the increase in luciferase units, which indicates that the luciferase units correspond to apoptosis. Furthermore, induction of cleaved PARP, an indicator for apoptosis was greater in all 24 hour combination treated lysates, compared to the single-agent treated or untreated samples (Figure 4B) (13).

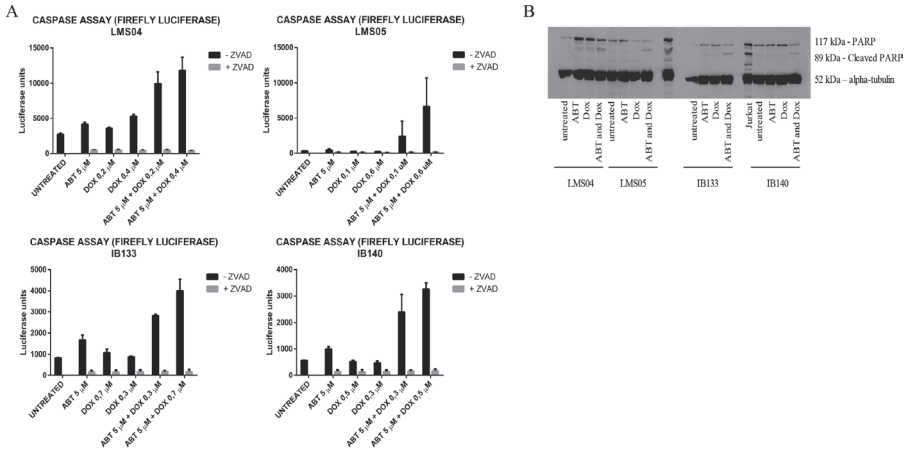


Figure 4. Caspase Glo3/7 assay and PARP western blots to evaluate apoptosis after single-agent and combination treatments. (**A**) Amount of luciferase units measured in combined treated cells was significantly higher compared to single and untreated cells. For each condition also the pan-caspase inhibitor (Z-VAD-FMK) was included which show low or no expression of luciferase units. (**B**) Cleaved PARP expression was confirmed in all combined treated cells by western blot.

Discussion

In this study we found high expression of Bcl-2 family members in leiomyosarcomas and demonstrate a role for these proteins in the response of leiomyosarcomas to conventional chemotherapy. More specifically, inhibition of Bcl-2 family members using the ABT-737 BH3 mimetic rendered leiomyosarcoma cell lines more sensitive to doxorubicin, suggesting new therapeutic options for patients with metastatic or inoperable disease.

We demonstrated a high protein expression of Bcl-xL and Bcl-w in leiomyosarcomas compared to benign uterine smooth muscle tumours. Bcl-2 expression was higher in leiomyomas compared to leiomyosarcomas, which was also reported by others (15, 16). Some studies showed a correlation between high Bcl-2 expression and longer disease-specific survival in uterine leiomyosarcomas (15-18). In contrast, we observed a trend for high Bcl-2 expression with shorter survival. The difference might be due to differences in biological behavior between uterine and soft tissue leiomyosarcomas as our series contains only three uterine leiomyosarcomas.

These results are consistent with observations in other tumour types, including sarcomas, in which *in vitro* studies have also demonstrated an important role for the Bcl-2 family members in chemoresistance, and that

tumour cells could be sensitized to doxorubicin by inhibition of these proteins, using ABT-737 (19). The addition of doxorubicin after ABT-737 treatment resulted in a complete loss of cell viability in chondrosarcoma. In other tumour cell lines, including castration-resistant prostate cancer (CRPC), malignant peripheral nerve sheath tumour (MPNST), acute promyelocytic leukemia (APL) and acute myeloid leukemia (AML), ABT-737 also showed synergy in combination with chemotherapeutic agents (20-23).

Treatment with ABT-737 alone resulted only at a high concentration in a small reduction of the cell viability in the IB133 and IB140 cell lines. Therefore, we concluded that *in vitro* treatment with ABT-737 as single agent has almost no influence on the cell viability. Other studies in chondrosarcomas and small cell lung carcinomas also revealed limited activity of ABT-737 monotherapy (24). To explore a possible role of the Bcl-2 family members in response to conventional chemotherapy, we combined ABT-737 with doxorubicin, which is commonly used as single agent in the treatment of high grade soft tissue sarcomas (9, 10). Indeed, a synergistic effect was seen as cell viability decreased and apoptosis could be shown. Interestingly, single treatment with doxorubicin decreased cell viability, but had no impact on apoptosis. This suggests that even though cells stopped proliferating and lost their viability, they failed to activate their apoptotic machinery at the doses tested, most probably due to expression of Bcl-2 family members. This is in accordance with the low response rates and the poor impact of chemotherapeutic agents on overall survival of patients.

The four leiomyosarcoma cell lines were analyzed using the AmpliSeq™ Cancer Hotspot Panel v2. We detected a *CTNNB1* S45C variation in IB140, with a coverage of 61 reads. Revision of the corresponding primary tumour confirmed the diagnosis of leiomyosarcoma as well as the presence of the same mutation (Suppl fig 2). Nuclear staining for β-catenin was absent, so the significance of the S45C *CTNNB1* alteration in this tumour remains unclear. Two cell lines showed alterations in *TP53*. The leiomyosarcoma cell lines which showed the highest synergy (LMS05 and IB140) have a wild type *TP53* gene, whereas the cell line with the lowest synergy (LMS04) did not. TP53 is a major protein in the apoptotic signaling pathway and when *TP53* is mutated the apoptotic response is impaired (25). Furthermore, wild type *TP53* increases the responsiveness to chemotherapy by down-regulating multidrug resistance-1 expression (26). Even though the numbers are small, a mutation in this key protein might explain the difference in treatment response for LMS04 and IB133.

Furthermore, the cell lines showed Mcl-1 expression at the same level or stronger as compared to the HeLa control, and it has been reported that Mcl-1 in HeLa cells plays an important role in the sensitization to ABT-737 (27). High Mcl-1 levels are associated with resistance of cell lines to ABT-737 and downregulation of Mcl-1 sensitizes carcinoma cells to doxorubicin (27-29). This suggests that Mcl-1 expression might be important in the anti-apoptotic mechanisms in the LMS cell lines, and modulation of this protein level might be a target to increase the effect of ABT-737 and doxorubicin treatment. Treatment with ABT-737, doxorubicin or a combination did not change the expression levels of Bcl-xL, Bcl-w or Bcl-2. This is comparable to other studies in which there were only slight differences in the expression of Bcl-2 and Bcl-xL after treatment (21, 30). Thus, ABT-737 and doxorubicin do not down regulate the expression of the Bcl-2 family members at the protein level, and may instead block their inhibitory effect on Bak/Bax proteins in the mitochondrial membrane (31).

The effect of ABT-263 (navitoclax®, Abbott), a structural analogue of ABT-737, has been analyzed in toxicity phase I and II studies in small cell lung carcinomas. Thrombocytopenia was the most frequently observed adverse effect. However, marrow compensation platelet counts showed partial recovery when dosing was sustained, and in the week without dosing, recovery to baseline was seen. Diarrhea, vomiting, nausea and fatigue were other common adverse effects (24, 32). These adverse events are also frequently seen after chemotherapeutic treatment but are most of the time not life-threatening, suggesting that the preclinical evidence generated in this report may be realistically translated to clinical application.

In conclusion, our results show that inhibition of Bcl-2 family members using ABT-737 renders leiomyosarcoma cell lines more sensitive to doxorubicin, especially in the presence of wildtype *TP53*, suggesting new potential therapeutic options for leiomyosarcoma patients with metastatic or inoperable disease.

Acknowledgements

The authors thank Inge Briaire-de Bruijn and Yvonne de Jong for expert technical assistance.

Conflict of interest

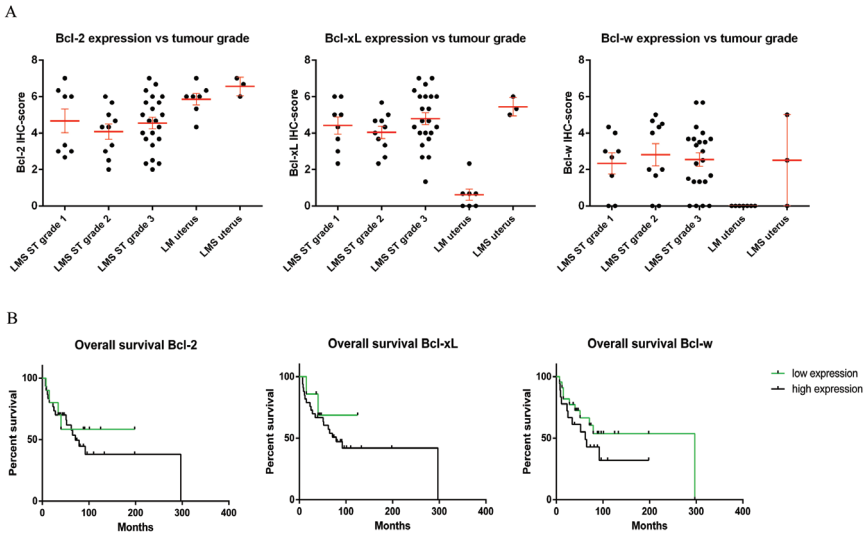
The authors declare no conflict of interest.

References

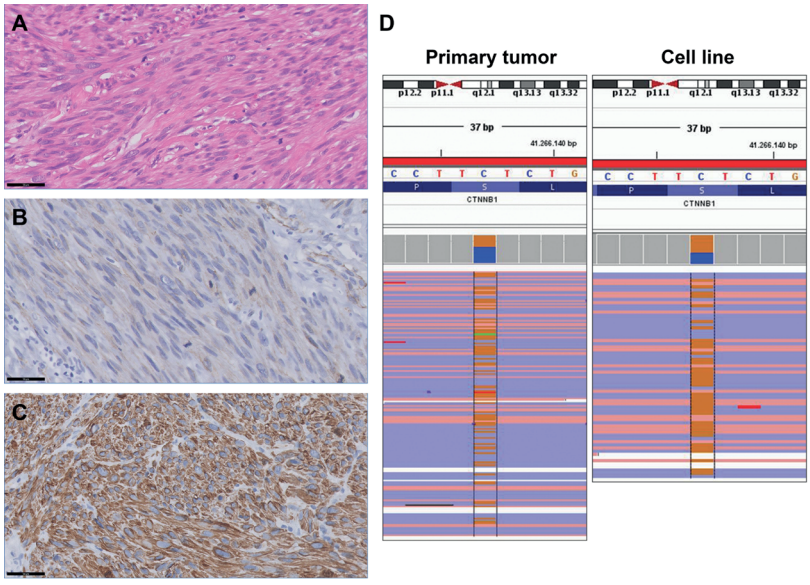
1. Fletcher CDM, Bridge JA, Hogendoorn PCW, et al. World Health Organisation Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. 4th ed. Lyon: IARC Press; 2013.
2. Villar VH, Vogler O, Martinez-Serra J, et al. Nilotinib counteracts P-glycoprotein-mediated multidrug resistance and synergizes the antitumoral effect of doxorubicin in soft tissue sarcomas. PLoS One. 2012;7(5):e37735.
3. Abdullah LN, Chow EK. Mechanisms of chemoresistance in cancer stem cells. Clin Transl Med. 2013;2(1):3.
4. Tacar O, Sriamornsak P, Dass CR. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. J Pharm Pharmacol. 2013;65(2):157-70.
5. Jiang X, Wang X. Cytochrome C-mediated apoptosis. Annu Rev Biochem. 2004;73:87-106.
6. Win TT, Yusuf Y, Jaafar H. Apoptotic activities in soft tissue sarcoma: immunohistochemical study and their association with tumour characteristics. Malays J Med Sci. 2013;20(2):10-6.
7. Hong T, Shimada Y, Uchida S, et al. Expression of angiogenic factors and apoptotic factors in leiomyosarcoma and leiomyoma. Int J Mol Med. 2001;8(2):141-8.
8. Oltersdorf T, Elmore SW, Shoemaker AR, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature. 2005;435(7042):677-81.
9. Grobmyer SR, Maki RG, Demetri GD, et al. Neo-adjuvant chemotherapy for primary high-grade extremity soft tissue sarcoma. Ann Oncol. 2004;15(11):1667-72.
10. Kraybill WG, Harris J, Spiro IJ, et al. Phase II study of neoadjuvant chemotherapy and radiation therapy in the management of high-risk, high-grade, soft tissue sarcomas of the extremities and body wall: Radiation Therapy Oncology Group Trial 9514. J Clin Oncol. 2006;24(4):619-25.
11. de Graaff MA, Cleton-Jansen AM, Szuhai K, et al. Mediator complex subunit 12 exon 2 mutation analysis in different subtypes of smooth muscle tumors confirms genetic heterogeneity. Hum Pathol. 2013;44(8):1597-604.
12. Hoekstra AS, de Graaff MA, Briaire-de Bruijn IH, et al. Inactivation of SDH and FH cause loss of 5hmC and increased H3K9me3 in paraganglioma/pheochromocytoma and smooth muscle tumors. Oncotarget. 2015;6(36):38777-88.
13. Shrivastav S, Bonar RA, Stone KR, et al. An in vitro assay procedure to test chemotherapeutic drugs on cells from human solid tumors. Cancer Res. 1980;40(12):4438-42.
14. Wong M, Tan N, Zha J, et al. Navitoclax (ABT-263) reduces Bcl-x(L)-mediated chemoresistance in ovarian cancer models. Mol Cancer Ther. 2012;11(4):1026-35.
15. Bodner K, Bodner-Adler B, Kimberger O, et al. Bcl-2 receptor expression in patients with uterine smooth muscle tumors: an immunohistochemical analysis comparing leiomyoma, uterine smooth muscle tumor of uncertain malignant potential, and leiomyosarcoma. J Soc Gynecol Investig. 2004;11(3):187-91.
16. Zhai YL, Nikaido T, Toki T, et al. Prognostic significance of bcl-2 expression in leiomyosarcoma of the uterus. British journal of cancer. 1999;80(10):1658-64.
17. Lusby K, Savannah KB, Demicco EG, et al. Uterine leiomyosarcoma management, outcome, and associated molecular biomarkers: a single institution's experience. Annals of surgical oncology. 2013;20(7):2364-72.
18. Leiser AL, Anderson SE, Nonaka D, et al. Apoptotic and cell cycle regulatory markers in uterine leiomyosarcoma. Gynecologic oncology. 2006;101(1):86-91.
19. van Oosterwijk JG, Herpers B, Meijer D, et al. Restoration of chemosensitivity for doxorubicin and cisplatin in chondrosarcoma in vitro: BCL-2 family members cause chemoresistance. Ann Oncol. 2012;23(6):1617-26.
20. Lee SJ, Park HJ, Kim YH, et al. Inhibition of Bcl-xL by ABT-737 enhances chemotherapy sensitivity in neurofibromatosis type 1-associated malignant peripheral nerve sheath tumor cells. Int J Mol Med. 2012;30(2):443-50.
21. Parrondo R, de Las PA, Reiner T, et al. ABT-737, a small molecule Bcl-2/Bcl-xL antagonist, increases antimitotic-mediated apoptosis in human prostate cancer cells. PeerJ. 2013;1:e144.

22. Ugarenko M, Nudelman A, Rephaeli A, et al. ABT-737 overcomes Bcl-2 mediated resistance to doxorubicin-DNA adducts. Biochem Pharmacol. 2010;79(3):339-49.
23. Baev DV, Krawczyk J, O'Dwyer M, et al. The BH3-mimetic ABT-737 effectively kills acute myeloid leukemia initiating cells. Leuk Res Rep. 2014;3(2):79-82.
24. Rudin CM, Hann CL, Garon EB, et al. Phase II study of single-agent navitoclax (ABT-263) and biomarker correlates in patients with relapsed small cell lung cancer. Clin Cancer Res. 2012;18(11):3163-9.
25. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell. 1997;88(3):323-31.
26. Zhan M, Yu D, Lang A, et al. Wild type p53 sensitizes soft tissue sarcoma cells to doxorubicin by down-regulating multidrug resistance-1 expression. Cancer. 2001;92(6):1556-66.
27. van Delft MF, Wei AH, Mason KD, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell. 2006;10(5):389-99.
28. Long J, Ji Z, Jiang K, et al. miR-193b Modulates Resistance to Doxorubicin in Human Breast Cancer Cells by Downregulating MCL-1. Biomed Res Int. 2015;2015:373574.
29. Konopleva M, Contractor R, Tsao T, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. Cancer Cell. 2006;10(5):375-88.
30. Rooswinkel RW, van de Kooij B, Verheij M, et al. Bcl-2 is a better ABT-737 target than Bcl-xL or Bcl-w and only Noxa overcomes resistance mediated by Mcl-1, Bfl-1, or Bcl-B. Cell Death Dis. 2012;3:e366.
31. DeMatteo RP, Lewis JJ, Leung D, et al. Two hundred gastrointestinal stromal tumors: recurrence patterns and prognostic factors for survival. Ann Surg. 2000;231(1):51-8.
32. Gandhi L, Camidge DR, Ribeiro de OM, et al. Phase I study of Navitoclax (ABT-263), a novel Bcl-2 family inhibitor, in patients with small-cell lung cancer and other solid tumors. J Clin Oncol. 2011;29(7):909-16.

Supplementary Information



Supplementary figure 1. Immunohistochemistry score in relation with histological grade and overall survival. (**A**) The overall score was not correlated with histological grade. (**B**) Leiomyosarcoma patients were divided into two groups (high >3 and low ≤3) based on the immunohistochemical score of Bcl-2, Bcl-xL and Bcl-w. A shorter overall survival was seen for patients with a high expression of Bcl-2, Bcl-xL and Bcl-w, although the difference was not significant.



Supplementary figure 2. Histology, immunohistochemistry and NGS analysis of the IB140 cell line and the corresponding primary tumour. (**A**) H&E staining of the primary tumour showed high mitotic activity, (**B**) no nuclear β-catenin expression, and (**C**) a strong and diffuse staining for caldesmon. The

morphology and immunohistochemistry are highly characteristic of leiomyosarcoma. (**D**) NGS analysis of the primary tumour and the cell line showed *CTNNB1 c.134C>G* (p.S45C) variation in the cell line (total reads 61; 24 C (39%), 37 G (61%)) and primary tumour (total reads 2787; 1642 C (59%), 1135 G 41%)).

Supplementary table 1. Clinicopathological data of leiomyosarcoma patients.

Sample	Age	Gender	P/R/M	Tumour localisation	Grade
1	39	M	P	Jejunum	1
2	60	F	P	Rectum	1
3	55	F	P	Retroperitoneum	1
4	76	F	R	Retroperitoneum	1
5	41	M	P	Soft tissue jaw	1
6	73	M	R	Duodenum	1
7	48	F	P	Rectus abdominis	1
8	32	M	P	Musculus psoas	1
9	23	M	R	Lower extremity	2
10	74	M	P	Upper extremity	2
11	56	F	P	Lower extremity	2
12	79	M	P	Subcutis back	2
13	43	M	P	Upper extremity	2
14	80	F	P	Lower extremity	2
15	30	M	P	Colon	2
16	76	F	P	Lower extremity	2
17	65	F	P	Lower extremity	2
18	55	M	M	Liver	2
19	80	F	P	Lower extremity	3
20	86	M	P	Lower extremity	3
21	43	F	P	Pelvis	3
22	67	F	P	Upper extremity	3
23	68	F	P	Lower extremity	3
24	70	M	P	Lower extremity	3
25	65	F	P	Lower extremity	3
26	70	M	P	Lower extremity	3
27	56	F	P	Lower extremity	3
28	75	M	P	Lower extremity	3
29	67	F	P	Upper extremity	3
30	41	F	P	Lower extremity	3
31	70	F	P	Retroperitoneum	3
32	84	F	P	Upper extremity	3
33	74	F	M	Pleura	3
34	50	F	P	Arteria femoralis	3
35	58	M	P	Lower extremity	3
36	52	F	P	Lower extremity	3
37	48	M	P	Stomach	3
38	76	F	P	Lower extremity	3
39*	50	F	P	Retroperitoneum	3
40*	50	F	M	Abdomen	3
41	39	F	P	Uterus	NA

Supplementary table 1. Continued

Sample	Age	Gender	P/R/M	Tumour localisation	Grade
42	61	F	P	Uterus	NA
43	52	F	P	Uterus	NA

P=primary tumor; R=recurrence; M=metastasis; NA=non applicable; *primary and metastatic tumour sample from the same patient. None of the included samples were pretreated with chemotherapy or radiation.

Supplementary table 2. The characteristics of the four leiomyosarcoma cell lines.

Cell lines	Passage number	Diagnosis and grade	Patient	*TP53*[$]	Cancer Hotspot panel
LMS04	P45	LMS III	F/54	Homozygous deletion	KDR
LMS05	P34	LMS II ST	M/76	Wild type: exon 4-9	No mutations
IB133	P21	LMS III RP	F/56	Deletion: exon 2 and 3	No mutations
IB140	P22	LMS II ST	M/51	Wild type: exon 1-11	CTNNB1 S45C[#]

[#] The mutation was also found in the primary tumour, which upon revision demonstrated abundant mitotic activity, strong expression of heavy caldesmon, and lack of nuclear staining for beta-catenin, consistent with the diagnosis of leiomyosarcoma (Suppl fig 2).

$ determined by Sanger sequencing.

LMS: leiomyosarcoma, RP: retroperitoneum, ST: soft tissue.

Supplementary table 3. Details of antibodies used for immunohistochemistry.

Antibody	Manufacturer	Dilution	Antigen retrieval
Bcl-2	Abcam	1/6	Citrate, pre-incubation 5% Elk milk
Bcl-xL	Cell signaling	1/3	Citrate, DAB+
Bcl-w	Abcam	1/3	Citrate, DAB+

Supplementary table 4. Details of antibodies used for western blot.

Antibody/clone	Species	Manufacturer	Dilution
Bcl-2/50E3	Rabbit	Cell signaling technology, Leiden, The Netherlands	1:1000
Bcl-xL/54H6	Rabbit	Cell signaling technology	1:1000
Bcl-w/31H4	Rabbit	Cell signaling technology	1:1000
Mcl-1	Rabbit	Cell signaling technology	1:1000
PARP/46D11	Rabbit	Cell signaling technology	1:1000
Alpha-tubulin/DM1A	Mouse	Abcam, Cambridge, UK	1:1000

Chapter 5

NY-ESO-1 (CTAG1B) expression in mesenchymal tumors

Makoto Endo, Marieke A. de Graaff, Davis R. Ingram, Simin Lim, Dina C. Lev, Inge H. Briaire-de Bruijn, Neeta Somaiah, Judith V.M.G. Bovée, Alexander J. Lazar, Torsten O. Nielsen

Modern Pathology. 2015; 28(4): 587-595

Abstract

New York esophageal squamous cell carcinoma 1 (NY-ESO-1, CTAG1B) is a cancer-testis antigen and currently a focus of several targeted immunotherapeutic strategies. We performed a large-scale immunohistochemical expression study of NY-ESO-1 using tissue microarrays of mesenchymal tumors from three institutions in an international collaboration. A total of 1132 intermediate and malignant, and 175 benign mesenchymal lesions were enrolled in this study. Immunohistochemical staining was performed on tissue microarrays using a monoclonal antibody for NY-ESO-1. Among mesenchymal tumors, myxoid liposarcomas showed the highest positivity for NY-ESO-1 (88%), followed by synovial sarcomas (49%), myxofibrosarcomas (35%) and conventional chondrosarcomas (28%). Positivity of NY-ESO-1 in the remaining mesenchymal tumors was consistently low, and no immunoreactivity was observed in benign mesenchymal lesions. Based on these findings, nearly 90% of myxoid liposarcomas, as well as a significant proportion of synovial sarcomas, myxofibrosarcomas and conventional chondrosarcomas are good candidates for immunotherapy targeting NY-ESO-1.

Keywords:
NY-ESO-1, cancer-testis antigen, myxoid liposarcoma, mesenchymal tumor, sarcoma, immunohistochemistry, immunotherapy

Introduction

New York esophageal squamous cell carcinoma 1 (NY-ESO-1), encoded by the *CTAG1B* gene, is a cancer-testis antigen that was identified in 1997 from the serum of a patient with esophageal squamous cell carcinoma (1). Cancer-testis antigens such as NY-ESO-1 have attracted increasing attention as immunotherapeutic targets because, among normal tissues, they are expressed only in adult testis germ cells and are atypically re-expressed in many malignancies (2, 3). NY-ESO-1 is of particular interest to researchers and clinicians because it is highly immunogenic and is expressed in a variety of carcinomas, melanomas and sarcomas (4). A recent clinical trial of adoptive immunotherapy, using genetically modified T cells directed against NY-ESO-1, demonstrated objective clinical responses in a number of metastatic synovial sarcoma and melanoma patients with NY-ESO-1-positive tumors (5). The results of that clinical trial highlight the potential effectiveness of immunotherapy against tumors expressing NY-ESO-1, and several clinical trials (using antigen sensitization, adoptive T-cell transfer, and dendritic cell vaccine strategies) are currently underway.

Mesenchymal tumors arising from bone or soft tissues comprise many histologic subtypes, which even taken together occur at a much lower rate than the more common carcinomas, creating a practical barrier to developing new drug therapies. However, the well-defined biology of many sarcomas suggests they may be particularly susceptible to appropriate targeted strategies. Recent studies have reported NY-ESO-1 expression in an especially large proportion of myxoid liposarcomas and synovial sarcomas (2, 6-9). These studies also revealed the absence of NY-ESO-1 expression in their histologic mimics such as myxoma, myxofibrosarcoma, extraskeletal myxoid chondrosarcoma and low-grade fibromyxoid sarcoma, and concluded that immunohistochemical staining of NY-ESO-1 is useful not only for candidate selection in immunotherapy, but also for histologic differential diagnosis. A limitation of these previous studies is that the sample sizes were too small to be conclusive and lacked independent external validation.

The goals of this study are to validate published reports of NY-ESO-1 expression in myxoid liposarcoma and synovial sarcoma patients on a larger sample set, and to investigate NY-ESO-1 expression in other mesenchymal tumors not represented in previous studies. To do so, we undertook a multi-

institutional NY-ESO-1 expression survey on tissue microarrays encompassing a wide variety of malignant, intermediate and benign bone and soft tissue tumors, seeking to determine if NY-ESO-1 is indeed useful in the differential diagnosis among mesenchymal tumors and to identify subtypes that are most likely to benefit from NY-ESO-1 immunotherapy.

Materials and Methods

Tumor samples and tissue microarrays

In this study, formalin-fixed paraffin-embedded tissue microarrays from 3 institutions, the University of British Columbia (UBC), the MD Anderson Cancer Center (MDACC) and the Leiden University Medical Center (LUMC), with a diverse selection of benign, intermediate and malignant mesenchymal tumors were used. Each tissue microarray contains duplicate, triplicate or quadruplicate 0.6 mm (UBC, LUMC), 1.5 mm (LUMC) or 2.0 mm (MDACC, LUMC) cores derived from representative viable diagnostic areas identified by a specialized bone and soft tissue tumor pathologist (TON, AJL, JVMGB). Tissue microarrays from UBC included in this study are TMA 01-003 (10), TMA 03-008 (11), TMA 06-007 (12), TMA 06-001B (13), TMAs 08-019, 09-006, 10-009 (14), TMAs 12-004, 12-005, 12-006, 12-010 (15) and TMA MPNST (16). From MDACC, myxoid liposarcoma tissue microarrays contained untreated tumors and tumors pre-treated with chemotherapy, radiation or a combination of both (17, 18). From the LUMC, tissue microarrays were selected containing chondrosarcomas (19, 20), osteosarcomas (21), myxoid liposarcomas and a variety of benign and malignant soft tissue tumors (22, 23).

Immunohistochemistry

Immunohistochemical staining was performed with an autostainer for tissue microarrays of the UBC and MDACC following protocols as described previously (24). Briefly, antigen retrieval was performed using the Standard Cell Conditioning 1 (CC1) protocol (Ventana, Tucson, AZ, USA). Sections were incubated with mouse anti-NY-ESO-1 monoclonal antibody (clone E978, Invitrogen, Carlsbad, CA, USA) at 1:25 dilution for 2 hours at room temperature, and then with UltraMap HRP-conjugated anti-mouse IgG secondary antibody for 16 min. The ChromoMap DAB Kit (Ventana) provided the visualization

chromogen. Slides were then counterstained with hematoxylin and mounted. Immunohistochemical staining of the tissue microarrays of the LUMC was performed manually; 4 µm sections were transferred to adhesive coated slides (Leica Biosystems, Rijswijk, The Netherlands) and dried overnight at 37°C. Next slides were deparaffinized in xylene, rehydrated and incubated in 0.3% methanol/H_2O_2 at room temperature for 20 min to block the endogenous peroxidase activity. Antigen retrieval was performed by 10 min microwave treatment in tris/EDTA-solution (pH=9.0), followed by the addition of the primary NY-ESO-1 / CTAG1B antibody (1:100, clone E978, Invitrogen) and overnight incubation in a moist chamber at room temperature. Next, slides were incubated with PowerVision Poly-HRP (Leica), visualized with DAB+ Substrate Chromogen System (DAKO, Heverlee, Belgium) and counterstained with hematoxylin. All wash steps were done in PBS.

In both protocols the staining was preceded by a titration series on normal testis, and during the actual staining testis was included as a positive control, and as the tissue present in the negative control reaction (without the addition of the primary antibody).

Digital Images

Digital images of immunostained and H&E-stained tissue microarrays of UBC were acquired using a BLISS imager (Bacus Laboratories, Lombard, IL, USA). A relational database was constructed that correlates scoring and identification information with images of each core. This information is publicly accessible at http://www.gpecimage.ubc.ca (username: nyeso1; password: nyeso1).

Evaluation of immunohistochemistry

The tissue microarray slides were evaluated under a light microscope or on the scanned images by two to five independent observers (ME, MAG, TON, JVMGB, AJL), including three experienced bone and soft tissue tumor subspecialty pathologists (TON, JVMGB, AJL). NY-ESO-1 immunopositivity was scored in a semiquantitative manner for the intensity (0 = negative, 1 = weak positive, 2 = moderate positive, 3 = strong positive) and the percentage of positive tumor cells (0 = 0%, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%). Each core on the tissue microarrays was scored separately and the average score from cores of the same tumor were calculated. Tumor samples with more than 50% of positive tumor cells and a staining intensity of moderate or strong (2 or 3) were

defined as "positive". This cutoff value was chosen to be consistent with criteria used in an ongoing clinical trial of NY-ESO-1 immunotherapy (5). In addition, cross comparison of manual and autostaining revealed the manual method generated a weak background stain not seen with the automated platform. However, the scoring system, which considered weak (intensity 1) staining as negative meant that the cases defined as "positive" were consistently categorized and equally discernible with both staining methods. Comparison of TMA to whole section was performed on a comparison set of eleven cases, and the staining results were found to be concordant.

Statistical Analysis

A chi-square test was used to assess the differences between histological subtypes; statistical significance was defined as $p < 0.05$. Data analysis was performed with JMP software (version 9.0.2, JMP Software, Cary, NC, USA).

Table 1. Immunohistochemical NY-ESO-1 (CTAG1B) expression in intermediate and malignant bone and soft tissue tumors.

	TMAs UBC and MDACC			TMAs LUMC			Total		
Malignant and intermediate bone and soft tissue tumors	**cases**	**N positive**	**% pos cases**	**cases**	**N positive**	**% pos cases**	**cases**	**N positive**	**% pos cases**
SOFT TISSUE									
Adipocytic tumors									
Liposarcoma, dedifferentiated	5	0	0.0	2	1	50.0	7	1	14.3
Liposarcoma, myxoid	**128**	**110**	**85.9**	**30**	**29**	**96.7**	**158**	**139**	**88.0**
Liposarcoma, myxoid, post-treatment	**43**	**18**	**41.9**				**43**	**18**	**41.9**
Liposarcoma, pleomorphic	7	0	0.0	4	0	0.0	11	0	0.0
Liposarcoma, well-differentiated	13	1	7.7	8	0	0.0	21	1	4.8
Fibroblastic / myofibroblastic tumors									
Desmoid-type fibromatosis	8	0	0.0				8	0	0.0
Dermatofibrosarcoma protuberans	17	2	11.8	1	0	0.0	18	2	11.1
Solitary fibrous tumor	15	0	0.0	2	0	0.0	17	0	0.0
Fibrosarcoma	8	1	12.5				8	1	12.5
Myofibroblastic sarcoma				2	0	0.0	2	0	0.0
Myxofibrosarcoma				**17**	**6**	**35.3**	**17**	**6**	**35.3**
Low grade fibromyxoid sarcoma	5	0	0.0				5	0	0.0
Sclerosing epithelioid fibrosarcoma	1	0	0.0				1	0	0.0
Smooth muscle tumors									
Leiomyosarcoma	5	0	0.0	50	8	16.0	55	8	14.5
Skeletal muscle tumors									
Rhabdomyosarcoma, alveolar	9	0	0.0				9	0	0.0
Rhabdomyosarcoma, embryonal	12	0	0.0	2	1	50.0	14	1	7.1
Rhabdomyosarcoma, pleomorphic				3	1	33.3	3	1	33.3
Vascular tumors of soft tissue									
Angiosarcoma				1	0	0.0	1	0	0.0
Epithelioid angiosarcoma	4	0	0.0				4	0	0.0
Epithelioid hemangioendothelioma	1	0	0.0				1	0	0.0
Gastrointestinal stromal tumors									
Gastrointestinal stromal tumor	143	0	0.0	3	0	0.0	146	0	0.0
Nerve sheath tumors									
Malignant peripheral nerve sheath tumor	75	2	2.7	7	0	0.0	82	2	2.4

Table 1. continued.

	TMAs UBC and MDACC			TMAs LUMC			Total		
Malignant and intermediate bone and soft tissue tumors	**cases**	**N positive**	**% pos cases**	**cases**	**N positive**	**% pos cases**	**cases**	**N positive**	**% pos cases**
Tumors of uncertain differentiation									
Alveolar soft part sarcoma	8	0	0.0				8	0	0.0
Angiomatoid fibrous histiocytoma	2	0	0.0				2	0	0.0
Chondrosarcoma, extraskeletal myxoid	5	0	0.0				5	0	0.0
Clear cell sarcoma	8	0	0.0				8	0	0.0
Desmoplastic small round cell tumor	2	0	0.0				2	0	0.0
Epithelioid sarcoma	7	0	0.0				7	0	0.0
Malignant myoepithelioma	1	0	0.0				1	0	0.0
Synovial sarcoma	**63**	**30**	**47.6**	**6**	**4**	**66.7**	**69**	**34**	**49.3**
Undifferentiated / unclassified sarcomas									
Undifferentiated pleomorphic sarcoma	5	1	20.0	22	2	9.1	27	3	11.1
Undifferentiated spindle cell sarcoma				5	0	0.0	5	0	0.0
Endometrial stromal sarcoma									
Endometrial stromal sarcoma	58	3	5.2				58	3	5.2
BONE									
Chondrogenic tumors									
Chondrosarcoma, conventional	**29**	**2**	**6.9**	**113**	**38**	**33.6**	**142**	**40**	**28.2**
Chondrosarcoma, clear cell				18	0	0.0	18	0	0.0
Chondrosarcoma, dedifferentiated	4	0	0.0	26	3	11.5	30	3	10.0
Chondrosarcoma, mesenchymal	3	0	0.0	15	0	0.0	18	0	0.0
Osteogenic tumors									
Osteosarcoma	4	1	25.0	76	4	5.3	80	5	6.3
Miscellaneous tumors									
Ewing sarcoma	21	0	0.0				21	0	0.0

Bold indicates entities with a significant fraction of positive cases.

Table 2. Immunohistochemical NY-ESO-1 expression in benign bone and soft tissue tumors.

	TMAs UBC and MDACC			TMAs LUMC			Total		
Benign bone and soft tissue tumors	**cases**	**N positive**	**% pos cases**	**Scorable cases**	**N positive**	**% pos cases**	**Scorable cases**	**N positive**	**% pos cases**
SOFT TISSUE									
Atypical fibroxantoma	2	0	0.0				2	0	0.0
Congenital mesoblastic nephroma	2	0	0.0				2	0	0.0
Fetal rhabdomyoma	2	0	0.0				2	0	0.0
Fibroma/fibromatosis	36	0	0.0	4	0	0.0	40	0	0.0
Granuloma annulare	1	0	0.0				1	0	0.0
Leiomyoma				6	0	0.0	6	0	0.0
Lipoma	7	0	0.0	7	0	0.0	14	0	0.0
Lipoma, spindle cell	10	0	0.0				10	0	0.0
Myofibroma and Myofibromatosis	6	0	0.0				6	0	0.0
Myositis ossificans	2	0	0.0				2	0	0.0
Myxoma				3	0	0.0	3	0	0.0
Neurofibroma	33	0	0.0				33	0	0.0
Nodular fasciitis	1	0	0.0				1	0	0.0
Perineurioma	3	0	0.0				3	0	0.0
Schwannoma	21	0	0.0	3	0	0.0	24	0	0.0
Tenosynovial giant cell tumor				1	0	0.0	1	0	0.0
BONE									
Chondroblastoma	4	0	0.0				4	0	0.0
Chondromyxoid fibroma	3	0	0.0				3	0	0.0
Enchondroma	6	0	0.0	5	0	0.0	11	0	0.0
Osteochondroma				6	0	0.0	6	0	0.0
Synovial chondromatosis				1	0	0.0	1	0	0.0

Table 3. Immunohistochemical NY-ESO-1 expression in non-mesenchymal tumors and in several non-tumorous lesions.

	TMAs UBC and MDACC			TMAs LUMC			Total		
	cases	**N positive**	**% pos cases**	**Scorable cases**	**N positive**	**% pos cases**	**Scorable cases**	**N positive**	**% pos cases**
Non-mesenchymal tumors									
Hepatoblastoma	2	0	0.0				2	0	0.0
Melanoma	7	0	0.0				7	0	0.0
Melanoma, desmoplastic	10	1	10.0				10	1	10.0
Squamous cell carcinoma	8	0	0.0				8	0	0.0
Wilms tumor	23	0	0.0				23	0	0.0
Non-tumorous lesions									
Granuloma annulare	1	0	0.0				1	0	0.0
Reumatoid nodule	5	0	0.0				5	0	0.0
Normal fat	8	0	0.0				8	0	0.0

Results

In total, specimens from 1132 malignant and intermediate bone and soft tissue tumors, 175 benign bone and soft tissue tumors, 50 non-mesenchymal tumors, 6 non-tumorous lesions and 8 samples of normal fat were available for evaluation (Tables 1 - 3). There were 38 subtypes of malignant and intermediate tumors and 21 different benign mesenchymal lesions in this cohort. The subtypes with more than 50 samples available included myxoid liposarcoma (n=201 in total; 158 untreated cases and 43 tumors which received treatment prior to resection), gastrointestinal stromal tumor (n=146), conventional chondrosarcoma (n=142), malignant peripheral nerve sheath tumor (n=82), osteosarcoma (n=80), synovial sarcoma (n=69), endometrial stromal sarcoma (n=58) and leiomyosarcoma (n=55).

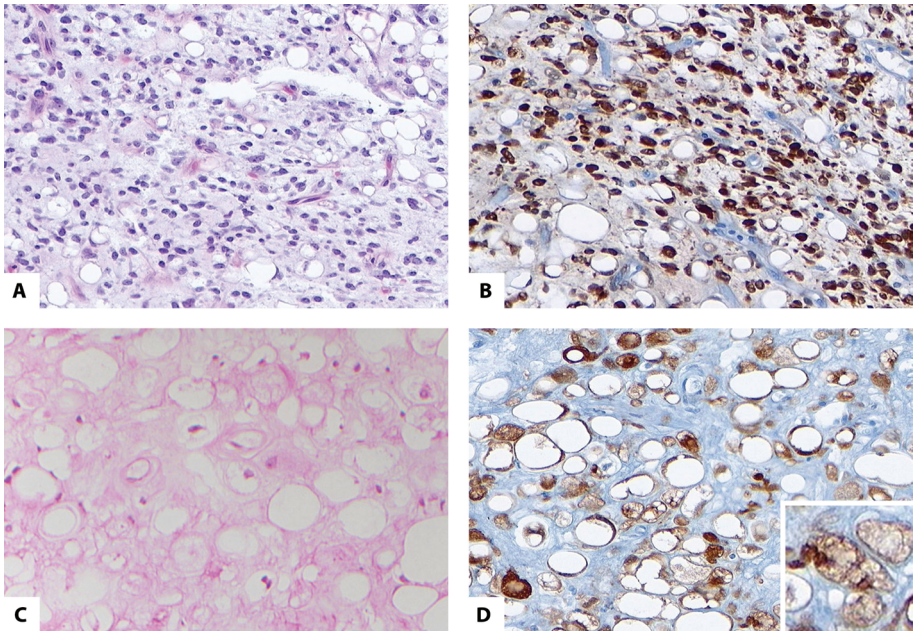


Figure 1. Immunohistochemical staining of NY-ESO-1 (CTAG1B) in myxoid and round cell liposarcomas.

Myxoid liposarcoma without treatment prior to resection revealed strong nuclear immunoreactivity in the majority of the tumor cells: (A) H&E, (B) NY-ESO-1 immunohistochemistry. Myxoid liposarcoma after neo-adjuvant treatment (with both radiation and chemotherapy) showed typical histologic changes of hyalinization (C) but retained nuclear and cytoplasmic staining (D).

Among a wide variety of mesenchymal tumors, myxoid liposarcomas that had not received neoadjuvant treatment prior to resection showed the highest positivity for NY-ESO-1 (CTAG1B): 88.0% (139/158). The high number of positive cases was observed both in the autostained cohort cases from UBC and MDACC (85.9%; 110/128) and in the manually stained cohort cases from LUMC (96.7%; 29/30). Even among myxoid liposarcomas which received chemotherapy or radiation prior to resection, NY-ESO-1 expression was still detectable in 41.9% (18/43) of cases, although this was significantly lower ($p<0.0001$) compared to the cases without preoperative treatment. Expression was seen both in myxoid as well as round cell areas. In an additional 37.2% (16/43) of these neoadjuvantly-treated tumors, weak staining in a small subset of the tumor cells was present; the remaining 20.9% (9/43) did not reveal any residual immunoreactivity. In comparison, the other liposarcomas – including well-differentiated (4.8% positivity; 1/21) and dedifferentiated (14.3%; 1/7) subtypes – showed NY-ESO-1 expression in only a minor subset of the cases; none of the tested pleomorphic liposarcomas revealed NY-ESO-1 expression (0%; 0/11). NY-ESO-1 positivity in myxoid liposarcomas was significantly higher than in the other liposarcomas ($p<0.0001$) or in the other malignant and intermediate bone and soft tissue tumors ($p<0.0001$).

NY-ESO-1 expression was observed in almost half (49.3%; 34/69) of the synovial sarcomas. In addition, positivity was found in conventional chondrosarcoma (28.2%; 40/142) and dedifferentiated chondrosarcoma (10.0%; 3/30). In the conventional chondrosarcomas there was no correlation of NY-ESO-1 expression and histological grade. Two of the positive dedifferentiated chondrosarcomas revealed NY-ESO-1 expression only in the dedifferentiated component and the third positive case showed immunoreactivity in the well-differentiated component.

Other sarcomas with a substantial percentage of positive tumors include myxofibrosarcoma (35.3%; 6/17), leiomyosarcoma (14.5%; 8/55), dermatofibrosarcoma protuberans (11.1%; 2/18 – including one of nine cases with fibrosarcomatous change) and undifferentiated pleomorphic sarcoma (11.1%; 3/27). Pleomorphic rhabdomyosarcoma (33.3%; 1/3) and fibrosarcoma (12.5%; 1/8) also presented positivity in a minority of the cases, although the sample size was small. The 175 benign bone and soft tissue tumors, the 6 non-tumorous lesions and the 8 normal fat samples were all negative for NY-ESO-1.

In the group of the non-mesenchymal tumors 10% (1/10) of the desmoplastic melanomas and none of the other melanomas showed NY-ESO-1 positivity.

Positive controls of formalin-fixed, paraffin-embedded testis sections revealed strong NY-ESO-1 expression among spermatogonia and spermatocytes in the seminiferous tubules. No immunoreactivity was observed in any of the negative controls. Representative results of a selection of malignant bone and soft tissue tumors are shown in Figures 1 - 3.

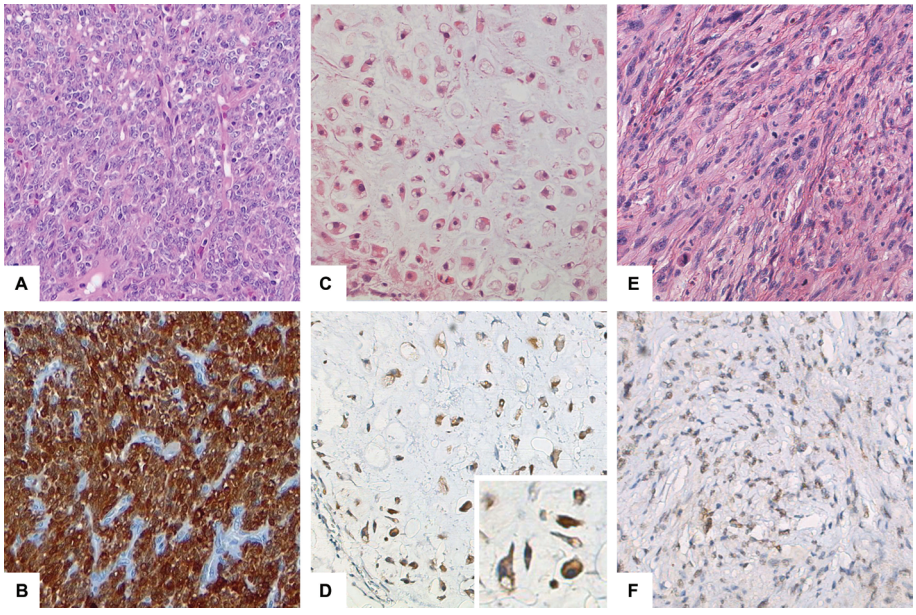


Figure 2. Other sarcomas with positive immunohistochemical staining for NY-ESO-1.

A+B: Synovial sarcoma with strong staining of all tumor cells. **C+D,** incl. inset: Conventional chondrosarcoma with prominent cytoplasmic staining. **E+F**: Myxofibrosarcoma with moderate immunoreactivity.

Discussion

To the best of our knowledge, this is the largest-scale NY-ESO-1 (CTAG1B) expression study in bone and soft tissue tumors, including over 1300 specimens representing 38 malignant and borderline and 21 benign histologic subtypes. Of special note is the inclusion in our study of 201 myxoid liposarcomas and 69 synovial sarcomas – two sarcoma subtypes that have been considered to be potentially good candidates for NY-ESO-1 immunotherapy. We employed a

cut-off value for immunohistochemical positivity by reference to the eligibility of an antecedent clinical trial of immunotherapy targeting NY-ESO-1 (5).

Myxoid liposarcoma represents 20-30% of all liposarcomas, mostly occurs in young adults and is characterized by a translocation t(12;16)(q13;p11) or, in a few percent of the cases, a t(12;22)(q13;q12), leading to the chimeric fusion product FUS-DDIT3 or EWSR1-DDIT3, respectively (25, 26). According to the literature, one third of myxoid liposarcoma patients develop distant metastases (18, 27, 28), so effective systemic therapy is needed to improve the prognosis of many patients. Previous studies reported NY-ESO-1 expression in 95% to 100% of myxoid liposarcomas; these results were based on sample sizes ranging from 25 to 38 cases (6, 9). Our larger study revealed that 139 out of the 158 (88.0%) untreated myxoid liposarcomas were positive for NY-ESO-1, and concurs with the rate of positivity reported in the previous studies. The slightly lower rate of positivity might be attributable to the fact that some tumors only display a focal NY-ESO-1 positivity, which might be missed due to the use of tissue microarrays. Hemminger et al. reported that other liposarcoma subtypes, lipomas and other myxoid mesenchymal tumors were negative for NY-ESO-1, suggesting that NY-ESO-1 could be a sensitive and specific immunohistochemical marker to differentiate myxoid liposarcoma from tumors with overlapping histomorphological features (9). In this study several mesenchymal tumors with myxoid or lipogenic differentiation were evaluated, including well-differentiated liposarcoma (4.8%; 1/21), dedifferentiated liposarcoma (14.3%; 1/7), spindle cell lipoma (0%; 0/10), lipoma (0%; 0/14), normal fat tissue (0%; 0/8), myxoma (0%; 0/3) and myxofibrosarcoma (35.3%; 6/17). Comparing those mimics, myxoid liposarcoma shows remarkably strong immunoreactivity for NY-ESO-1 (88.0%) and therefore its expression helps to discriminate myxoid liposarcoma from tumors in the differential diagnosis.

Interestingly, chemotherapy- or radiation-pretreated myxoid liposarcomas showed a lower positivity for NY-ESO-1. The biological mechanism behind the decreasing NY-ESO-1 positivity after the preoperative treatment is unknown; however, some of these samples at histological examination showed a low tumor cell density with hyalinization, reactive matrix and weak NY-ESO-1 staining below the intensity required to label the tumor as positive. NY-ESO-1 expression is still more frequent even in treated myxoid liposarcomas than in other sarcomas; therefore NY-ESO-1-targeted immunotherapy combined with established therapies remains an attractive potential strategy. This observed

reduction in NY-ESO-1 positivity in post-treatment myxoid liposarcoma was not seen in a set of eight additional synovial sarcoma specimens, in which all retained strongly positive expression following neoadjuvant treatment (radiation +/- chemotherapy).

Synovial sarcoma is a translocation-associated sarcoma characterized by the fusion gene, *SS18-SSX1*, *-SSX2* or *-SSX4* (29). More than half of the patients are teenagers and young adults. The development of novel systemic therapies is important because metastasis of synovial sarcoma is common (overall 5-year metastasis-free survival rate 51.1%)(30). Jungbluth et al. first investigated NY-ESO-1 expression in synovial sarcoma and found that 80% (20/25) of the samples expressed NY-ESO-1 (31). Recently, Lai et al. investigated 50 synovial sarcomas and reported that 76% (38/50) of synovial sarcomas expressed NY-ESO-1 in a strong and diffuse pattern (7). Our study revealed 49.3% positivity across 69 cases of synovial sarcoma, somewhat lower than that of the previous papers, possibly due to decreased sensitivity using the tissue microarray platform. Considering the combined results from our and previous studies, approximately half to three quarters of synovial sarcomas show NY-ESO-1 expression.

In a recent clinical trial using genetically engineered autologous T lymphocytes reactive with NY-ESO-1, four of the six advanced synovial sarcoma patients who received immunotherapy showed documented partial responses (5); worth noting is that this response lasted at least 18 months in one patient. This result has encouraged the development of NY-ESO-1-targeted immunotherapy not only for synovial sarcomas, but also for other NY-ESO-1 expressing sarcomas including myxoid liposarcomas. Public information on clinicaltrials.gov lists at least fourteen clinical trials employing vaccine, autologous T-cell or dendritic cell transduction immunotherapy strategies that test patients with NY-ESO-1 expressing sarcomas, mostly in active recruitment phases in 2014. Some vaccine studies use a low NY-ESO-1 positivity cutoff value of >5%, hoping that the vaccine can still elicit a immune response in these patients.

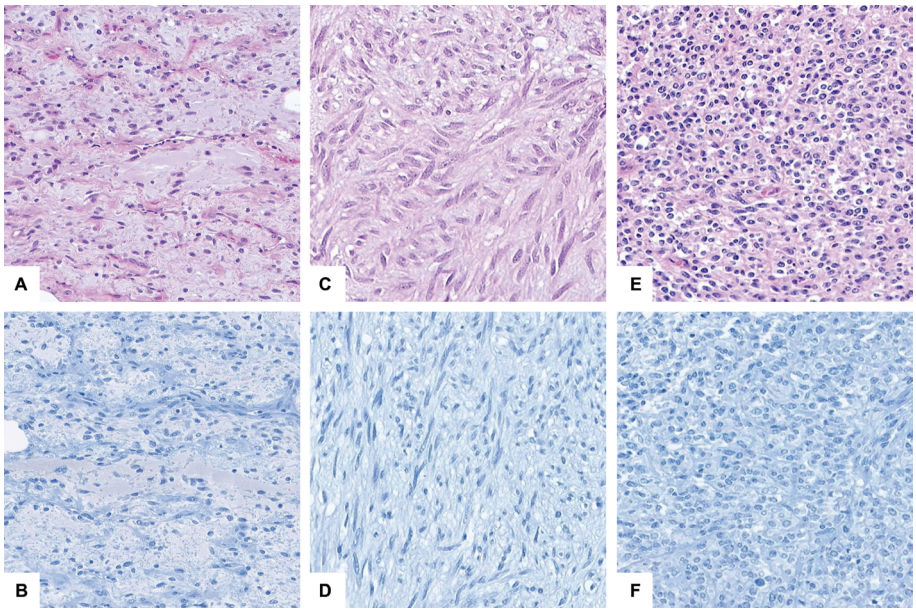


Figure 3. Soft tissue sarcomas without NY-ESO-1 immunoreactivity.

A+B: Myxoid liposarcoma. **C+D**: Gastrointestinal stromal tumor. **E+F**: malignant peripheral nerve sheat tumor.

Primary central chondrosarcoma is a bone sarcoma, accounting for approximately 20% of malignant bone tumors (28). As it is highly resistant to existing chemotherapeutic agents, the search for new targeted therapies is ongoing. Our study revealed positivity for NY-ESO-1 in 28.2% (40/142) of conventional chondrosarcoma and 10.0% (3/30) of dedifferentiated chondrosarcoma specimens, while clear cell and mesenchymal chondrosarcomas were all negative. Lai et al. reported that one out of two tested chondrosarcomas showed immunoreactivity for NY-ESO-1, and a relatively low NY-ESO-1 mRNA expression level was found in four out of eleven cases in another study (7, 32). Although the number of positive cases is relatively low, immunotherapy may be of significance in a subset of these patients, who are not included in the eligibility criteria for some of the existing clinical trials.

Apart from six of seventeen tested myxofibrosarcomas and one of three pleomorphic rhabdomyosarcomas, the other tested types of mesenchymal tumors were almost uniformly negative for NY-ESO-1 expression. Although our study included over 1132 cases, the numbers of some rare sarcomas are too limited to draw final conclusions on NY-ESO-1 expression in these subtypes.

In summary, we confirm that NY-ESO-1 can be reliably detected by immunohistochemistry. Using previously described scoring criteria, but based on much larger numbers of samples than have been assessed previously, we confirm very high frequencies of NY-ESO-1 expression in myxoid liposarcomas, high frequencies in synovial sarcomas, and we find similar levels of expression in 28.2% of conventional chondrosarcomas. Clinical trials of NY-ESO-1 targeted therapies should be open to patients with positive histologies, and NY-ESO-1 expression by immunohistochemistry should be considered as a potential entry criteria or integrated stratification as a potential predictive marker for such therapeutic strategies. NY-ESO-1 positivity in the remaining subtypes of mesenchymal tumors was rare. Benign bone and soft tissue lesions showed no positivity for NY-ESO-1, supporting the limited level of expression of this biomarker in normal tissues and the possibility that targeted therapies will have limited side effects.

Acknowledgements

We thank Christine Chow for her excellent technical assistance. We thank Anne-Marie Cleton-Jansen, Dorien van der Geest, Marieke Kuijjer, Danielle Meijer, Jolieke van Oosterwijk and Heidi van Paassen for the help with the construction of the LUMC tissue micro arrays and the collection of the clinical data. We would like to thank EuroBoNeT partners Nick Athanasou, the University of Oxford, UK; Soren Daugaard, Department of Pathology, RH, Denmark, Bernadette Liegl, Department of Pathology, Medizinische Universität Graz, Austria and Pierro Picci, Department of Pathology, Rizzoli Institute, Bologna, Italy for providing cases of rare chondrosarcoma subtypes. We also thank Samuel Leung for building the online relational database. Specimen access was provided through the BC Bone and Soft Tissue Tumour Bank (protocol H08-01717). This work was supported by grants from the Canadian Cancer Society Research Institute (Grant #701582), the Japan Society for the Promotion of Science KAKENHI (Grant #25893168, #26713046), a grant from the Fukuoka Foundation for Sound Health and an International Collaborative Grant from the Liddy Shriver Sarcoma Initiative.

Disclosure/Conflict of Interest:

The authors declare no conflict of interest.

References

1. Chen YT, Scanlan MJ, Sahin U, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc Natl Acad Sci U S A. 1997;94(5):1914-8.
2. Jungbluth AA, Chen YT, Stockert E, et al. Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. Int J Cancer. 2001;92(6):856-60.
3. Scanlan MJ, Gure AO, Jungbluth AA, et al. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. Immunol Rev. 2002;188:22-32.
4. Nicholaou T, Ebert L, Davis ID, et al. Directions in the immune targeting of cancer: lessons learned from the cancer-testis Ag NY-ESO-1. Immunol Cell Biol. 2006;84(3):303-17.
5. Robbins PF, Morgan RA, Feldman SA, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol. 2011;29(7):917-24.
6. Pollack SM, Jungbluth AA, Hoch BL, et al. NY-ESO-1 is a ubiquitous immunotherapeutic target antigen for patients with myxoid/round cell liposarcoma. Cancer. 2012;118(18):4564-70.
7. Lai JP, Robbins PF, Raffeld M, et al. NY-ESO-1 expression in synovial sarcoma and other mesenchymal tumors: significance for NY-ESO-1-based targeted therapy and differential diagnosis. Mod Pathol. 2012;25(6):854-8.
8. Hemminger JA, Ewart TA, Scharschmidt TJ, et al. The cancer-testis antigen NY-ESO-1 is highly expressed in myxoid and round cell subset of liposarcomas. Mod Pathol. 2013;26(2):282-8.
9. Hemminger JA, Iwenofu OH. NY-ESO-1 is a sensitive and specific immunohistochemical marker for myxoid and round cell liposarcomas among related mesenchymal myxoid neoplasms. Mod Pathol. 2013;26(9):1204-10.
10. Nielsen TO, Hsu FD, O'Connell JX, et al. Tissue microarray validation of epidermal growth factor receptor and SALL2 in synovial sarcoma with comparison to tumors of similar histology. Am J Pathol. 2003;163(4):1449-56.
11. Ng TL, Gown AM, Barry TS, et al. Nuclear beta-catenin in mesenchymal tumors. Mod Pathol. 2005;18(1):68-74.
12. Cheng H, Dodge J, Mehl E, et al. Validation of immature adipogenic status and identification of prognostic biomarkers in myxoid liposarcoma using tissue microarrays. Hum Pathol. 2009;40(9):1244-51.
13. Steigen SE, Straume B, Turbin D, et al. Clinicopathologic factors and nuclear morphometry as independent prognosticators in KIT-positive gastrointestinal stromal tumors. J Histochem Cytochem. 2008;56(2):139-45.
14. Pacheco M, Nielsen TO. Histone deacetylase 1 and 2 in mesenchymal tumors. Mod Pathol. 2012;25(2):222-30.
15. Endo M, Su L, Nielsen TO. Activating transcription factor 2 in mesenchymal tumors. Hum Pathol. 2014;45(2):276-84.
16. Terry J, Saito T, Subramanian S, et al. TLE1 as a diagnostic immunohistochemical marker for synovial sarcoma emerging from gene expression profiling studies. Am J Surg Pathol. 2007;31(2):240-6.
17. Demicco EG, Torres KE, Ghadimi MP, et al. Involvement of the PI3K/Akt pathway in myxoid/round cell liposarcoma. Mod Pathol. 2012;25(2):212-21.
18. Hoffman A, Ghadimi MP, Demicco EG, et al. Localized and metastatic myxoid/round cell liposarcoma: clinical and molecular observations. Cancer. 2013;119(10):1868-77.
19. Waaijer CJ, de Andrea CE, Hamilton A, et al. Cartilage tumour progression is characterized by an increased expression of heparan sulphate 6O-sulphation-modifying enzymes. Virchows Arch. 2012;461(4):475-81.
20. Meijer D, Gelderblom H, Karperien M, et al. Expression of aromatase and estrogen receptor alpha in chondrosarcoma, but no beneficial effect of inhibiting estrogen signaling both in vitro and in vivo. Clin Sarcoma Res. 2011;1(1):5.
21. Mohseny AB, Szuhai K, Romeo S, et al. Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. J Pathol. 2009;219(3):294-305.

22. de Graaff MA, Cleton-Jansen AM, Szuhai K, et al. Mediator complex subunit 12 exon 2 mutation analysis in different subtypes of smooth muscle tumors confirms genetic heterogeneity. Hum Pathol. 2013;44(8):1597-604.
23. Willems SM, Schrage YM, Baelde JJ, et al. Myxoid tumours of soft tissue: the so-called myxoid extracellular matrix is heterogeneous in composition. Histopathology. 2008;52(4):465-74.
24. Sun Y, Turbin DA, Ling K, et al. Type I gamma phosphatidylinositol phosphate kinase modulates invasion and proliferation and its expression correlates with poor prognosis in breast cancer. Breast Cancer Res. 2010;12(1):R6.
25. Crozat A, Aman P, Mandahl N, et al. Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. Nature. 1993;363(6430):640-4.
26. Powers MP, Wang WL, Hernandez VS, et al. Detection of myxoid liposarcoma-associated FUS-DDIT3 rearrangement variants including a newly identified breakpoint using an optimized RT-PCR assay. Mod Pathol. 2010;23(10):1307-15.
27. Kilpatrick SE, Doyon J, Choong PF, et al. The clinicopathologic spectrum of myxoid and round cell liposarcoma. A study of 95 cases. Cancer. 1996;77(8):1450-8.
28. Fletcher CDM, Bridge JA, Hogendoorn PCW, et al. World Health Organisation Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. 4th ed. Lyon: IARC Press; 2013.
29. Clark J, Rocques PJ, Crew AJ, et al. Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. Nat Genet. 1994;7(4):502-8.
30. Ferrari A, Gronchi A, Casanova M, et al. Synovial sarcoma: a retrospective analysis of 271 patients of all ages treated at a single institution. Cancer. 2004;101(3):627-34.
31. Jungbluth AA, Antonescu CR, Busam KJ, et al. Monophasic and biphasic synovial sarcomas abundantly express cancer/testis antigen NY-ESO-1 but not MAGE-A1 or CT7. Int J Cancer. 2001;94(2):252-6.
32. Pollack SM, Li Y, Blaisdell MJ, et al. NYESO-1/LAGE-1s and PRAME are targets for antigen specific T cells in chondrosarcoma following treatment with 5-Aza-2-deoxycitabine. PLoS One. 2012;7(2):e32165.

Chapter 6

Establishment and characterization of a new human myxoid liposarcoma cell line (DL-221) with the FUS-DDIT3 translocation

Marieke A. de Graaff, Jamie S.E. Yu, Hannah C. Cheung, Davis R. Ingram, Theresa Nguyen, Jeffrey Juehui Liu, Svetlana Bolshakov, Károly Szuhai, Pierre Åman, Keila E. Torres, Dina Lev, Torsten O. Nielsen, Judith V.M.G. Bovée, Alexander J. Lazar, Neeta Somaiah

Laboratory Investigation. 2016; 96(8): 885-894.

Abstract

Myxoid liposarcoma has the pathognomonic fusion oncogene *FUS-DDIT3* encoding a chimeric transcription factor. Metastatic risk is higher with an increased round cell component and has been linked to aberrations involving the IGFR/PI3K/AKT pathway. These molecular insights have yet to translate to targeted therapies and the lack of experimental models is a major hindrance. We describe the initial in-depth characterization of a new cell line (DL-221) and establishment of a mouse xenograft model.

The cell line DL-221 was derived from a metastatic pleural lesion showing myxoid and round cell histology. This newly established cell line was characterized for phenotypic properties and molecular cytogenetic profile, using PCR, COBRA-FISH and western blot. Next-generation whole exome sequencing was performed to further characterize the cell line and the parent tumor. NOD-SCID-IL2R gamma knockout mice were xenograft hosts.

DL-221 cells grew an adhering monolayer and COBRA-FISH showed an aneuploid karyotype with t(12;16)(q13;p11) and several other rearrangements; RT-PCR demonstrated a *FUS-DDIT3* fusion transcript type 1. Both the cell line and the original tumor harbored a *TP53* compound heterozygous mutation in exon 4 and 7 and were wild type for *PIK3CA*. Moreover, among the 1254 variants called by whole exome sequencing, there was 77% concordance between the cell line and parent tumor. The recently described hotspot mutation in the *TERT* promoter region in myxoid liposarcomas was also found at C228T in DL-221. Xenografts suitable for additional pre-clinical studies were successfully established in mice after subcutaneous injection.

The established DL-221 cell line is the only published available myxoid liposarcoma cell line that underwent spontaneous immortalization, without requiring SV40 transformation. The cell line and its xenograft model are unique and helpful tools to study the biology and novel potential targeted treatment approaches for myxoid liposarcoma.

Keywords:
Myxoid liposarcoma, round cell liposarcoma, translocation, FUS-DDIT3, TP53, PIK3CA, HDAC, TERT, CTAG1B, exome sequencing, targeted therapy.

Introduction

Myxoid liposarcoma (MLS) is a malignant mesenchymal neoplasm, representing one third of the liposarcomas and accounting for 5% of all adult soft tissue sarcomas (1). Histologically MLS is composed of a variably cellular ovoid cell proliferation with signet-ring lipoblasts set in a myxoid matrix with characteristic plexiform vasculature. Areas of undifferentiated small round cells with scarce cytoplasm and no matrix (round cell variant) are associated with a higher metastatic potential and a worse prognosis (2-8). Other poor prognostic factors are the presence of necrosis, male gender, increasing age and multifocal disease (2, 8-10).

Localized MLS can be effectively treated with surgery or the combination of surgery and radiation, but 35-50% of patients develop recurrence or metastases. The metastatic pattern is unconventional with spread to sites in soft tissue or bone sometimes occurring before the tumor metastasizes to the lungs (1, 6, 11). Although MLS is relatively sensitive to radiotherapy and certain chemotherapeutic agents, treatment of extensive disease is challenging (12).

MLS is characterized by a reciprocal translocation generating a fusion transcription factor oncogene. In >95% a translocation t(12;16)(q13;p11) is present, fusing *FUS* (fused in sarcoma; a.k.a. *TLS*) with *DDIT3* (DNA-damage-inducible transcript 3; a.k.a. *CHOP*) (13, 14). At least eleven different *FUS-DDIT3* fusion types have been described and the fusion type does not impact clinical outcome (2, 13, 15). Less than 5% of the cases harbour a t(12;22)(q13;q12) leading to an *EWSR1-DDIT3* fusion, of which four different transcripts are described (1, 15, 16).

Although the exact mechanism via which the chimeric transcription factor exerts its oncogenic effects remains to be elucidated, it is postulated that it acts as an aberrant transcriptional regulator, stimulating proliferation while inhibiting adipogenic differentiation (17-19). The chimeric product is highly expressed and interferes with heterodimerization of DDIT3 with CCAAT/enhancer-binding protein-β (C/EBPβ). The activity of the transcription factors C/EBPα and PPARγ is inhibited and fat differentiation is blocked (19).

Exome sequencing and biomarker analysis of MLS specimens has identified alterations in the IGF/Akt/mTOR axis, implicated in cellular processes such as cell survival, proliferation and growth. Overexpression of the receptor tyrosine kinases AXL, RET and IGF1R, and the ligand IGF1, are negative

prognostic biomarkers (8, 20, 21). Activating mutations in *PIK3CA* are found in 14-18% of MLS and loss of expression of PTEN is found in 12% of the tumors and is mutually exclusive from *PIK3CA* mutations (22, 23). Increased PI3K/Akt signalling has been demonstrated by high expression of downstream targets like phosphorylated 4EBP1, PRAS40 and S6. The *PIK3CA* mutation rate, IGFR expression and loss of PTEN were higher in tumors with a round cell component suggesting that this pathway might be involved in round cell transformation and tumor progression. *TP53* mutations and reduced protein expression of p16INK4/p14ARF have been identified in a subset of tumors, most frequently in round cell components (24). Hotspot mutations in the *TERT* (telomerase reverse transcriptase) promoter region were recently reported in MLS (23% to 74%) (25, 26). These mutations led to increased protein expression of TERT and have been implicated in telomerase dysregulation and the resultant proliferative capability of tumor cells (27). The cancer-testis antigen NY-ESO-1 (a.k.a. CTAG1B) has recently shown to be almost universally expressed in MLS (89-100%)(28-30). NY-ESO-1 expression is normally limited to germ cells making it a good cancer immunotherapeutic target (31, 32).

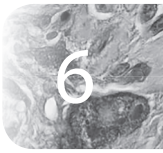
Over the last few years, we have gained increasing insight into the molecular pathogenesis of MLS; however, translating this knowledge into specific therapies has been challenging. Reliable *in vitro* and *in vivo* models are crucial to investigate novel therapies and to study the mechanisms of action. Although MLS is a common sarcoma subtype, so far only two MLS cell lines (402-91 and 1765-92) were established, both immortalized by transfection with the SV40 large T-antigen, and have been made available worldwide (33, 34).

In this study, we present the establishment and characterization of a new MLS cell line (DL-221), which to our knowledge is the first spontaneously immortalized cell line of this tumor type. This was undertaken as part of the International Myxoid Liposarcoma Consortium and our motivation was to facilitate preclinical research by having representative models to test new therapies.

Materials and Methods

Myxoid liposarcoma primary culture and cell lines

With approval of the Institutional Review Board (IRB) of The University of Texas MD Anderson Cancer Center and with patient's written informed consent, isolation of tumor cells was performed as described previously (35). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gemini bio-products, West Sacramento, CA) and 1% penicillin-streptomycin (100U/ml), and placed in a humidified 5% CO_2 incubator at 37°C. The previously established human MLS cell lines 402-91 and 1765-92 were cultured in RPMI1640 (Thermo Fisher Scientific, Waltham, MA) supplemented with FBS and antibiotics. The population doubling time was calculated during the exponential growth phase using the algorithm provided by Doubling Time software (http://www.doubling-time.com). All cell lines were tested on a regular basis for mycoplasma infection. Short Tandem Repeat (STR) DNA fingerprinting was performed on the cell lines, the metastatic tumor tissue from which the DL-221 cell line was derived and from xenografted tissue (AmpFLSTR Identifiler PCR Amplification kit; Applied Biosystems, Foster City, CA). The data was analyzed with GeneMapper (v4.0, Applied Biosystems).



Fluorescence *in situ* hybridization (FISH)

Two color *FUS* split apart FISH was performed on interphase cells using a probe mix containing bacterial artificial chromosome (BAC) clone RP11-196G11 direct labeled with FITC (FITC-dUTP) (Roche diagnostics GmbH, Mannheim, Germany) and clone RP11-120K18 direct labeled with Cy3 (Roche). Both clones flank the *FUS* gene and are separated by 120 kbp. Scoring of the split-apart FISH was done by counting at least 100 nuclei; signals were considered split-apart when their distance was larger than the size of a single hybridization signal. Harvested metaphase cells were used for COBRA-FISH. Pictures were taken with a DMRA microscope (Leica, Rijswijk, The Netherlands) and karyotypes analyzed with Colorproc (v2.0, LUMC, Leiden, The Netherlands). A detailed description of the harvesting, hybridization, probe labeling and COBRA-FISH protocols has been published (36, 37).

FUS-DDIT3 fusion type characterization

Total RNA was extracted from cells using RNeasy Mini Kit (Venio, The Netherlands) as per manufacturer instructions. cDNA was generated using the SuperScript III reverse transcriptase kit (Life Technologies, Carlsbad, CA) and PCR was performed with Taq polymerase (Life Technologies). Sanger sequencing of the amplified product was performed; Chromas (Technelysium, South Brisbane, QLD, Australia) and the BLAST Multiple Alignment tool (NCBI) were used to depict the sequence and analyze the breakpoints, respectively (23). A detailed description of PCR conditions has been published previously (see Table S1 for primers)(13).

Immunoprecipitation, SDS-PAGE and western blot

Immunoprecipitation was performed on protein lysates from near-confluent cells. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested by scraping in ice-cold lysis buffer containing 15 mM Tris (pH 8.0), 100 mM NaCl, 1% Triton X-100, supplemented with protease inhibitor cocktail (P2714, Sigma-Aldrich, St. Louis, MO). Lysates were incubated on ice for 30 min, clarified by centrifugation at 4°C, followed by protein estimation using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Overnight immunoprecipitation with anti-DDIT3 (sc-793, Santa Cruz Biotechnology, Dallas, TX) was performed using Dynabeads® Protein G (Life Technologies, Carlsbad, CA) as per manufacturer instructions. Next, SDS-PAGE separation and western blot were performed using anti-FUS (sc-373698, Santa Cruz) and anti-DDIT3 (ab10444, Abcam, Cambridge, UK) for detection. The following Cell Signaling Technology (Danvers, MA) antibodies were also used for western blot: anti-AKT (#2920), anti-phospho-AKT (Ser473) (#9271), anti-AXL (#4566) and PTEN (#9559). Antibodies of Abcam were used for western blot analysis of HDAC1 (ab19845), HDAC2 (ab32117) and HDAC3 (ab32369)(38).

Molecular characterization: mutation analysis and myxoid liposarcoma associated biomarkers

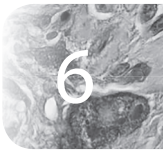
Mutation analysis was performed for exon 4 to 10 of *TP53,* exons 9 and 20 of *PIK3CA* and the *TERT* promoter region by conventional Sanger sequencing as described elsewhere (Table S1)(23). Detailed procedures of NY-ESO-1 immunohistochemistry have been described previously (30).

Cell viability assay

In vitro cell viability assays were performed as described previously (39). Briefly, metabolic activity was measured by a WST-1 colorimetric assay as read-out for the cell viability after doxorubicin treatment. Cells were treated with increasing doxorubicin concentrations for 72hr in quadruplicates and experiments were performed 2 to 4 times. GraphPad Prism (v6.05, La Jolla, CA) was used to calculate the IC_{50}-values.

In vivo animal model

All animal procedures and care were approved by the MD Anderson Cancer Center Institutional Animal Care and Usage Committee. Animals received humane care as per the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals". Mouse studies were performed as described previously (40). Briefly, $3x10^6$ cells were injected subcutaneously in the flank of (n=2/cell line) 6 weeks old female NOD-SCID-ILR2 gamma mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, The Jackson Laboratory, Bar Harbour, ME). Tumor growth was assessed twice weekly with a digital caliper (Mitutuyo, Chicago, IL); tumor volume was calculated by using the formula (tumor volume (mm^3) = length x width x height x 0.5). Mice injected with DL-221 cells were sacrificed at 79 and 92 days post injection due to ulcerated tumor burden. The tumors were resected, weighed (Ohaus Adventurer® Pro Scale), fixed in formalin and embedded in paraffin. Sections were H&E stained to evaluate tumor histology. *FUS* split-apart FISH was performed to confirm the presence of the translocation.



Sequencing

Whole exome sequencing was performed on DNA isolated from the DL-221 metastatic tumor tissue and its derivative cell line at passage 33/34. Coding regions were captured with baits from the Agilent SureSelect Human All Exon V4 (Agilent Technologies, Inc., Santa Clara, CA). After selecting for 200 bp median fragment sizes, the samples were run on an Illumina HiSeq 2000 (Illumina Inc., CA) to generate 76 bp paired-end reads. Duplicate reads were removed using Picard and mapping was done using BWA (41), giving an overall mean coverage of 69X for the metastatic tumor and 62X for the derived cell line. The percentages of reads having at least 50X mean coverage were 75% and 65%, respectively.

Variants were called separately for each sample using VarScan1 (42) against the human reference genome. Further comparisons between samples were done by using MuTect (43) and Pindel (44) with each one of either the tumor or cell line being set as the "reference" with 3% allele frequency cutoff for making calls. To confirm the translocation, Breakdancer (45) and Meerkat (46) were used. Visual comparisons of allele frequency patterns were made using R: Vioplot (47) scatterplot, and 2D kernel density plots. Annotations were obtained using AnnoVar, including protein predictors Mutation Assessor and Polyphen2. Pathway analysis was performed using DAVID (48, 49). The exome sequence data has been deposited in the European Genome-phenome Archive (EGA).

Results

Patient history and establishment of the DL-221 cell line

The cell line was generated from a metastatic tumor deposit from the pleura of a 42-year-old male patient (Figure 1A). The patient was originally diagnosed with myxoid liposarcoma of the right anterior thigh that was treated with surgical resection and adjuvant radiation therapy. One year later he was diagnosed with metastatic disease involving the right periorbital area and chest wall (10^{th} rib) for which he received adriamycin/ifosfamide followed by right lateral orbitotomy and chest wall resection. Subsequently, the patient developed multiple metastatic lesions involving the thoracic, lumbar spine and sacrum for which he received multiple chemotherapy regimens. The pleural lesion that was utilized to establish the cell line had been treated with trabectedin prior to resection. The pathology report indicated that the metastatic lesion was found to have a myxoid and sclerotic component. In addition, round cell change was identified. He developed additional metastatic disease involving the lungs, pleura, posterior diaphragmatic region, pelvic muscles and presacral space for which he was again treated with trabectedin followed by single agent doxorubicin. He ultimately succumbed to the disease.

Isolated tumor cells from the metastatic pleura tumor were originally slow growing and passaged once a month, until after 14 months they underwent spontaneous immortalization and a fast growing clone emerged. This clone was isolated and expanded, and after 24 consecutive months of culturing, the

cell line has reached over 90 passages. The cell line has a population doubling time of 30 hours calculated at passage 62; this was stable at least through passage 90. Tumor cells grow in an adherent monolayer fashion; phase contrast microscopy revealed spindle cell morphology in monolayer culture (Figure 1B). Cells are mononuclear and the enlarged nuclei contain one or more clearly discernible nucleoli. The cells do not exhibit contact inhibition when reaching confluence. A genetic profile of the cell line was generated by STR typing and was consistent with the metastatic lesion (Table S2).

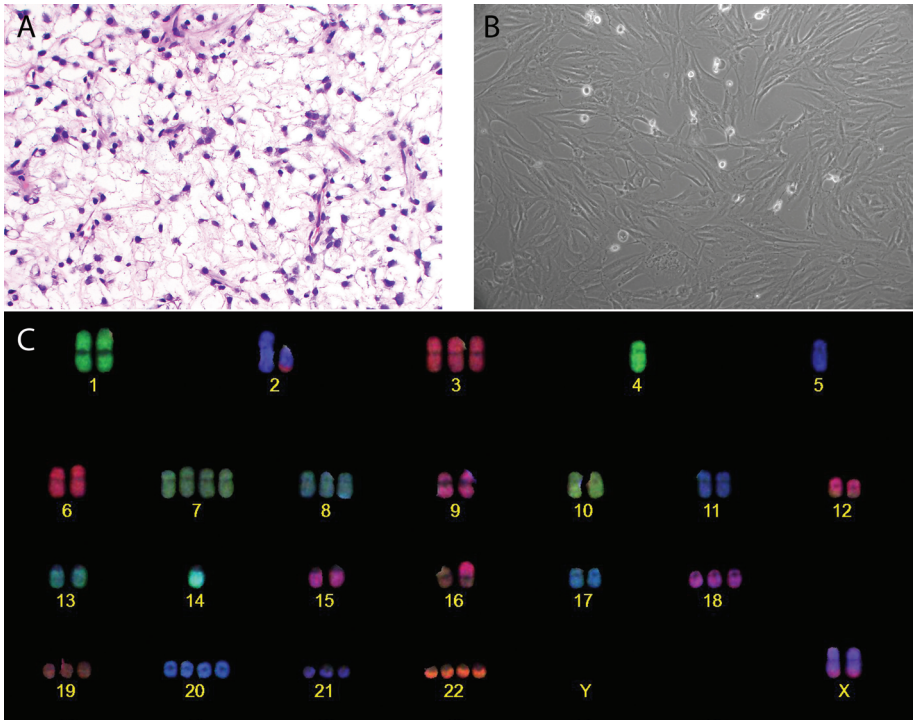


Figure 1. Establishment of the myxoid liposarcoma DL-221 cell line with the characteristic translocation t(12;16)(q13;p11).

(A) Representative H&E-section of the tumor of the pleura of which the tumor cells were isolated for the establishment of the DL-221 cell line (magnification 40x). (B) Tumor cells in culture showing spindle-shaped morphology and nuclei with large nucleoli. (C) COBRA-FISH karyogram of DL-221 cell line showing a near triploid karyotype with several numerical and structural aberrations.

COBRA-fluorescence *in situ* hybridization

The presence of the diagnostic *FUS-DDIT3* translocation was monitored with *FUS* split-apart FISH at several times during the culture process to assure a

pure culture of tumor cells. The composite karyotype of the DL-221 cell line at passage p31 and p68 was 71~75<3n±>,der(X)t(X;15)(q;q)x2,-Y,der(2)t(X;2)(q;p)t(2;3)(q;p)x2,-4,+5,der(7)t(7;X)(q;q),+i(7)(q10),+7,+8,+8,-9,+11,t(12;16)(q13;p11),-13,+14,-15,der(17)t(10;17)(p;p),+18,+19,+20,+21,idic(22)[10]/71~75<3n±>,der(X)t(X;15)(q;q)x2,-Y,der(2)t(X;2)(q;p)t(2;3)(q;p)x2,der(4)t(2;4)(q;p),-4,+5,der(7)t(5;7)(p;q),der(7)t(7;X)(q;q),+8,+8,-9,+10,+11,t(12;16)(q13;p11)x2,-13,+14,-15,+18,+19,+20,+21,+22[6] (Figure 1C). Several additional non-clonal rearrangements were found. The cell line has at least two main subpopulations based on the presence of one or two copies of the translocation t(12;16)(q13;p11) and with two or one copies of chromosome 12 and 16 to complement the triploid genome, respectively. The two different clones are still present in the same proportion, two copies of the translocation versus one copy of the translocation in a ratio of 1.0:1.7, after long term in culture. All chromosomal aberrations that are found at least two times are included in the composite karyotype. The karyotypes are made according to the guidelines of the International System for Human Cytogenetic Nomenclature (50).

Fusion-type characterization

The *FUS-DDIT3* fusion type was determined with reverse transcriptase-PCR and sequencing. A type 1 fusion was found in the DL-221 cell line, fusing the first seven exons of *FUS* to the entire reading frame of the *DDIT3* gene. The 402-91 and 1765-92 cell lines were included as positive controls in Figure 2A and show as expected type 1 and type 8 fusions, respectively. After electrophoresis the chimeric products reveal a band at the position corresponding to the size of the fusion product. A band was observed around 136 bp in 402-91 and DL-221, consistent with a type 1 fusion product, whereas a larger band at 730 bp was found for 1765-92 due to additional *FUS* exons in the type 8 fusion product.

Immunoprecipitation

To prove the presence of the FUS-DDIT3 chimeric fusion oncoprotein in the myxoid liposarcoma cell lines, immunoprecipitation experiments were performed with a DDIT3 antibody on protein lysates from 402-91, 1765-92 and DL-221. Further analysis with SDS-PAGE and immunoblotting with antibodies against DDIT3 and FUS revealed one band for DDIT3 and two bands picked up by the FUS antibody in all samples. The estimated molecular weight of the protein detected with anti-DDIT3 is around 75 kDa in 402-91 and DL-221 and

around 100 kDa in 1765-92, corresponding to the estimated weight of the chimeric fusion proteins of 75 kDa and 100 kDa for type 1 and 8 fusion proteins, respectively. Two bands picked up by the anti-FUS antibody were detected in all three cell lines; one band was present at the same level as the DDIT3 band (i.e. FUS-DDIT3) whereas the other band with a weight of 68 kDa represents native FUS protein. The abundant presence of normal FUS is expected based on the high transcriptional activity of this gene; as expected, no expression of the stress-induced native DDIT3 was detected (Figure S1)(51). Merging of the immunoblots of FUS (green) and DDIT3 (red) revealed a yellow band in each of the three cell lines, confirming that it represents the fusion oncoprotein (Figure 2B).

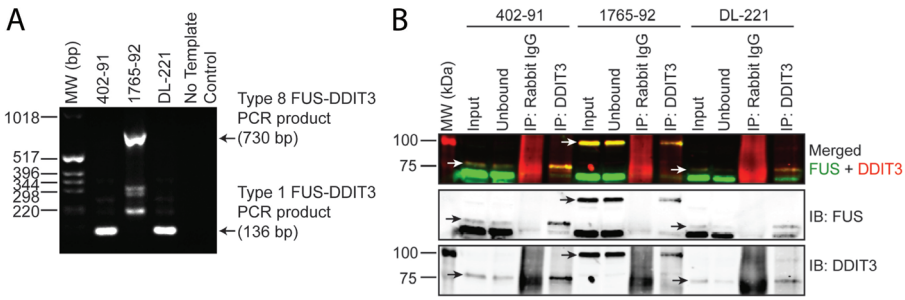


Figure 2. Fusion type characterization of the DL-221 cell line and proving the presence of the FUS-DDIT3 chimeric fusion oncoprotein.

(A) RT-PCR was performed on the three myxoid liposarcoma cell lines. This confirms a type 1 fusion product (136 bp) in both DL-221 and 402-91, while the longer fusion product of 730 bp in 1765-92 was consistent with the type 8 fusion product. (B) Immunoprecipitation of FUS-DDIT3 from the myxoid liposarcoma cell lines 402-91, 1765-92 and DL-221 using an anti-DDIT3 antibody. FUS-DDIT3 (arrow) was detected in all three cell lines at around 75 kDa in 402-91 and DL-221 (expressing type 1 FUS-DDIT3) and around 100 kDa (expressing the larger type 8 FUS-DDIT3).

Molecular characterization: mutation analysis and myxoid liposarcoma associated biomarkers

Target panel sequencing revealed two different *TP53* mutations in the cell line, c.374C>G, p.T125R in exon 4 and c.715A>G, p.N239D in exon 7. The original tumor also harbored these pathogenic variations (52). Mutational analysis of the hotspot regions in exons 9 and 20 of *PIK3CA* did not identify an aberration in the tumor or cell line, nor any other mutations in this gene as confirmed with whole exome sequencing. No *TP53* nor *PIK3CA* aberrations were found in 402-91 or 1765-92. Mutational analysis of the *TERT* promoter region revealed a C228T mutation in DL-221 (Figure S2). Next, we confirmed the presence of the

previously reported C228T hotspot mutation in 402-91 and 1765-92. None of the cell lines harbored the second reported hotspot mutation in MLS, C250T (26).

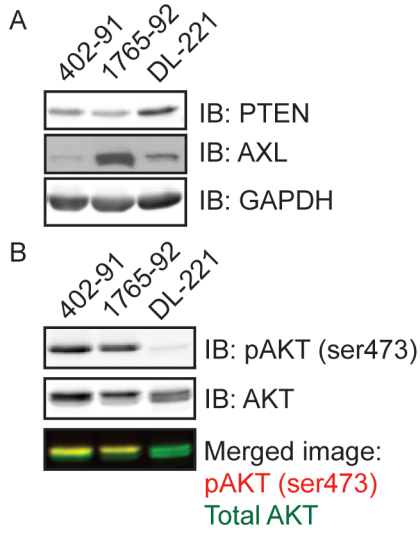


Figure 3. PTEN and pAKT protein expression.

Western blot analysis of (A) PTEN and AXL expression, with GAPDH expression as a loading control, and (B) pAKT (ser473) expression relative to total AKT expression in the myxoid liposarcoma cell lines. The bottom panel shows the merged image of the pAKT (ser473) signal in red and total AKT signal in green. An overlap of both signals produces a yellow band

PTEN expression was retained in all three cell lines (Figure 3A). Analysis of the expression of MLS associated biomarkers with western blots showed a strong expression of total Akt in all three cell lines, while phospho-Akt (pAkt-473) was only found in 402-91 and 1765-92 (Figure 3B). The presence of p53 protein in DL-221 was confirmed with western blot analysis, which must be a mutated protein. The p53 protein was abundantly present in 402-91 and 1765-92, but the expression levels are not necessarily relevant in these two cell lines due to the SV40-immortalisation, as reported previously (53). AXL was highly expressed in 1765-92, moderately expressed in DL-221 and minimally expressed in 402-91 (Figure 3A). The protein expression of HDAC-1, -2 and -3 was evaluated. All three histone deacetylase family members were expressed in 402-91, all except HDAC-3 in 1765-92 and, HDAC-2 and -3 in DL-221 (Figure S3). The 402-91 had a moderate immunohistochemical expression of NY-ESO-1; both DL-221 and 1765-92 showed strong expression. The biomarker status of the three MLS cell lines is shown in Table 1.

Table 1. Molecular biomarkers in myxoid liposarcoma cell lines (402-91, 1765-92, DL-221).

Cell line	FUS-DDIT3 fusion	TP53	P53 (WB)	PIK3CA	TERT	p-AKT473/ Total AKT (WB)	AXL (WB)	HDAC 1/2/3 (WB)	NY-ESO-1 (IHC)
402-91	Type 1	WT	Positive	WT	C228T	+/++	+	+/++/+	+
1765-92	Type 8	WT	Positive	WT	C228T	+/++	+++	+/+/-	++
DL-221	Type 1	T125R, N239D	Positive	WT	C228T	-/++	++	-/+/+	++

WB = western blot; WT = wild type; IHC = immunohistochemistry

Cytotoxicity experiments with colorimetric assays were performed to determine the effect of treatment on cell viability. The conventional chemotherapeutic doxorubicin was highly toxic for all three MLS cell lines. For DL-221 the IC_{50} values were in a range of 35 to 89 nM (average 62 nM) and the IC_{50} values for 402-91 and 1765-92 were 24 nM (range 21-29 nM) and 47 nM (range 31-55 nM) respectively after 72 hour treatment (Figure 4).

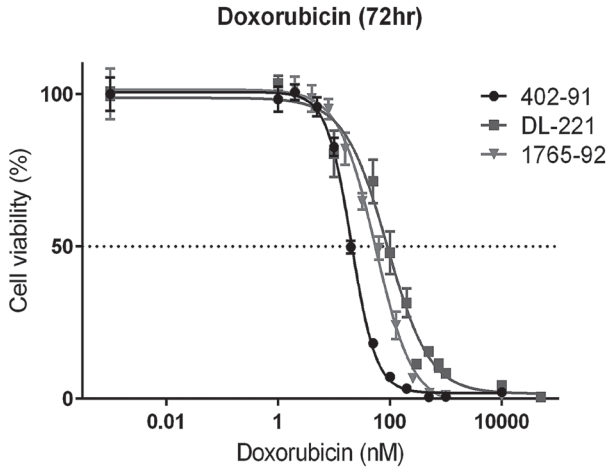


Figure 4. Dose-response curves of MLS cell lines to doxorubicin treatment *in vitro.*

All three cell lines were treated with doxorubicin for 72 hour. For each cell line, one representative experiment of consecutive experiments is depicted.

Mouse studies

DL-221 cells from passage 15 were injected subcutaneously in the flank of two NOD-SCID-ILR2 gamma mice. Tumor development was observed in both mice

and they were sacrificed at day 79 and 92 post-injection, due to ulcerated tumor burden (Figure 5A). No metastatic lesions were found. Histological evaluation of the xenografts revealed a similar morphology, consisting of a moderately cell-rich, myxoid lesion with short spindle-shaped, moderately pleomorphic tumor cells. Marked nuclear atypia with hyperchromatic and heterogeneous nuclei was present and large nucleoli were visible in the majority of the cells (Figure 5B). A *FUS*-split apart FISH assay was performed on FFPE sections from both xenografts and revealed a segregation of the signals, consistent with the expected rearrangement at the *FUS* locus (Figure 5C). Cells obtained from both first generation resections were successfully passaged. The STR profile of the xenografts revealed the same profile as the original tumor and the cell line (Table S2). Injection of the two other available MLS cell lines, 402-91 and 1765-92, did not lead to tumor formation *in vivo* in 16 months.

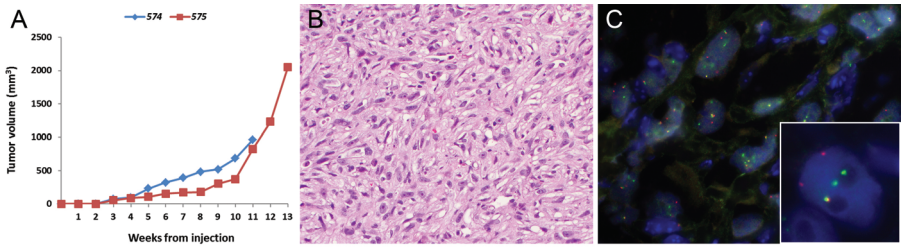


Figure 5. Xenograft model of DL-221 cell line.

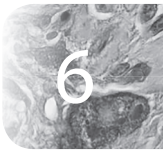
(A) tumor growth in two mice, (B) H&E picture of xenograft tumor (magnification 40x) and (C) *FUS* split-apart FISH reveals a break in the *FUS* gene in the tumor cells in mice, the gain of the green signal was consistent with the karyotype image shown in figure 1.

Next generation sequencing

The metastatic tumor and its derivative cell line both underwent whole exome sequencing to an average mean coverage of 60X. Using Varscan1 to compare each sample to the hg19 reference genome, a total of 1254 exonic variants were called (1230 nonsynonymous, 23 stopgains, 1 stoploss), 960 of which were shared between the two samples. Among those shared, 38 genes overlap with the Sanger Cancer Gene Census, 10 of which were "Probably damaging" by Polyphen2 and 7 with "Medium Impact" by Mutation Assessor. Analysis of these alleles using DAVID showed significant *p*-values from both Panther ($p = 5.2 \text{ X } 10^{-5)}$ and KEGG ($p = 4.8 \text{ X } 10^{-4}$) for the Wnt signaling pathway, which includes Polyphen2-predicted "probably damaging" variants in *APC* (p.R99W), *APC2*

(p.G323R), and *TP53* (p.N239D, p.T125R) (Table S3). Ninety percent (863/960) of the alleles retained insignificant allele frequency changes of <0.2 between the metastatic tumor and cell line (Figure S4). Driver mutations such as the two *TP53* missense mutations p.T125R and p.N239D that were characterized in the primary tumor were confirmed in both samples and retained consistent allele frequencies (metastatic p.T125R 0.43 allele freq, depth = 54, cell line 0.46 allele freq, depth = 54; p.N239D metastatic 0.4 allele freq, depth = 37, cell line 0.32 allele freq, depth = 50). These two variants are not found in any of the population databases ESP6500 and 1000G. As well, the *FUS-DDIT3* translocation was confirmed from split reads found using Meerkat and a call found with Breakdancer that was ~1 kb away from the actual breakpoint (Table S4 and S5)(46).

By MuTect, 63 exonic variants were found in the metastatic tumor that were absent from the cell line, while 32 exonic variants were present in the cell line but not in the tumor. A small group of 28 nonsynonymous alleles were selected for during cell line establishment, as evidenced by increases in allele frequencies of >0.5 (Table S6). There are several possible explanations for these frequency changes. First, all of these alleles come from just three chromosomes: chr2 (9/28), chr4 (6/28), and chr9 (13/28), which could be due to copy number events. The losses of both chromosomes 4 and 9 seen by FISH karyotyping of the cell line would explain the loss of heterozygosity effects that resulted in allele frequencies of 1.00 in the cell line for these two chromosomes. Other possibilities are that clonal selection or purification of the tumor from normal cell contamination resulted in higher allele frequencies, which may explain those alleles from chromosome 2. Of these 28 alleles, five are predicted "Probably damaging" by Polyphen2 (*FAT4* p.P1313S; *DSPP* p.D1152N; *SP140L* p.C181R; *IFIH1* p.L125M; and *IPPK* p.R130W).



Discussion

Myxoid liposarcoma is a relatively common sarcoma subtype, yet there are only two previously established cell lines that are widely available (33). Representative models for preclinical testing, including cell lines and functioning xenografts, are essential to understanding the underlying molecular determinants of round cell and metastatic progression in this disease and to identify and develop targeted therapeutic strategies.

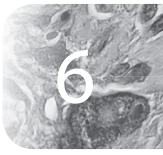
Here we report the establishment of a new MLS cell line (DL-221) and demonstrate that this cell line can be successfully used in a xenograft model. The cell line was grown from isolated myxoid liposarcoma cells from a metastatic pleura lesion. After 14 months of culturing, a fast growing clone emerged and was expanded. DL-221 has been cultured for more than 24 months and shows a stable doubling time of around 30 hours. In contrast to the two other established MLS cell lines, 402-91 and 1765-92, which were both immortalized by induction of the large T-cell SV40 antigen (33), DL-221 is the first published MLS cell line that underwent spontaneous immortalization. SV40 suppresses the transcriptional activity of the tumor suppressor p53 and hence affects the p53 pathway in the cells. SV40 immortalization also involves the inactivation of other growth suppressors as pRB and SEN6 (54). This has to be kept in mind when studying the *in vitro* effect of new treatment options in these cell lines, as it is not fully representative of the oncogenic process that occurs in human patient tumors. One of the reasons for the limited MLS models could be the difficulty of culturing them *in vitro*, which has been our experience as well. This might be related to a lack of necessary cytokines, growth factors, adhesion molecules or endothelial cells in the extracellular environment, difficulty in adjusting to a monolayer culture or a combination of these factors. Most primary cultures from MLS tumor tissues slow down after several weeks or months in culture, and finally result in terminal fat differentiation or senescence.

Exome sequencing of the original tumor and the DL-221 cell line was performed to search for new genetic aberrations that are common between the cell line and original tumor and those that arise in the cell line specific to the stress of growing in culture. The *FUS-DDIT3* translocation and the *TP53* mutations were present in both samples.

The DL-221 cell line was regularly tested for the presence of the chimeric fusion oncogene, which was confirmed by *FUS*-split apart FISH on interphase cells. COBRA-FISH karyotyping of metaphase cells revealed a near triploid karyotype with several numerical and structural aberrations including the characteristic balanced t(12;16)(q13;p11). The tumor cell population consists of two clones, which differ in the number of copies per cell of this translocation. The most common clonal cell population contains one copy of the translocation and the other clone two (with the ratio of the clones being 1.7:1.0), and this was stable across multiple passages. It is as yet unknown if this has any consequences on the level of FUS-DDIT3 fusion product in the different

clones. Fusion type characterization revealed a type 1 product, fusing exon 7 of *FUS* to the complete coding sequence of *DDIT3*. This is the same fusion as in the cell line 402-91; while the 1765-92 cell line carries the longer type 8 fusion. No cell line is available with the most common (65%) type 2 fusion; by comparison type 1 fusions are encountered in 22% of patients, whereas type 8 fusions are rare (2, 13).

MLS can contain several additional molecular genetic alterations, like *TP53*, *PIK3CA* and *TERT* mutations, which directly influence tumor cell biology and might be involved in round cell transformation, migration capacity and differential response to drugs. Alterations of the p53 pathway have been described in several studies on MLS and the percentage of cases with an alteration varies from <5% to 83% (2, 8, 24, 55, 56). Whole exome sequencing of the DL-221 cell line revealed two *TP53* missense mutations, T125R in exon 4 and N239D in exon 7; both mutations lead to the substitution of an amino acid of a different class and are expected to be deleterious. Sanger sequencing of exon 4 to 10 did confirm the mutations in exons 4 and 7. *TP53* alterations are most frequently encountered in the hotspot exons 5 to 8; these two mutations have both been reported before in carcinomas (cosmic database). *PIK3CA* mutations have been identified in 14-18% of the MLS cases and are associated with round cell progression and shorter disease-specific survival (22, 23). Loss of PTEN expression, which is mutually exclusive with *PIK3CA* mutations, and IGF1R overexpression are other mechanisms to activate the PI3K/Akt pathway. Immunohistochemical expression of the downstream pathway member, phosphorylated 4EBP1, is significantly correlated to pathway activating events, and p-4EBP1 is highly expressed in 60% of MLS. All three cell lines are wildtype for *PIK3CA* and retain PTEN expression. Total-Akt was expressed in all three cell lines, whereas p-Akt473 was only present in 402-91 and 1765-92, possibly implying that the PI3K pathway is activated in the 402-91 and 1765-92 cell lines but not in the DL-221 cell line. A minor response has been reported in two MLS patients treated with mTOR inhibitors (everolimus and temsirolimus), a downstream target of the PI3K pathway (57). These findings warrant testing of the several new inhibitors of PI3K, Akt and mTOR in the preclinical and clinical setting for MLS. Expression of the receptor tyrosine kinase AXL is associated with metastasis and worse outcome in several malignancies, and has been identified as a prognosticator of disease-specific survival in MLS patients (8). AXL was present in DL-221 and 1765-92 but is only weakly expressed in 402-91.



This might indicate that AXL inhibition is a candidate treatment in a subset of MLS. Point mutations in the telomerase reverse transcriptase (*TERT*) promotor region have been identified in 23% (3/13) to 74% (29/39) of MLS (25, 26). This broad range in the mutation rate warrants investigation of more patients to elucidate the role of *TERT* mutations in MLS. The mutation creates a new transcription factor-binding site leading to an upregulation of TERT and finally lengthening of the telomeres. DL-221 carries the C228T mutation, as do both the other cell lines we tested. NY-ESO-1, a cancer testis antigen expressed in the majority of the myxoid liposarcomas (30), is strongly expressed in 1765-92 and DL-221 cells. The patients from whom these cell lines originated could have been candidates for NY-ESO-1 directed immunotherapeutic approaches and the cell lines might serve as good models to test its efficacy (58).

HDAC inhibitors have shown inhibitory and cytotoxic effects on several soft tissue sarcoma tumor cell models grown *in vitro* and *in vivo*, including the 402-91 cell line (59). The MLS fusion gene can affect gene transcription processes by interacting with C/EBP family members; in addition, the *EWSR1-DDIT3* fusion gene has shown to act on at an epigenetic level and enhance histone deacetylation and DNA methylation (60). Epigenetic alterations might be involved in the transcriptional regulation of MLS. Expression of HDAC members -1, -2 and -3 was investigated. HDAC-2 was the only one showing moderate to strong expression in all three cell lines, thereby making this a potential target for treatment (61). HDAC-1 and -3 were moderately expressed in 2 out of 3 cell lines. A recent phase II trial with a pan-HDAC inhibitor SB939 as a single agent tested in 22 translocation associated sarcoma patients (5 of whom had MLS) revealed no objective responses but 2 out of 4 evaluable MLS patients achieved stable disease (62). Those patients with high HDAC-2 expression appeared to benefit the most (61-63); however extensive pre-clinical selection of the most promising HDAC-inhibitors might be worthwhile in specific soft tissue sarcoma subtypes. DL-221, similar to the other two cell lines displays sensitivity to the commonly used chemotherapeutic doxorubicin; we are in the process of performing a high throughput drug screen to look for activity with novel targeted agents.

In summary, DL-221, a new spontaneously immortalized myxoid liposarcoma cell line and generated xenograft, represents a much-needed addition to the *in vitro* and *in vivo* models available to study this disease. We also report whole exome sequencing to reflect the concordant abnormalities

between the cell line and original tumor, data informative for preclinical testing and selecting novel targeted therapeutics that might be translated to the clinic. We hope this cell line and xenograft model will serve to accelerate the pace of preclinical and translational research in MLS, leading to new treatment options to prevent or treat metastatic disease.

Acknowledgements
This work is supported by the Liddy Shriver Sarcoma Initiative.

Disclosures
The authors have no conflicts of interest to declare.

Supplementary tables 3, 4, 5 and 6 are available at *Laboratory Investigation*'s website.

References

1. Fletcher CDM, Bridge JA, Hogendoorn PCW, et al. World Health Organisation Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. 4th ed. Lyon: IARC Press; 2013.
2. Antonescu CR, Tschernyavsky SJ, Decuseara R, et al. Prognostic impact of P53 status, TLS-CHOP fusion transcript structure, and histological grade in myxoid liposarcoma: a molecular and clinicopathologic study of 82 cases. Clin Cancer Res. 2001;7(12):3977-87.
3. Fiore M, Grosso F, Lo VS, et al. Myxoid/round cell and pleomorphic liposarcomas: prognostic factors and survival in a series of patients treated at a single institution. Cancer. 2007;109(12):2522-31.
4. Haniball J, Sumathi VP, Kindblom LG, et al. Prognostic factors and metastatic patterns in primary myxoid/round-cell liposarcoma. Sarcoma. 2011;2011:538085.
5. Kilpatrick SE, Doyon J, Choong PF, et al. The clinicopathologic spectrum of myxoid and round cell liposarcoma. A study of 95 cases. Cancer. 1996;77(8):1450-8.
6. Moreau LC, Turcotte R, Ferguson P, et al. Myxoid\round cell liposarcoma (MRCLS) revisited: an analysis of 418 primarily managed cases. Ann Surg Oncol. 2012;19(4):1081-8.
7. ten Heuvel SE, Hoekstra HJ, van Ginkel RJ, et al. Clinicopathologic prognostic factors in myxoid liposarcoma: a retrospective study of 49 patients with long-term follow-up. Ann Surg Oncol. 2007;14(1):222-9.
8. Hoffman A, Ghadimi MP, Demicco EG, et al. Localized and metastatic myxoid/round cell liposarcoma: clinical and molecular observations. Cancer. 2013;119(10):1868-77.
9. Nishida Y, Tsukushi S, Nakashima H, et al. Clinicopathologic prognostic factors of pure myxoid liposarcoma of the extremities and trunk wall. Clin Orthop Relat Res. 2010;468(11):3041-6.
10. de Vreeze R, de Jong D, Nederlof P, et al. Multifocal myxoid liposarcoma-metastasis or second primary tumor? A molecular biological analysis. J Mol Diagn. 2010;12(2):238-43.
11. Fuglo HM, Maretty-Nielsen K, Hovgaard D, et al. Metastatic pattern, local relapse, and survival of patients with myxoid liposarcoma: a retrospective study of 45 patients. Sarcoma. 2013;2013:548628.
12. Katz D, Boonsirikamchai P, Choi H, et al. Efficacy of first-line doxorubicin and ifosfamide in myxoid liposarcoma. Clin Sarcoma Res. 2012;2(1):2.
13. Powers MP, Wang WL, Hernandez VS, et al. Detection of myxoid liposarcoma-associated FUS-DDIT3 rearrangement variants including a newly identified breakpoint using an optimized RT-PCR assay. Mod Pathol. 2010;23(10):1307-15.
14. Crozat A, Aman P, Mandahl N, et al. Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. Nature. 1993;363(6430):640-4.
15. Bode-Lesniewska B, Frigerio S, Exner U, et al. Relevance of translocation type in myxoid liposarcoma and identification of a novel EWSR1-DDIT3 fusion. Genes Chromosomes Cancer. 2007;46(11):961-71.
16. Sandberg AA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: liposarcoma. Cancer Genet Cytogenet. 2004;155(1):1-24.
17. Tan AY, Manley JL. The TET family of proteins: functions and roles in disease. J Mol Cell Biol. 2009;1(2):82-92.
18. Goransson M, Andersson MK, Forni C, et al. The myxoid liposarcoma FUS-DDIT3 fusion oncoprotein deregulates NF-kappaB target genes by interaction with NFKBIZ. Oncogene. 2009;28(2):270-8.
19. Perez-Mancera PA, Bermejo-Rodriguez C, Sanchez-Martin M, et al. FUS-DDIT3 prevents the development of adipocytic precursors in liposarcoma by repressing PPARgamma and C/EBPalpha and activating eIF4E. PLoS One. 2008;3(7):e2569.
20. Cheng H, Dodge J, Mehl E, et al. Validation of immature adipogenic status and identification of prognostic biomarkers in myxoid liposarcoma using tissue microarrays. Hum Pathol. 2009;40(9):1244-51.
21. Negri T, Virdis E, Brich S, et al. Functional mapping of receptor tyrosine kinases in myxoid liposarcoma. Clin Cancer Res. 2010;16(14):3581-93.
22. Barretina J, Taylor BS, Banerji S, et al. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. Nat Genet. 2010;42(8):715-21.

23. Demicco EG, Torres KE, Ghadimi MP, et al. Involvement of the PI3K/Akt pathway in myxoid/round cell liposarcoma. Mod Pathol. 2012;25(2):212-21.
24. Oda Y, Yamamoto H, Takahira T, et al. Frequent alteration of p16(INK4a)/p14(ARF) and p53 pathways in the round cell component of myxoid/round cell liposarcoma: p53 gene alterations and reduced p14(ARF) expression both correlate with poor prognosis. J Pathol. 2005;207(4):410-21.
25. Saito T, Akaike K, Kurisaki-Arakawa A, et al. TERT promoter mutations are rare in bone and soft tissue sarcomas of Japanese patients. Molecular and clinical oncology. 2016;4(1):61-4.
26. Koelsche C, Renner M, Hartmann W, et al. TERT promoter hotspot mutations are recurrent in myxoid liposarcomas but rare in other soft tissue sarcoma entities. J Exp Clin Cancer Res. 2014;33(1):33.
27. Vinagre J, Almeida A, Populo H, et al. Frequency of TERT promoter mutations in human cancers. Nat Commun. 2013;4:2185.
28. Hemminger JA, Ewart TA, Scharschmidt TJ, et al. The cancer-testis antigen NY-ESO-1 is highly expressed in myxoid and round cell subset of liposarcomas. Mod Pathol. 2013;26(2):282-8.
29. Pollack SM, Jungbluth AA, Hoch BL, et al. NY-ESO-1 is a ubiquitous immunotherapeutic target antigen for patients with myxoid/round cell liposarcoma. Cancer. 2012;118(18):4564-70.
30. Endo M, de Graaff MA, Ingram DR, et al. NY-ESO-1 (CTAG1B) expression in mesenchymal tumors. Mod Pathol. 2015;28(4):587-95.
31. Chen YT, Boyer AD, Viars CS, et al. Genomic cloning and localization of CTAG, a gene encoding an autoimmunogenic cancer-testis antigen NY-ESO-1, to human chromosome Xq28. Cytogenet Cell Genet. 1997;79(3-4):237-40.
32. Jungbluth AA, Antonescu CR, Busam KJ, et al. Monophasic and biphasic synovial sarcomas abundantly express cancer/testis antigen NY-ESO-1 but not MAGE-A1 or CT7. Int J Cancer. 2001;94(2):252-6.
33. Aman P, Ron D, Mandahl N, et al. Rearrangement of the transcription factor gene CHOP in myxoid liposarcomas with t(12;16)(q13;p11). Genes Chromosomes Cancer. 1992;5(4):278-85.
34. Thelin-Jarnum S, Lassen C, Panagopoulos I, et al. Identification of genes differentially expressed in TLS-CHOP carrying myxoid liposarcomas. Int J Cancer. 1999;83(1):30-3.
35. Lahat G, Zhu QS, Huang KL, et al. Vimentin is a novel anti-cancer therapeutic target; insights from in vitro and in vivo mice xenograft studies. PLoS One. 2010;5(4):e10105.
36. Szuhai K, Bezrookove V, Wiegant J, et al. Simultaneous molecular karyotyping and mapping of viral DNA integration sites by 25-color COBRA-FISH. Genes Chromosomes Cancer. 2000;28(1):92-7.
37. Szuhai K, Tanke HJ. COBRA: combined binary ratio labeling of nucleic-acid probes for multi-color fluorescence in situ hybridization karyotyping. Nat Protoc. 2006;1(1):264-75.
38. Lopez G, Song Y, Lam R, et al. HDAC Inhibition for the Treatment of Epithelioid Sarcoma: Novel Cross Talk Between Epigenetic Components. Mol Cancer Res. 2016;14(1):35-43.
39. Willems SM, Schrage YM, Bruijn IH, et al. Kinome profiling of myxoid liposarcoma reveals NF-kappaB-pathway kinase activity and casein kinase II inhibition as a potential treatment option. Mol Cancer. 2010;9:257.
40. Jin Z, Lahat G, Korchin B, et al. Midkine enhances soft-tissue sarcoma growth: a possible novel therapeutic target. Clin Cancer Res. 2008;14(16):5033-42.
41. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754-60.
42. Koboldt DC, Chen K, Wylie T, et al. VarScan: variant detection in massively parallel sequencing of individual and pooled samples. Bioinformatics. 2009;25(17):2283-5.
43. Cibulskis K, Lawrence MS, Carter SL, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat Biotechnol. 2013;31(3):213-9.
44. Ye K, Schulz MH, Long Q, et al. Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. Bioinformatics. 2009;25(21):2865-71.
45. Chen K, Wallis JW, McLellan MD, et al. BreakDancer: an algorithm for high-resolution mapping of genomic structural variation. Nat Methods. 2009;6(9):677-81.
46. Yang L, Luquette LJ, Gehlenborg N, et al. Diverse mechanisms of somatic structural variations in human cancer genomes. Cell. 2013;153(4):919-29.

47. Hintze JL, Nelson RD. Violin Plots: A Box Plot-Density Trace Synergism. The American Statistician. 1998;52(2):181-4.
48. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1):44-57.
49. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37(1):1-13.
50. Shaffer LG, McGowan-Jordan J, Schmid M. ISCN (2013): An International System for Human Cytogenetic Nomenclature. Basel: S. Karger; 2013 2013.
51. Ron D, Habener JF. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes & development. 1992;6(3):439-53.
52. Shihab HA, Gough J, Cooper DN, et al. Predicting the functional consequences of cancer-associated amino acid substitutions. Bioinformatics. 2013;29(12):1504-10.
53. Stahlberg A, Kabjorn GC, Engtrom K, et al. Normal and functional TP53 in genetically stable myxoid/round cell liposarcoma. PLoS One. 2014;9(11):e113110.
54. Jha KK, Banga S, Palejwala V, et al. SV40-Mediated immortalization. Exp Cell Res. 1998;245(1):1-7.
55. Pilotti S, Lavarino C, Mezzelani A, et al. Limited role of TP53 and TP53-related genes in myxoid liposarcoma. Tumori. 1998;84(5):571-7.
56. Dei Tos AP, Piccinin S, Doglioni C, et al. Molecular aberrations of the G1-S checkpoint in myxoid and round cell liposarcoma. Am J Pathol. 1997;151(6):1531-9.
57. Sanfilippo R, Dei Tos AP, Casali PG. Myxoid liposarcoma and the mammalian target of rapamycin pathway. Curr Opin Oncol. 2013;25(4):379-83.
58. Robbins PF, Kassim SH, Tran TL, et al. A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. Clin Cancer Res. 2015;21(5):1019-27.
59. Liu S, Cheng H, Kwan W, et al. Histone deacetylase inhibitors induce growth arrest, apoptosis, and differentiation in clear cell sarcoma models. Mol Cancer Ther. 2008;7(6):1751-61.
60. Suzuki K, Matsui Y, Higashimoto M, et al. Myxoid liposarcoma-associated EWSR1-DDIT3 selectively represses osteoblastic and chondrocytic transcription in multipotent mesenchymal cells. PLoS One. 2012;7(5):e36682.
61. Pacheco M, Nielsen TO. Histone deacetylase 1 and 2 in mesenchymal tumors. Mod Pathol. 2012;25(2):222-30.
62. Chu QS, Nielsen TO, Alcindor T, et al. A phase II study of SB939, a novel pan-histone deacetylase inhibitor, in patients with translocation-associated recurrent/metastatic sarcomas-NCIC-CTG IND 200dagger. Ann Oncol. 2015;26(5):973-81.
63. Cassier PA, Lefranc A, Amela EY, et al. A phase II trial of panobinostat in patients with advanced pretreated soft tissue sarcoma. A study from the French Sarcoma Group. Br J Cancer. 2013;109(4):909-14.

Supplementary Information

Supplementary Table 1: Primers for *PIK3CA*, *TERT* and *TP53* mutational analysis and *FUS-DDIT3* fusion variant determination.

Gene	Exon	Forward sequence	Reverse sequence
PIK3CA	9	TGACAAAGAACAGCTCAAAGC	TTAGCACTTACCTGTGACTCCA
PIK3CA	20	TGATGACATTGCATACATTCG	TGTGTGGAAGATCCAATCCA
TERT	Pro-moter	GTAAAACGACGGCCAGCAGCGCT GCCTGAAACTC	GTCCTGCCCCTTCACCTT
TP53	4a	CCATCTACAGTCCCCCTTG	ATGATGACAGGGGCCAGGAG
TP53	4b	GCACCAGCAGCTCCTACAC	ATGAGCCCCTCAGGGCAACTG
TP53	5	TTTCAACTCTGTCTCCTTCCTCTT	AGCCCTGTCGTCTCTCCAG
TP53	6	AGGCCTCTGATTCCTCACTG	ATGAGAGACCCCAGTTGCAAACC
TP53	7	CTTGGGCCTGTGTTATCTCC	ATGAGTGTGCAGGGTGGCAAG
TP53	8	TGCTTCTCTTTTCCTATCCTGAG	ATGAGCTTCTTGTCCTGCTTGC
TP53	9	ACCTTTCCTTGCCTCTTTCC	ATGAAGAAAACGGCATTTTGAGTG
TP53	10	CTTCTCCCCCTCCTCTGTTG	ATGAGAAGGGGCTGAGGTCACTC
FUS	5	GCAGAACCAGTACAACAGCA	NA
FUS	6	GAACCCAGAGGTCGTGGAG	NA
FUS	7	GGTGGCTTCAATAAATTTGG	NA
FUS	12	ATGAATGCAACCAGTGTAAGGCCC	NA
FUS	13	TAAACCAGATGGCCCAGGAG	NA
DDIT3	2	NA	TGCTTTCAGGTGTGGTGATG
DDIT3	3	NA	GGAGAAAGGCAATGACTCAG

NA = not applicable.

Supplementary Table 2. STR typing results of metastatic tumor, xenograft tumor, DL-221 cell line and 402-91 and 1765-92 cell lines.

Sample	AMEL	CSF1PO	D13S317	D16S539	D5S818	D7S820	TH01	TPOX	vWA
DL-221 Tumor	X,Y	10,13	8,11	12,13	12	10,12	8,9	8	14,17
DL-221 Xenograft	X	10,13	8,11	12,13	12	10,12	8,9	8	14,17
DL-221 Cell line	X	10,13	8,11	12,13	12	10,12	8,9	8	14,17
402-91	X,Y	11,12	11,12	11,12	11,14	10,12	6,9.3	8,11	14,18
1765-92	X	11,12	11,12	12	11,12	9,13	7,9.3	8	15,18

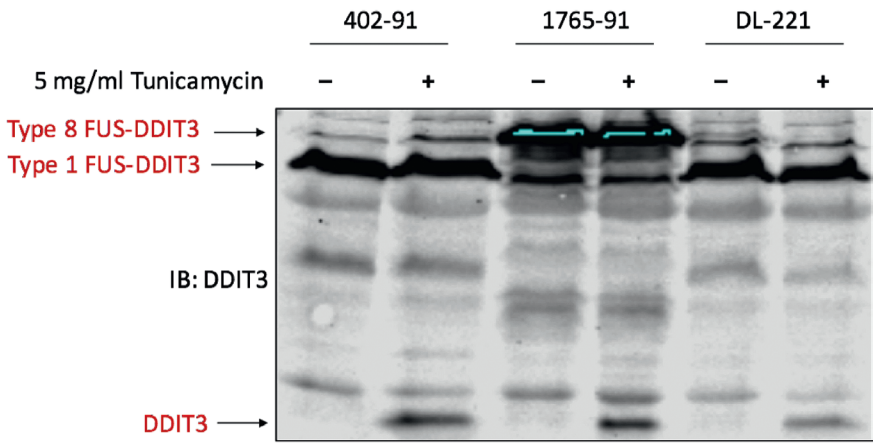


Figure S1. DDIT3 expression.

Myxoid liposarcoma cell lines 402-91, 1765-92 and DL-221 do not express the 29 kDa native DDIT3 under regular culture conditions. Tunicamycin treatment at 5 mg/ml was included as a positive control to cause endoplasmic reticulum stress, and induce DDIT3 protein expression. The higher molecular weight FUS-DDIT3 fusion proteins were also observed in all three cell lines at a higher level of expression compared to native DDIT3.

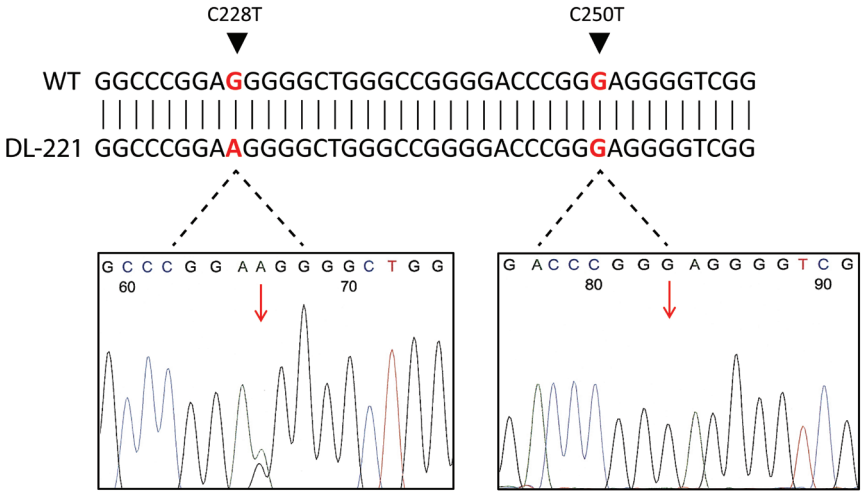


Figure S2. *TERT* hotspot mutation in DL-221.

Mutational analysis for promoter hotspot mutations in *TERT*, showing the C228T mutation in DL-221.

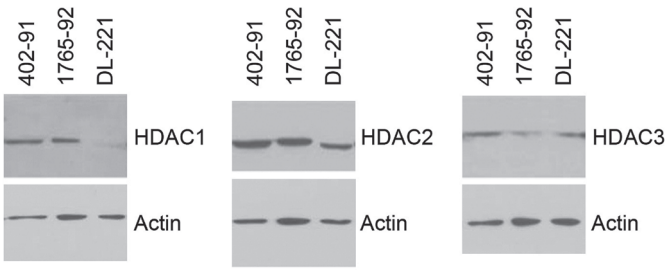


Figure S3. HDAC expression.

Expression levels of the HDAC-1, -2 and -3 proteins in the three myxoid liposarcoma cell lines.

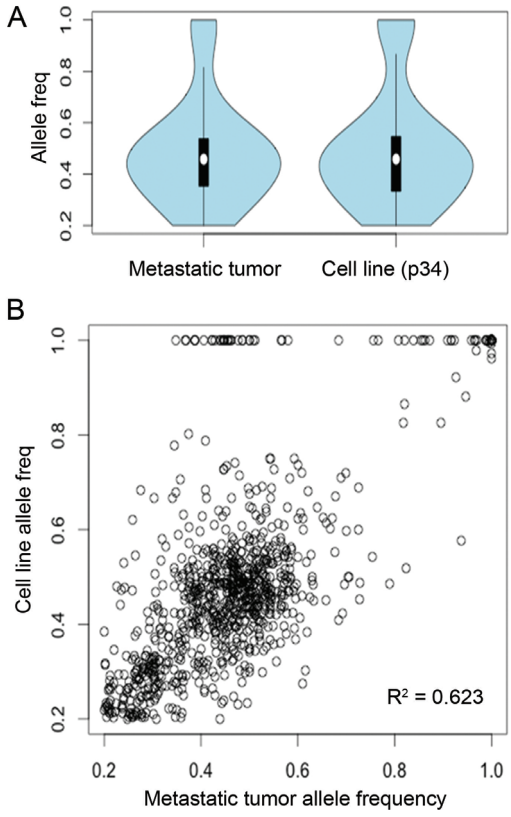


Figure S4. Comparison of the VarScan1 variant calls between DL-221 and the parental metastatic lesion.

(A) The R packag vioplot was used to graph violin density plots of all VarScan1 calls from both the metastatic tumor and the derivative cell line. The similarity of the plots indicates high identity between the two samples. 960/1255 variants are shared between the two, with 63 exonic variants present in the metastatic tumor but not in the cell line and 32 exonic variants present in the cell line but not in the metastatic tumor. (B) 2D allele frequency plot showing that 90% of changes in allele frequency between the metastatic lesion and the cell line are small (<0.2). The two samples have a high correlation in variants ($r^2 = 0.623$).

Chapter 7

High-throughput screening of myxoid liposarcoma cell lines reveals survivin as a potential novel druggable target

Marieke A. de Graaff, Shruti Malu, Irma Guardiola, Alwine B. Kruisselbrink, Yvonne de Jong, Willem E. Corver, H. Gelderblom, Patrick Hwu, Torsten O. Nielsen, Alexander J. Lazar, Neeta Somaiah, Judith V.M.G. Bovée

Submitted

Abstract

Myxoid liposarcoma is a soft tissue sarcoma characterized by a recurrent t(12;16) translocation. Although tumours are initially radio- and chemosensitive, the management of inoperable or metastatic MLS can be challenging. Therefore, our aim was to identify novel targets for systemic therapy. We performed an *in vitro* high-throughput drug screen using three myxoid liposarcoma cell lines (402-91, 1765-92, DL-221), which were treated with 273 different drugs, including various targeted inhibitors, FDA approved drugs, natural products and chemotherapeutic agents, at four different concentrations. Cell lines and tissue microarrays were used for validation. As expected, all cell lines revealed a strong growth inhibition to conventional chemotherapeutic agents, such as anthracyclines and taxanes. A good response was observed to compounds interfering with Src and the mTOR pathway, which are known to be affected in these tumours. Moreover, we identified survivin (BIRC5) as a potential novel target, since a strong inhibitory effect was seen at low concentration using the survivin inhibitor YM155. Sensitivity was confirmed in 402-91 and 1765-92 cells in dose response curves and siRNA for BIRC5 decreased cell viability. Survivin is multifunctional protein involved in the correct segregation of sister chromatids during mitosis. Secondly, it has an anti-apoptotic function. Interestingly, immunohistochemistry revealed abundant expression of survivin restricted to the nucleus in all 32 tested primary tumor specimens. Inhibition of survivin in 402-91 and 1765-92 by YM155 increased the percentage S-phase but did not induce apoptosis. In conclusion, using a 273 compound drug screen we confirmed previously identified targets (mTOR, Src) in myxoid liposarcoma. In addition, we identified survivin as a potential novel target for therapy. However, although a strong decrease in cell viability is observed using the survivin inhibitor YM155, the increased percentage S-phase combined with the lack of apoptosis warrant further investigation before application in the treatment of metastatic myxoid liposarcoma.

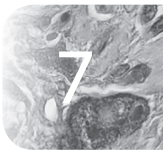
Keywords: myxoid liposarcoma, soft tissue sarcoma, drug screen, survivin, YM155, mTOR, targeted therapy

Introduction

Myxoid liposarcoma (MLS) is a malignant soft tissue tumour accounting for 20-30% of the liposarcomas and roughly 5% of all soft tissue sarcomas (1). These tumours are histopathologically characterized by a proliferation of stellate spindle cells with monomorphic ovoid nuclei, embedded in a myxoid matrix with a plexiform vasculature (1). High grade tumours are defined by having more than 5% of closely packed small blue round cells with high nuclear/cytoplasm ratio and scant stroma. Myxoid liposarcoma is genetically characterized by a reciprocal translocation t(12;16)(q13;p11), generating a fusion product of FUS and DDIT3. The chimeric fusion oncoprotein acts as an aberrant transcription factor, and is known to influence the expression of several genes, including inhibition of adipogenic transcription factors C/EBPα and PPARγ (2, 3).

MLS tumours are initially sensitive to conventional chemo- and radiation therapy, but despite adequate local treatment, up to 40% can progress to local or distant relapse (4-7). Myxoid liposarcoma exhibits a unique metastatic pattern, as tumour cells tend to spread to other soft tissue sites, before metastasizing to the lungs. The disease can become quite extensive and management of metastatic or otherwise inoperable tumours often is challenging. This is reflected by the variable 5-year survival rates reported in several studies, which range from 8% for advanced disease to around 83% to 93% for cases with purely myxoid and localized tumours (5-9).

In addition to doxorubicin and ifosfamide, recently eribulin, a microtubule-dynamics inhibitor, was shown to offer a survival benefit when compared with dacarbazine in the third-line setting in liposarcomas and is now FDA approved(10). Moreover, myxoid liposarcoma was shown to be sensitive to trabectedin (ET-743, Ecteinascidin), a natural alkylating agent derived from a marine tunicate (11). The drug has a complex mechanism of action that is not entirely elucidated, but involves binding to the DNA-minor groove, interaction with DNA repair complexes and additional effects on the tumour microenvironment (12). Unfortunately, similar to other systemic therapies, resistance develops and the antitumour effect of trabectedin has been shown to diminish after some time on treatment (13). Therefore new therapeutic approaches are warranted to improve the outcome of advanced or metastatic myxoid liposarcoma.



Over the past decades, therapeutic progress has been hampered by the sparse availability of representative preclinical models. For many years only two published cell lines (403-91 and 1765-92) were widely available, both of which were SV40 immortalized (14, 15). Recently, we reported on the generation of a novel myxoid liposarcoma cell line (DL-221) and ancillary mouse xenograft model (16). This newly established cell line is so far the only known myxoid liposarcoma cell line that underwent spontaneous immortalization.

Here we used all three available myxoid liposarcoma cell lines in an *in vitro* high-throughput drug screen to search for novel therapeutic agents that have the potential to enter future clinical trials. Drug screens are regularly used and contribute to the discovery of new candidate targets in cancer therapies (17, 18); furthermore, the pathways targeted by effective drugs can yield insights into tumor biology. In addition to the conventional chemotherapeutic agents used in daily practice, such as anthracyclines and taxanes, we found that YM155, a survivin inhibitor, also strongly decreased tumour growth. Strong nuclear accumulation of survivin was observed in 100% of myxoid liposarcomas, and confirmed to be essential for tumour growth. Inhibitors of survivin may therefore represent novel and promising therapeutic options in myxoid liposarcoma.

Materials and Methods

Cell culture

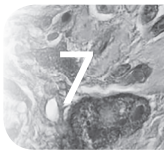
The myxoid liposarcoma cell lines 402-91 and 1765-92 (both kindly provided by Pierre Åman, Sahlgrenska Cancer Centre, Department of Pathology, Institute of Biomedicine, University of Gothenburg, Sweden) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Landsmeer, The Netherlands) and 1% penicillin-streptomycin (100U/ml). DL-221 was cultured with DMEM supplemented with 10% FBS and 1% pen/strep. All included cell lines have been well characterized for possible alterations in myxoid liposarcomas. All three cell lines are *PIK3CA* wild type, 402-91 and 1765-92 are *TP53* wild type and only DL-221 has two *TP53* mutations (T125R and N239D)(16). Cells were maintained in a humidified 5% CO_2 incubator at 37°C. Cell lines were tested on a regular basis for mycoplasma infections. STR (short tandem repeat) typing was performed before and after the experiments

to confirm cell line identity using the Cell ID GenePrint 10 system (Promega, Leiden, The Netherlands).

Drug screen

A high throughput drug screen was performed in which a selection of 273 drugs (Suppl. Table S1) out of 2100 of the Bioactive Compound Library L1700 (Selleckchem, Houston, TX, USA) were tested on the three myxoid liposarcoma cell lines. Selection was based on potential clinical relevance of drugs. Drug stocks were dissolved in DMSO and stored in aliquots at -80°C. Cells were seeded in 96-well plates 24 hour before treatment. Addition of the drugs was performed with the Freedom EVO® 200 liquid handling platform (Tecan, Männedorf, Switzerland) and final treatment concentrations were 1, 10, 100 and 1000 nM, each in triplicate. Cells were treated for 72 hours and thereafter CellTiter-Blue Cell Viability Assay (#G8081, Promega, Madison, WI) was added and incubated for 3 or 4 hours. Plates were read at room temperature using Fluoroskan Ascent™ FL fluorometer (Thermo Fisher Scientific Inc., Pittsburgh, PA), measuring the fluorescence at 530 Ex/604 Em. Data was analyzed by Tableau (Tableau, Seattle, WA, USA). A compound was considered effective if able to reduce the cell viability >50% at a drug concentration of 100 nM in at least two cell lines.

Compounds and cell viability assays



For single agent validation studies, the survivin suppressant YM155, sepantronium bromide (S1130, Selleckchem), everolimus (S1120, Selleckchem), panobinostat (LBH-589, 13280, Cayman Chemical, Ann Arbor, MI, USA) and trabectedin (ET-743, PharmaMar, Madrid, Spain) were dissolved in DMSO according to the manufacturers' instructions. Z-vad-FMK (550377, BD bioscience) was used as a general caspase inhibitor and also dissolved in DMSO. Myxoid liposarcoma cell lines were plated in 96-well plates 24 hr before treatment. Cells were treated with YM155 using concentrations from 0.01 till 5000 nM, in 11 steps, for 72 hours. Cell viability experiments were performed three times, in triplicate. As myxoid liposarcomas are known to respond well to trabectedin, combination treatments with YM155 were performed to investigate possible synergism or antagonism of both drugs. Concentrations used for combination treatment were for both drugs selected around the IC_{25}, IC_{50} and IC_{75} values of single drug treatment for each cell line.

This resulted in nine different dose combinations per cell line, and drugs were added simultaneously. After 72 hours incubation a Presto blue assay (Life-Technologies, Scotland, UK) was performed. Fluorescence was measured at 590nm on a fluorometer (Victor^{3}V 1420 multilabel reader, Perkin Elmer, The Netherlands). Combination experiments were performed twice, in triplicate. To evaluate whether the combination treatments were synergistic, a simplified version of the Bliss independence model was applied (19).

siRNA knockdown

For siRNA knockdown experiments, validated smartpool siRNAs targeting BIRC5 and control siRNAs (siGAPDH and siPLK1) were purchased from Dharmacon (GE Life Sciences, Landsmeer, the Netherlands). Reversed siRNA (50nM) transfection was carried out using DharmaFECT3 transfection reagent (Thermo Fisher Scientific Inc.) in triplo using 5000-10000 cells/well, the experiments were performed twice. After 24 hours evaluation of gene knockdown qRT-PCR were performed and expression levels were normalized towards housekeeping genes CPSF6, GPR108 and CYPA (20). After 72 hours Presto Blue assay and protein analysis by Western blot analysis were performed.

Immunohistochemistry

Survivin expression was investigated in myxoid liposarcoma tissue samples using a previously constructed tissue micro-array with tumour samples from 32 patients diagnosed in the Leiden University Medical Center (21). The tumours of 15 patients consisted of a purely myxoid (M), intermediate cell density (I) or round cell (RC) morphology. The other 17 patients had tumours containing a combination of two histological areas (M+I, M+RC or I+RC); in these cases both areas were included, resulting in a total of 49 tumour samples. All samples were handled in a coded fashion, and all procedures were performed according to the ethical guidelines, "Code for Proper Secondary Use of Human Tissue in the Netherlands" (Dutch Federation of Medical Scientific Societies). Cell pellets of the three untreated myxoid liposarcoma cell lines were fixed in formalin and embedded in paraffin by using Shandon™ Cytoblock™ (Thermo Fisher Scientific Inc.). Sections were incubated with rabbit anti-survivin monoclonal antibody (71G4B7, #2808, Cell Signaling Technology, Leiden, The Netherlands) in a 1:100 dilution. Staining was visualized with DAB+ substrate Chromogen System (DAKO, Heverlee, Belgium). Placenta tissue was included as a control.

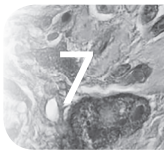
Both nuclear and cytoplasmic expression was scored separately by two independent observers (JVMGB, MAG). A semi-quantitative scoring system was used, combining the staining intensity (0=negative, 1=weak, 2=moderate, 3=strong) and the percentage of positive tumour cells (0=0%, 1=1-25%, 2=25-50%, 3=51-75% and 4=76-100%) as described previously (22).

RNA isolation and quantitative real-time PCR

RNA was isolated from myxoid liposarcoma cell lines using TRIzol (Invitrogen, Carlsbad, CA, USA) followed by a purification procedure using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to manufacturer's instructions. Q-PCR was performed for the isoforms wild-type (WT) survivin, survivin 2b and survivin Δex3 with primers described previously (23). Expression levels were normalized towards housekeeping genes HPRT, GAPDH and TBP. Results are depicted relative to survivin WT expression for each cell line.

Apoptosis

Induction of apoptosis was assessed using the Caspase-Glo® 3/7 assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, MLS cells were plated into white walled 96 well plates (Corning, Fisher Scientific, Landsmeer, The Netherlands) and incubated with YM155 at the IC_{75} concentrations, as determined by cell viability assays. After 24 hr treatment the substrate was added in a 1:1 dilution and incubated for 30 minutes at room temperature. Luminescence was measured with a luminometer (Victor^{3}V 1420 multilabel reader). Experiments were performed twice.



Western blot

After 24 hours YM155 treated and untreated MLS cells or 72 hours after siRNA transfection MLS cells were collected in hot-SDS buffer (1% SDS, 10 mM Tris/EDTA with complete inhibitor and phosSTOP) for protein analysis as described previously (24). Briefly, 5ug or 10 µg protein of each sample was size-fractioned on 12% (TGX Stain-Fast Cast Acrylamide kit, BIO-RAD Laboratories) separating gels. Jurkat cell lysate treated with 25 µM etoposide (Cell Signaling (#2043)) was used as positive control for PARP and cleaved PARP detection. Proteins were transferred to a PVDF membrane and incubated overnight at 4°C with primary antibodies (all from Cell Signalling); PARP antibody (clone 46D11), survivin antibody (clone 71G4B7) or GAPDH antibody (clone D16H11). As a

loading control, α-tubulin staining (clone DM1A, Sigma-Aldrich, Zwijndrecht, The Netherlands) was included. After incubation with the secondary HRP-conjugated antibody for 30 minutes at room temperature, membranes were treated with ECL2 substrate (Pierce™, Thermo Scientific) as per manufacturer's instructions, and chemiluminescence was captured using ECL hyperfilm (Amersham, GE Healthcare Life Sciences, Eindhoven, The Netherlands).

Cell cycle analysis

Cell lines were cultured in T25 flasks in amounts ensuring 70%-90% confluency when harvested and treated with the YM155 IC_{50} concentrations. After 48hr treatment cells were prepared for flow cytometric analysis. In short, cells were harvested, counted and fixed in ice-cold methanol. Next, cells were washed, treated with RNase and stained for DNA using propidium iodide (PI) and stored one over night at 4°C. Next day, cells were analysed using an LSRII flowcytomer (BD biosciences). A blue 488 nm, 20 mW laser was used for excitation. PI fluorescence was collected using a 610/20 band pass filter. Data analysis was performed by using WinList 8 remotely connected to ModFit LT 4 (Verity Software House, Topsham, ME) (25). Each data file contained at least 10,000 single cell events. A one-compartment polynomial model was used for calculating the percentage G_1, S and G_2M phase of the cell cycle. This statistical model showed the best fit.

Statistical analysis

Dose response curves and IC_{50} values were determined using GraphPad Prism (version 6.05, GraphPad Software, La Jolla, CA). Mann-Whitney testing was performed to investigate differences in survivin expression (GraphPad Prism).

Results

Identification of 27 compounds effective against myxoid liposarcoma tumour cells

Out of 273 tested compounds, 27 drugs were shown to be effective in myxoid liposarcoma tumour cell lines *in vitro*, which was defined as a loss of >50% cell viability in two or all three cell lines at a drug concentration of 100 nM (Figure 1, Supplementary Table 1). All three myxoid liposarcoma cell lines

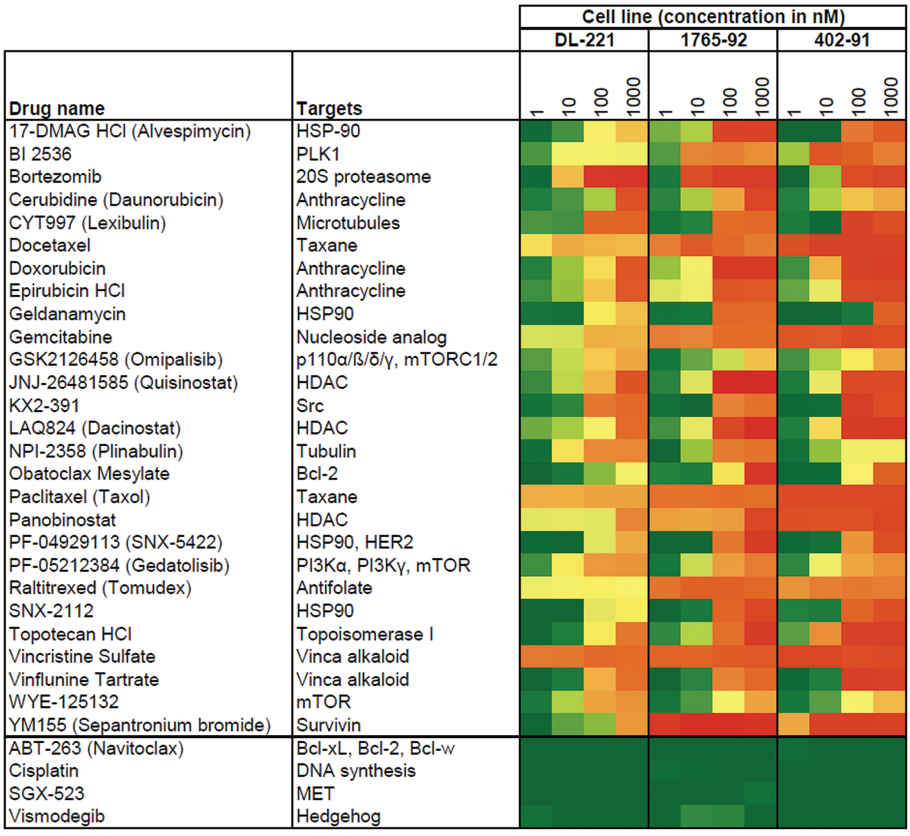
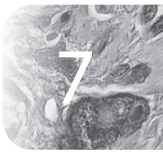


Figure 1. Hits of high throughput *in vitro* drug screen of three MLS cell lines.

List of 27 drugs with a reduction in cell viability of >50% in two or all three cell lines at a drug concentration of 100 nM. Strong inhibitory effect of the survivin inhibitor YM155 is observed in two out of three MLS cell lines. Also a good response is observed to several conventional chemotherapeutics, like doxorubicin, gemcitabine and paclitaxel. Per drug four concentrations (1, 10, 100 and 1000 nM) are tested, green boxes correspond to a high cell viability (~100%) and red boxes to a loss of cell viability (~0%). For comparison, at the bottom, four compounds are randomly shown that did not meet the criteria.



showed a strong response (>80% loss of cell viability) to treatment with the anthracyclines doxorubicin and epirubicin at dose concentrations of both 100 nM and 1000 nM (402-91 and 1765-92) and 1000 nM (DL-221) (Figure 1). The tested taxanes, docetaxel and paclitaxel, as well as a member of another class of mitotic inhibitors, vincristine, revealed a strong decrease in cell viability at the lowest concentration of 1 nM (average cell viabilities of the three cell lines respectively ~22%, ~24% and ~19% after 72 hours of treatment). In the drug screen YM155, a survivin suppressant, showed in the 402-91 and 1765-92 cell

line a very strong decrease in cell viability (average ~13% cell viability) at all four drug concentrations. In the DL-221 cell line there was a moderate reduction of the cell viability after treatment with YM155 at concentrations of 10, 100 and 1000 nM (ranging from 81% to 30% cell viability). Other interesting agents in the list are 17-DMAG HCl, geldanamycin, SNX-5422 and SNX-2112, all targeting HSP90. The list also contains several drugs affecting the PI3K/AKT/mTOR pathway, including omipalisib, gedatolisib and WYE-132. The HDAC inhibitors quisinostat, dacinostat and panobinostat showed a strong inhibitory effect in all three cell lines. KX2-391, an agent interfering with the proto-oncogene Src, also was effective in all three cell lines.

Validation of selected hits using cell viability assays

We selected the the survivin inhibitor YM155, the mTOR inhibitor everolimus, and the broad-spectrum HDAC inhibitor panobinostat (LBH-589) for further validation using cell viability assays. YM155 was of particular interest because this was the drug with the best antitumor effect at the lowest treatment dose in 2 of the 3 cell lines. The 402-91 and 1765-92 cell lines were confirmed to be highly sensitive to YM155 and showed IC_{50} values of 6.3 nM and 5.1 nM respectively (Figure 2A). The IC_{50} value of DL-221 was 194 nM, which is consistent with the results observed in the drug screen. In all three cell lines an inhibition of cell viability (~50%) was observed at a low dose concentration (~5 nM) of everolimus (Supplementary Figure 1A). However, with increasing dose no further decrease of cell viability was observed. The tested cell lines 402-91 and DL-221 demonstrated an IC50 value of 28 nM and 49 nM respectively after treatment with panobinostat (Supplementary Figure 1B).

To confirm the importance of BIRC5 in myxoid liposarcoma cell survival, siRNA experiments were performed. Knockdown of the survivin gene by smartpool siBIRC5 resulted in a partial decrease of viability in all three cell lines (402-91 69%, 1765-92 55% and DL-221 69%). The transfection was successful as all three myxoid liposarcoma cell lines and the control siRNA GAPDH showed a minor reduction of cell viability, whereas transfection with the positive control siPLK1 resulted in all three cell lines in a pronounced decrease in cell viability. Moreover, western blot analysis after transfection with siBIRC5 confirmed a decrease in survivin protein in all three cell lines (Supplementary Figure 2).

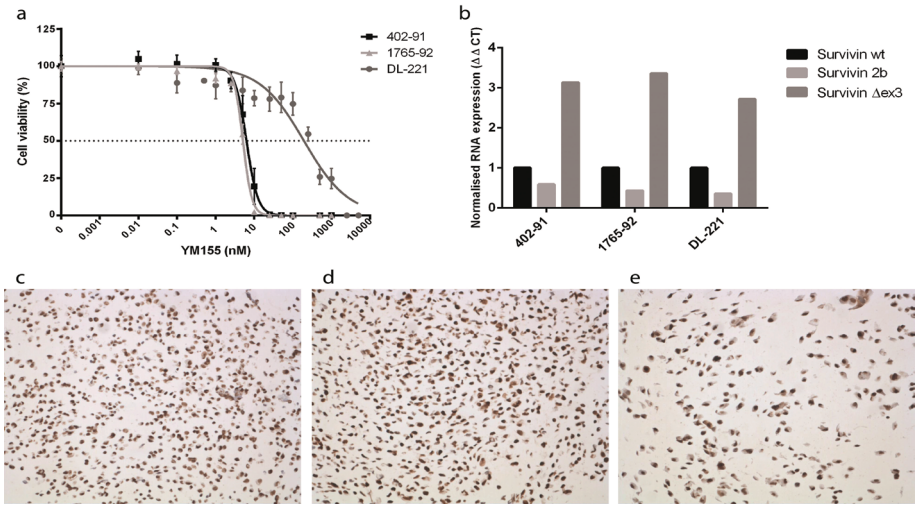


Figure 2. Role of survivin in myxoid liposarcoma cell lines.

Dose response curves for YM155 (72 hours) in myxoid liposarcoma cell lines. Error bars represents three experiments performed in triplicates (a). Normalised RNA expression of three survivin isoforms in cell lines showing relative abundance of the Δex3 isoform (b). Survivin immunohistochemistry of FFPE cell pellets revealed a strong nuclear survivin expression in the cell lines 402-91 (c), 1765-92 (d) and DL-221 (e) (20x magnification).

No synergistic effect of everolimus or YM155 and trabectedin

As trabectedin (ET-743) is effective and therefore often used in the treatment of myxoid liposarcoma, we investigated possible synergy between trabectedin and two of the validated screen hits, YM155 or Panobinostat. Combination treatment of trabectedin with YM155 showed no statistically significant difference between the expected and the observed cell viability in all three tested cell lines, indicating absence of synergism or antagonism for this drug combination (Supplementary Figure 3A, C, E). The combination of trabectedin and panobinostat was evaluated in the 402-91 and DL-221 cell lines, but again no statistical significant synergism or antagonism was observed (Supplementary Figure 3B, D).

Strong nuclear survivin expression in all myxoid liposarcoma cell lines and primary tumours

Since YM155 was most efficient in decreasing myxoid liposarcoma cell viability, we evaluated the protein expression of survivin in the myxoid liposarcoma cell lines. The FFPE cell pellets of the three cell lines showed strong nuclear survivin

expression and a weak cytoplasmic expression in all three cell lines (Figure 2C-E). No difference in the expression levels was observed.
Moreover, we examined the relative distribution of the three most common survivin isoforms: survivin WT, survivin 2b and survivin Δex3 by qRT-PCR. All cell lines revealed a similar mRNA expression pattern, with survivin Δex3 being most highly expressed, followed by survivin WT, while the lowest expression was found for survivin 2b (Figure 2B).

Next we studied a cohort of myxoid liposarcoma primary tumour samples. The tumour samples of all 32 patients (100%) on the tissue microarrays demonstrated strong nuclear expression of survivin protein (Figure 3A). No statistically significant differences were observed between the myxoid, intermediate and round cell components ($p>0.05$) (Figure 3B,C). Cytoplasmic staining was absent, with the exception of a single round cell liposarcoma with moderate cytoplasmic staining.

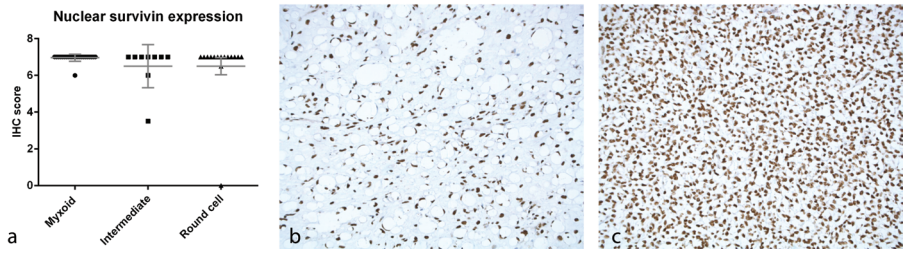


Figure 3. High nuclear expression of survivin in myxoid liposarcomas.

Immunohistochemical analysis of nuclear survivin expression in 32 MLS patients showing high expression in tumour components with myxoid, intermediate and round cell morphology (a). High nuclear survivin expression in tumour with myxoid morphology (b). High nuclear survivin expression in tumour with round cell morphology (c)(20x magnification).

YM155 does not induce apoptosis but increases the S-phase in two out of three MLS cell lines

To further evaluate the mechanism of action of YM155 in myxoid liposarcoma cell lines, we evaluated PARP expression in all three myxoid liposarcoma cell lines using Western blot analysis, but no cleaved PARP was found in the cell lines (Figure 4A) after 24 hours of YM155 treatment. Caspase 3/7 activity after treatment with the survivin inhibitor YM155 at IC_{75} concentration also revealed no significant increase in caspase 3/7 activity after 24 hours (data not shown).

These results indicate that YM155 does not induce apoptosis in myxoid liposarcoma.

We subsequently performed cell cycle analysis to evaluate a possible effect of YM155 on cell cycle regulation. After 48 hours, YM155 treated cells revealed an increase in the S-phase fraction and a decrease in the G1-fraction as compared to untreated 402-91 and 1765-92 cells. In DL-221 no clear changes were observed in cell cycle distribution after YM155 treatment (Figure 4B).

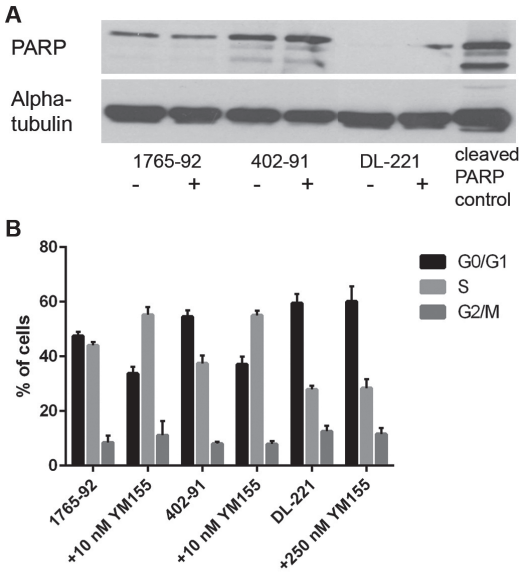


Figure 4. Effect of YM155 on apoptosis and cell cycle.

YM155 does not cause PARP dependent apoptosis, but increases S-phase in 2 of the myxoid liposarcoma cell lines. Western blot analysis for PARP and cleaved PARP expression in myxoid liposarcoma cell lines (a). FACS cell cycle analysis of myxoid liposarcoma cell lines treated with YM155 for 48 hours, measured in two independent experiments. Two cell lines, 402-91 and 1765-92, show a decrease in G_1 and an increase in S-phase after treatment. DL-221 does not show a difference in cell cycle distribution (b).

Discussion

Myxoid liposarcoma initially often responds well to treatment with radiotherapy, conventional chemotherapeutics, or trabectedin, however after several treatment cycles, the effect of treatment is observed to decrease. Therefore, for patients with locally advanced or metastatic disease new therapeutic options are highly warranted in order to improve survival. In this study our aim

was to identify novel candidate targets for treatment by performing a high throughput drug screen with multiple targeted (tyrosine kinase) inhibitors as well as substances of other classes.

The 27 most effective compounds (Figure 1) include the conventional chemotherapeutic agents used in daily practice, such as anthracyclines and taxanes, as anticipated. In addition, the results included three potent inhibitors of the PI3K/AKT/mTOR pathway (omipalisib, gedatolisib and WYE-125132). A subset of myxoid liposarcomas demonstrate dysregulation of this pathway by either *PIK3CA* mutation, loss of PTEN expression or Akt activation (26-28). No large series on mTOR inhibition in myxoid liposarcoma patients are available, although in two cases a minor response has been observed (29) and a clinical trial is ongoing (COSYMO www.clinicaltrials.gov NCT02821507). In the current study we found a decrease of cell viability down to 50% at low dose treatment with everolimus. As a plateau phase was reached, this drug is only able to kill a part of the cell population, suggesting that in clinical studies the drug will need to be combined with another (chemotherapeutic) drug.

The Src inhibitor KX2-391 is another interesting hit confirming previous preclinical results, as two previous studies reported that the Src pathway is highly active in myxoid liposarcoma (30, 31). KX2-391 is the first clinical Src inhibitor targeting the peptide substrate-binding site, with higher *in vitro* potency than dasatinib (32). In a small phase I study with several solid malignancies favourable pharmacokinetics and antitumour activity were observed (33).

We demonstrate that inhibition of survivin by YM155 results in a significant decrease of cell viability in two out of three cell lines (402-91 and 1765-92). YM155 blocks BIRC5 at the promoter region. In addition to this inhibition at the transcriptional level, also at the post-transcriptional level survivin can be downregulated by the addition of CDK or HSP90 inhibitors which are known to interact with the expression of BIRC5 in the cells (34). Interestingly, four of the 27 hits emerging from the compound screen target HSP90 (Figure 1), and demonstrate a strong inhibitory effect on cell viability. A recent study also demonstrates an important role for HSP90 inhibitor 17-DMAG resulting in decreased phosphorylation of several receptor tyrosine kinases and demonstrating massive tumour cell death in a xenograft model (35).

Survivin (BIRC5) is a member of the inhibitor of apoptosis family and is a multifunctional protein that is involved in several important cellular processes

in both the nucleus and the cytoplasm. Survivin interacts with aurora B kinase and both are part of the chromosomal passenger complex, which forms at the kinetochore in the nucleus and regulates microtubule-kinetochore attachment ensuring proper segregation of the sister chromatids during mitosis (36, 37). Cytoplasmic survivin is involved in two processes, on one hand the binding of XIAP, which inhibits caspase-9 and prevents activation of the apoptotic pathway. In addition, survivin is able to activate AKT and to upregulate α5 integrin, resulting in stimulation of cell motility. Normally, survivin is expressed during fetal development and also in certain differentiated tissues. High expression of survivin has been found in several malignancies, including sarcomas such as malignant peripheral nerve sheath tumor, pleomorphic liposarcoma, uterine leiomyosarcoma, chondrosarcoma and Ewing sarcoma (38-43).

All primary myxoid liposarcoma tumor samples, as well as the three cell lines, showed high nuclear expression of survivin. There are multiple survivin isoforms described. Several studies show a particular overexpression of survivin Δex3 in several malignancies, including for example breast and colon carcinoma and soft tissue sarcomas (44, 45). The expression of this isoform is also associated with a worse prognosis. We here show that also in the myxoid liposarcoma cell lines the survivin Δex3 isoform is relatively abundant. Successful knockdown of BIRC5 by siRNA resulted in a decreased cell viability which confirms that survivin is indeed important for the survival of myxoid liposarcoma tumor cells.

In a previous study, p53 overexpressing chondrosarcomas displayed higher levels of nuclear survivin expression and *TP53* mutant chondrosarcoma cell lines were more sensitive to YM155 compared to *TP53* wild-type chondrosarcoma cell lines (40). The myxoid liposarcoma cell line DL-221 is the only one carrying a *TP53* mutation while 402-91 and 1765-92 are *TP53* wild-type (16). Also, we did not observe a difference in survivin expression among the three myxoid liposarcoma cell lines, and as such the mechanism in myxoid liposarcoma might be different as compared to chondrosarcoma. Inhibition of survivin by p53 is regulated via several signal transduction pathways and alterations in one of these pathways might underlie a possible difference (46). A possible explanation for the observed difference might be the interaction between HDAC proteins and p53 at the promoter region of survivin. Also the presence of methyl residues on the survivin promoter block binding of p53, resulting in induced survivin expression. Next, survivin expression is also

influenced by other transcription factors, of which NF-*k*B is an important one as NF-*k*B is deregulated in myxoid liposarcoma as it is influenced by the FUS-DDIT3 fusion protein. However, as there is only one TP53 mutant myxoid liposarcoma cell line available, this number is too small to draw any conclusions.

Previously we and others showed that inhibition of survivin by YM155 is effective *in vitro* in several other malignancies, including squamous cell carcinoma, gastrointestinal stromal tumor and chondrosarcoma (40, 47, 48). Evaluation of the effect of YM155 in *in vivo* models for gastric carcinoma and osteosarcoma demonstrated a suppression of tumor growth in mice (49, 50). Interestingly, dual treatment with YM155 and temsirolimus, an inhibitor of mTOR, demonstrated an improved antitumor effect in a renal cancer model (51). However, in most of these carcinoma models survivin inhibition induces apoptosis, while for myxoid liposarcoma as well as for chondrosarcoma (40) survivin expression was predominantly nuclear. This can be contributed to its role in maintaining proper sister chromatid segregation during mitosis. Strikingly, after treatment with YM155 we observed an increase in percentage of cells in S-phase in 402-91 and 1765-92 (wild-type *TP53*). In DL-221 (*TP53*^{mut}) phases of the cell cycle were left unchanged while the caspase 3/7 pathway seems not to be activated, possibly associated with the lack of cytoplasmic survivin staining. This should be further explored since a high S-phase is significantly associated with a poor prognosis (52, 53). Furthermore, survivin knock-downs by shRNA increased chromosomal instability, irrespective of p53 (54). As together with a high S-phase this might lead to clonal selection and outgrowth of resistant clones, this warrants further investigation.

In conclusion, using a large compound screen we identified 27 compounds that are effective in decreasing cell viability of myxoid liposarcoma cells. We identified survivin as a potential novel target in myxoid liposarcoma. The survivin protein is expressed in 100% of tumour samples and two of three tumour cell lines are highly sensitive for treatment with YM155. However, despite a strong decrease in cell viability, the S-phase increased, which warrants further investigation before treating advanced myxoid liposarcoma patients with YM155 targeting survivin.

Disclosure/conflict of interest

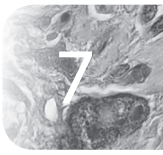
The authors have no disclosure or conflict of interest to declare.

References

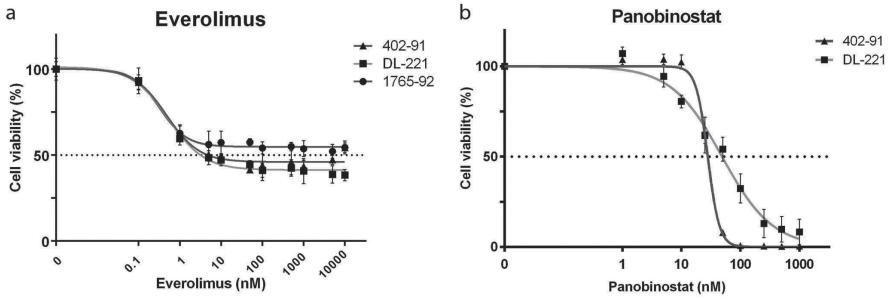
1. Fletcher CDM, Bridge JA, Hogendoorn PCW, et al. World Health Organisation Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. 4th ed. Lyon: IARC Press; 2013.
2. Perez-Mancera PA, Bermejo-Rodriguez C, Sanchez-Martin M, et al. FUS-DDIT3 prevents the development of adipocytic precursors in liposarcoma by repressing PPARgamma and C/EBPalpha and activating eIF4E. PLoS One. 2008;3(7):e2569.
3. Aman P, Dolatabadi S, Svec D, et al. Regulatory mechanisms, expression levels and proliferation effects of the FUS-DDIT3 fusion oncogene in liposarcoma. The Journal of pathology. 2016;238(5):689-99.
4. ten Heuvel SE, Hoekstra HJ, van Ginkel RJ, et al. Clinicopathologic prognostic factors in myxoid liposarcoma: a retrospective study of 49 patients with long-term follow-up. Ann Surg Oncol. 2007;14(1):222-9.
5. Fuglo HM, Maretty-Nielsen K, Hovgaard D, et al. Metastatic pattern, local relapse, and survival of patients with myxoid liposarcoma: a retrospective study of 45 patients. Sarcoma. 2013;2013:548628.
6. Moreau LC, Turcotte R, Ferguson P, et al. Myxoid\round cell liposarcoma (MRCLS) revisited: an analysis of 418 primarily managed cases. Ann Surg Oncol. 2012;19(4):1081-8.
7. Nishida Y, Tsukushi S, Nakashima H, et al. Clinicopathologic prognostic factors of pure myxoid liposarcoma of the extremities and trunk wall. Clin Orthop Relat Res. 2010;468(11):3041-6.
8. Hoffman A, Ghadimi MP, Demicco EG, et al. Localized and metastatic myxoid/round cell liposarcoma: clinical and molecular observations. Cancer. 2013;119(10):1868-77.
9. Baxter KJ, Govsyeyev N, Namm JP, et al. Is multimodality therapy necessary for the management of pure myxoid liposarcomas? A multi-institutional series of pure myxoid liposarcomas of the extremities and torso. Journal of surgical oncology. 2015;111(2):146-51.
10. Schoffski P, Chawla S, Maki RG, et al. Eribulin versus dacarbazine in previously treated patients with advanced liposarcoma or leiomyosarcoma: a randomised, open-label, multicentre, phase 3 trial. Lancet. 2016;387(10028):1629-37.
11. Gordon EM, Sankhala KK, Chawla N, et al. Trabectedin for Soft Tissue Sarcoma: Current Status and Future Perspectives. Advances in therapy. 2016;33(7):1055-71.
12. D'Incalci M, Galmarini CM. A review of trabectedin (ET-743): a unique mechanism of action. Mol Cancer Ther. 2010;9(8):2157-63.
13. Grosso F, Sanfilippo R, Virdis E, et al. Trabectedin in myxoid liposarcomas (MLS): a long-term analysis of a single-institution series. Ann Oncol. 2009;20(8):1439-44.
14. Aman P, Ron D, Mandahl N, et al. Rearrangement of the transcription factor gene CHOP in myxoid liposarcomas with t(12;16)(q13;p11). Genes Chromosomes Cancer. 1992;5(4):278-85.
15. Thelin-Jarnum S, Lassen C, Panagopoulos I, et al. Identification of genes differentially expressed in TLS-CHOP carrying myxoid liposarcomas. Int J Cancer. 1999;83(1):30-3.
16. de Graaff MA, Yu JS, Beird HC, et al. Establishment and characterization of a new human myxoid liposarcoma cell line (DL-221) with the FUS-DDIT3 translocation. Lab Invest. 2016;96(8):885-94.
17. Halvorson KG, Barton KL, Schroeder K, et al. A high-throughput in vitro drug screen in a genetically engineered mouse model of diffuse intrinsic pontine glioma identifies BMS-754807 as a promising therapeutic agent. PloS one. 2015;10(3):e0118926.
18. Eriksson A, Osterroos A, Hassan S, et al. Drug screen in patient cells suggests quinacrine to be repositioned for treatment of acute myeloid leukemia. Blood cancer journal. 2015;5:e307.
19. Wong M, Tan N, Zha J, et al. Navitoclax (ABT-263) reduces Bcl-x(L)-mediated chemoresistance in ovarian cancer models. Mol Cancer Ther. 2012;11(4):1026-35.
20. Hameetman L, Rozeman LB, Lombaerts M, et al. Peripheral chondrosarcoma progression is accompanied by decreased Indian Hedgehog signalling. The Journal of pathology. 2006;209(4):501-11.
21. Endo M, de Graaff MA, Ingram DR, et al. NY-ESO-1 (CTAG1B) expression in mesenchymal tumors. Mod Pathol. 2015;28(4):587-95.

22. Bovee JV, Cleton-Jansen AM, Kuipers-Dijkshoorn NJ, et al. Loss of heterozygosity and DNA ploidy point to a diverging genetic mechanism in the origin of peripheral and central chondrosarcoma. Genes Chromosomes Cancer. 1999;26(3):237-46.
23. Pavlidou A, Dalamaga M, Kroupis C, et al. Survivin isoforms and clinicopathological characteristics in colorectal adenocarcinomas using real-time qPCR. World journal of gastroenterology. 2011;17(12):1614-21.
24. Willems SM, Schrage YM, Baelde JJ, et al. Myxoid tumours of soft tissue: the so-called myxoid extracellular matrix is heterogeneous in composition. Histopathology. 2008;52(4):465-74.
25. van Haaften C, Boot A, Corver WE, et al. Synergistic effects of the sesquiterpene lactone, EPD, with cisplatin and paclitaxel in ovarian cancer cells. J Exp Clin Cancer Res. 2015;34:38.
26. Demicco EG, Torres KE, Ghadimi MP, et al. Involvement of the PI3K/Akt pathway in myxoid/round cell liposarcoma. Mod Pathol. 2012;25(2):212-21.
27. Negri T, Virdis E, Brich S, et al. Functional mapping of receptor tyrosine kinases in myxoid liposarcoma. Clin Cancer Res. 2010;16(14):3581-93.
28. Barretina J, Taylor BS, Banerji S, et al. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. Nat Genet. 2010;42(8):715-21.
29. Sanfilippo R, Dei Tos AP, Casali PG. Myxoid liposarcoma and the mammalian target of rapamycin pathway. Curr Opin Oncol. 2013;25(4):379-83.
30. Willems SM, Schrage YM, Bruijn IH, et al. Kinome profiling of myxoid liposarcoma reveals NF-kappaB-pathway kinase activity and casein kinase II inhibition as a potential treatment option. Mol Cancer. 2010;9:257.
31. Sievers E, Trautmann M, Kindler D, et al. SRC inhibition represents a potential therapeutic strategy in liposarcoma. Int J Cancer. 2015;137(11):2578-88.
32. Lau GM, Lau GM, Yu GL, et al. Expression of Src and FAK in hepatocellular carcinoma and the effect of Src inhibitors on hepatocellular carcinoma in vitro. Digestive diseases and sciences. 2009;54(7):1465-74.
33. Naing A, Cohen R, Dy GK, et al. A phase I trial of KX2-391, a novel non-ATP competitive substrate-pocket- directed SRC inhibitor, in patients with advanced malignancies. Invest New Drugs. 2013;31(4):967-73.
34. Chen X, Duan N, Zhang C, et al. Survivin and Tumorigenesis: Molecular Mechanisms and Therapeutic Strategies. Journal of Cancer. 2016;7(3):314-23.
35. Safavi S, Jarnum S, Vannas C, et al. HSP90 inhibition blocks ERBB3 and RET phosphorylation in myxoid/round cell liposarcoma and causes massive cell death in vitro and in vivo. Oncotarget. 2016;7(1):433-45.
36. McKenzie JA, Grossman D. Role of the apoptotic and mitotic regulator survivin in melanoma. Anticancer research. 2012;32(2):397-404.
37. van der Waal MS, Hengeveld RC, van der Horst A, et al. Cell division control by the Chromosomal Passenger Complex. Experimental cell research. 2012;318(12):1407-20.
38. Wurl P, Kappler M, Meye A, et al. Co-expression of survivin and TERT and risk of tumour-related death in patients with soft-tissue sarcoma. Lancet. 2002;359(9310):943-5.
39. Hingorani P, Dickman P, Garcia-Filion P, et al. BIRC5 expression is a poor prognostic marker in Ewing sarcoma. Pediatric blood & cancer. 2013;60(1):35-40.
40. de Jong Y, van Oosterwijk JG, Kruisselbrink AB, et al. Targeting survivin as a potential new treatment for chondrosarcoma of bone. Oncogenesis. 2016;5:e222.
41. Ghadimi MP, Liu P, Peng T, et al. Pleomorphic liposarcoma: clinical observations and molecular variables. Cancer. 2011;117(23):5359-69.
42. Ghadimi MP, Young ED, Belousov R, et al. Survivin is a viable target for the treatment of malignant peripheral nerve sheath tumors. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012;18(9):2545-57.
43. Lusby K, Savannah KB, Demicco EG, et al. Uterine leiomyosarcoma management, outcome, and associated molecular biomarkers: a single institution's experience. Annals of surgical oncology. 2013;20(7):2364-72.

44. Taubert H, Kappler M, Bache M, et al. Elevated expression of survivin-splice variants predicts a poor outcome for soft-tissue sarcomas patients. Oncogene. 2005;24(33):5258-61.
45. Necochea-Campion R, Chen CS, Mirshahidi S, et al. Clinico-pathologic relevance of Survivin splice variant expression in cancer. Cancer Lett. 2013;339(2):167-74.
46. Sah NK, Seniya C. Survivin splice variants and their diagnostic significance. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2015;36(9):6623-31.
47. Zhang W, Liu Y, Li YF, et al. Targeting of Survivin Pathways by YM155 Inhibits Cell Death and Invasion in Oral Squamous Cell Carcinoma Cells. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2016;38(6):2426-37.
48. Falkenhorst J, Grunewald S, Muhlenberg T, et al. Inhibitor of Apoptosis Proteins (IAPs) are commonly dysregulated in GIST and can be pharmacologically targeted to enhance the pro-apoptotic activity of imatinib. Oncotarget. 2016.
49. Cheng XJ, Lin JC, Ding YF, et al. Survivin inhibitor YM155 suppresses gastric cancer xenograft growth in mice without affecting normal tissues. Oncotarget. 2016;7(6):7096-109.
50. Zhang Z, Ma L, Wang J. YM155 exerts a growth inhibitory effect on human osteosarcoma in vitro and in vivo. Oncology reports. 2015;34(2):1074-80.
51. Carew JS, Espitia CM, Zhao W, et al. Targeting Survivin Inhibits Renal Cell Carcinoma Progression and Enhances the Activity of Temsirolimus. Mol Cancer Ther. 2015;14(6):1404-13.
52. Bagwell CB, Clark GM, Spyratos F, et al. DNA and cell cycle analysis as prognostic indicators in breast tumors revisited. Clinics in laboratory medicine. 2001;21(4):875-95.
53. Dayal JH, Sales MJ, Corver WE, et al. Multiparameter DNA content analysis identifies distinct groups in primary breast cancer. British journal of cancer. 2013;108(4):873-80.
54. Wiedemuth R, Klink B, Topfer K, et al. Survivin safeguards chromosome numbers and protects from aneuploidy independently from p53. Molecular cancer. 2014;13:107.

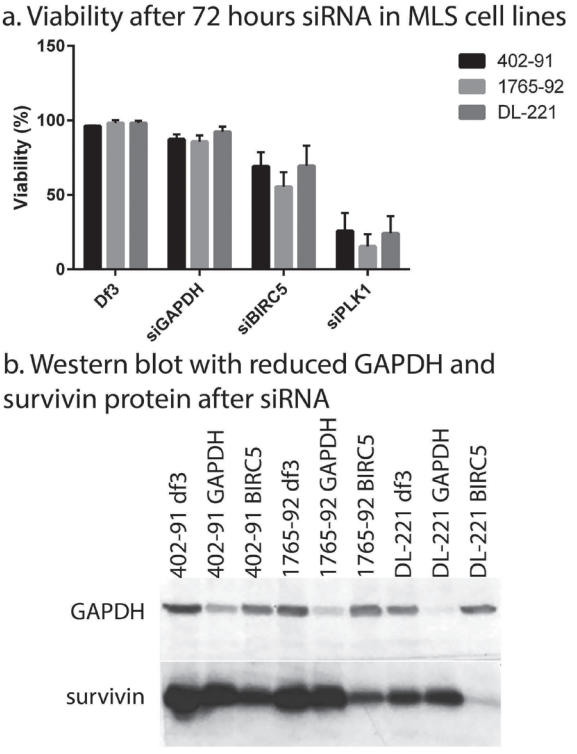


Supplementary Information



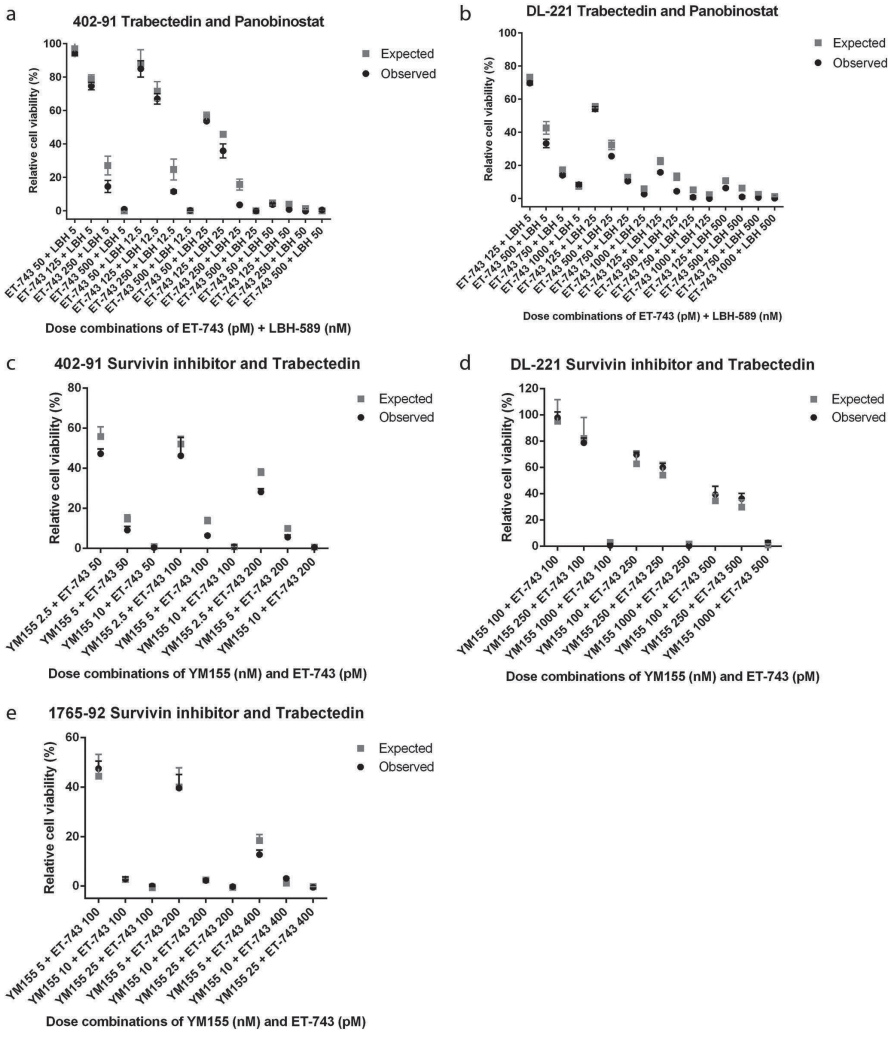
Supplementary Figure 1.

Dose response curves of myxoid liposarcoma cell lines after monotherapy with everolimus (a) and panobinostat (b) afther 72 hours of treatment.



Supplementary Figure 2. Knockdown BIRC5.

BIRC5 and PLK1 siRNA show a decrease in cell viability, whereas the transfec€tion agent alone or control siGAPDH hardly affect cell viability (a). A reduction in GAPDH and survivin protein expression was found after transfection with respectively siGAPDH and siBIRC5 in all three cell lines (b).



Supplementary Figure 3. Combination treatment of cell line 402-91 and DL-221 with panobinostat and trabectedin (a and b) showed no synergism or antagonism. Combination therapy of three MLS cell lines with trabectedin and YM155 also demonstrated no synergism or antagonism (c-e).

Chapter 8

Summary and concluding remarks

Summary and concluding remarks

This thesis focusses on the molecular pathology of two specific subtypes of soft tissue sarcomas, leiomyosarcoma of soft tissue and myxoid liposarcoma. As described in detail in the introduction, these malignant tumours each have their unique clinical features, histopathology and genetic profile. Soft tissue leiomyosarcomas are often high grade and generally have an aneuploid karyotype with multiple different and non-recurrent translocations. This complex genomic profile makes it hard to identify a common alteration that can be targeted in the search for new treatment strategies. Therefore, to gain insight in the aetiology of tumorigenesis the focus of research should be on the identification of genes and pathways driving and promoting tumour formation. The identified pathway alterations can be candidate targets for new therapeutic options interfering with members of these pathways. Myxoid liposarcomas, on the other hand, harbour a recurrent genetic aberration, most frequently resulting in the fusion protein FUS-DDIT3. This oncogenic fusion product acts as an aberrant transcription factor and influences gene expression resulting in malignant transformation and tumour growth. The presence of a specific genetic alteration makes the translocation product an ideal candidate for targeted treatment, however this is complicated by the fact that targeting a transcription factor is difficult and that probably not all target genes have been identified yet.

Mutations in exon 2 of the *mediator complex subunit 12* (*MED12*), located on chromosome Xq13.1, were first described in around 50%-70% of benign uterine smooth muscle tumours in 2011 (1). In **chapter 2** we investigated the presence of *MED12* exon 2 mutations in extra-uterine leiomyomas, including piloleiomyomas, abdominal leiomyomas and angioleiomyomas, and both soft tissue and uterine leiomyosarcomas, aiming to obtain more insight in the pathogenesis of these tumours. In line with the previously published study (1), we found a mutation rate of 58% in uterine leiomyomas. In contrast, only one abdominal leiomyoma harboured a *MED12* mutation and based on the expression of the oestrogen receptor, this lesion was most likely derived from the uterus. The absence of *MED12* mutations in all other extra-uterine leiomyomas highly suggests a different pathogenesis (2). Recently *MED12* mutations have been found in fibroadenomas of the breast and phyllodes tumours (3). As these mutations tend to occur in particular in hormone

sensitive tumours, it might be probable that *MED12* is only involved in tumours with activated hormone receptor signalling. We identified one uterine leiomyosarcoma with a *MED12* mutation, this low mutation rate is similar to what is described in literature (ranging from 2% to 30%) and supports the hypothesis that the majority of uterine leiomyosarcomas arise *de novo* while only rarely a leiomyosarcoma develops in a benign smooth muscle tumour (4). It has been postulated that in rare cases uterine leiomyomas create an environment that facilitates wild-type smooth muscle cells to acquire other genetic alterations and develop into a malignant phenotype, as has been described for *EXT*-mutated osteochondroma (5). We found nuclear β-catenin in half of the *MED12*-mutated uterine leiomyomas. When translocated to the nucleus, β-catenin directly interacts with MED12 and regulates transcription of target genes of the canonical Wnt-signalling pathway. In addition, in the same series we investigated fumarate hydratase (FH)-deficiency, which is mutually exclusive with *MED12* mutations. One uterine leiomyoma and one cutaneous leiomyoma from a patient with suspected hereditary leiomyomatosis and renal cell carcinoma syndrome, and 2 sporadic leiomyosarcomas were positive for S-(2-succinyl)cysteine (2SC) immunohistochemistry, which is a robust biomarker for *FH* mutations (6). Moreover, these FH deficient tumours showed increased H3K9me3 methylation compared to FH-wild type tumours, which was expected since alterations in several genes of the tricarboxylic acid cycle affect histone demethylases. The role of these epigenetic alterations, is not yet clarified in smooth muscle tumours.

Soft tissue leiomyosarcomas have a complex genetic profile with numerous non-recurrent aberrations resulting from a high degree of chromosomal instability. In the search for a better understanding of the pathogenesis of this tumour and to identify candidate targets for treatment, we performed COBRA-FISH karyotyping and found a t(6;14) in two cases, as described in **chapter 3**. Interestingly we identified in one of the tumours cryptic rearrangements around *HMGA1* and *RAD51L1*, genes that are also involved in a subset of leiomyomas. Detailed breakpoint mapping and in depth characterization with array-CGH profiling of both cases revealed different translocation breakpoints which, once more, underlines the complex and heterogeneous genetic profile of leiomyosarcomas. In this study a high grade leiomyosarcoma was encountered with a relatively simple karyotype, also in literature this has been described in a subset of tumours. Combination of

modern sequencing techniques might identify different subgroups in these complex heterogeneous tumours, like for example the 'muscle-enriched' group characterized by enhanced expression of muscle differentiation related genes. The recent discovery of the expression of LMOD1 in the 'muscle-enriched' group and ARL4C in another subgroup, associated with a better and worse disease specific survival, respectively, might be helpful in determination of the prognosis (7). However, additional research is needed to translate these findings into subtype-specific targeted therapy.

Soft tissue leiomyosarcomas are frequently resistant to chemotherapy and therefore the number of treatment options for this aggressive disease is limited. To gain insight in the mechanisms involved in chemoresistance and to search for new therapeutic targets, we investigated the role of the Bcl-2 family proteins in leiomyosarcomas, in **chapter 4**. The anti-apoptotic protein Bcl-2 was highly expressed in the majority of tumours and moreover a substantial number of tumours showed expression of Bcl-xL and Bcl-w. Single-agent treatment of leiomyosarcoma cell lines with ABT-737, a BH3 mimetic, resulted in only a slight reduction of cell viability. However, combination treatment with the conventional chemotherapeutic agent doxorubicin demonstrated synergism in all tested cell lines by inducing apoptosis. Therefore, inhibition of anti-apoptotic proteins by a BH3 mimetic sensitizes soft tissue leiomyosarcomas to chemotherapy. Before the results of this study can be translated to the practice of the clinical oncologist for the benefit of leiomyosarcoma patients, further validation in *in vivo* models need to be done. In case of positive and promising results, combination treatment with a Bcl-2 inhibitor and conventional chemotherapeutic agent might be a therapeutic option for patients with Bcl-2 positive tumours. The Bcl-2 inhibitor navitoclax has shown a favourable safety profile in several phase I trials. The antitumour effect of navitoclax monotherapy was disappointing in for example patients with small cell lung cancer and therefore clinical studies tend to focus on the combination with other chemotherapeutic agents (8, 9). A limited treatment effect might be attributed to expression MCL1 or galectin-3, which are both also antiapoptotic proteins, able to mimic the function of Bcl-2 and acts as a bypass mechanism to avoid apoptosis. A specific sphingosine kinase 2 inhibitor (ABC294640) promotes MCL1 degradation and induced apoptosis in combination with a Bcl-2 inhibitor in multiple myeloma (10). Therefore tumours expressing both Bcl-2 proteins as well as MCL1 or galectin-3, needs to be treated with a combination

of a Bcl-2 inhibitor and a compound that leads to a reduced function of MCL1 or galectin-3 to ensure successful suppression of the antiapoptotic function (11).

In **chapter 5** we investigated the protein expression of the cancer-testis antigen NY-ESO-1 (CTAG1B) in a large series of myxoid liposarcomas as well as its expression in several other benign and malignant bone and soft tissue neoplasms using immunohistochemical staining. Cancer-testis antigens are currently under the magnifying glass due to their unique combination of properties, which include expression exclusively in the testis in adults, aberrant expression in certain tumours and highly immunogenic features, rendering them an interesting target for immunotherapy. Strong expression of NY-ESO-1 was found in 88% of myxoid liposarcomas, which was the tumour with the highest proportion of positive cases. In the myxoid liposarcomas pre-treated with chemo- or radiotherapy prior to resection, NY-ESO-1 could be detected in 42% of cases. NY-ESO-1 was also positive in a subset of synovial sarcomas, myxofibrosarcomas and conventional chondrosarcomas, while all benign mesenchymal lesions were negative. Clinical trials investigate the effect of NY-ESO-1 targeted vaccine, autologous T-cell or dendritic cell immunotherapies strategies and these studies should be open to patients with NY-ESO-1 expression. A pilot clinical trial demonstrated an antitumour effect of NY-ESO-1 reactive T-cells in synovial sarcoma patients (12). At this moment several clinical trials are recruiting patients, including myxoid liposarcoma patients, to investigate NY-ESO-1 vaccine or T-cell therapy, either alone or in combination with another drug.

Establishing a cell line from primary myxoid liposarcoma tumour samples is a daunting task reflected by the presence of only two cell lines generated by SV40 virus transformation available world-wide, which hampers the research to this malignant tumour. **Chapter 6** focusses purely on myxoid liposarcoma and here we report the establishment and characterization of a new human myxoid liposarcoma cell line (DL-221) with the *FUS-DDIT3* translocation. The new cell line is studied in detail for mutations and pathway alterations frequently encountered in this tumour type, like for example the mutation status of *PIK3CA*, *TERT* and *TP53* and the protein expression of NY-ESO-1. Both the cell line as well as the ancillary xenograft model underwent next-generation whole exome sequencing and they are of pivotal importance to study the biology and novel potential-targeted treatment approaches for myxoid liposarcoma.

In **chapter 7** we continue the search for new candidate targets for the treatment of myxoid liposarcoma. A high-throughput drug screen was performed using three myxoid liposarcoma cell lines, including the one presented in chapter 6. As expected, a strong response to the chemotherapeutic agents of the classes of the anthracyclines and taxanes was observed. Survivin (BIRC5) was identified as an interesting novel target in the screen, as a strong decrease in cell viability was observed after treatment with the survivin inhibitor YM155. Survivin has a dual function in the cell; in the nucleus it is involved in cell cycle regulation and in the cytoplasm in the inhibition of apoptosis. We demonstrated that survivin is highly expressed in the nuclei of tumour samples supporting its role in cell cycle regulation in myxoid liposarcoma. Other interesting targets from the drug screen were drugs affecting mTOR, HDAC and HSP90. Further *in vivo* studies are required to investigate whether YM155 can be a promising therapeutic strategy for patients with advanced myxoid liposarcoma.

Concluding remarks and future perspectives

This thesis presents molecular data for soft tissue leiomyosarcoma, a tumour known for its complex genome and, as a consequence, the difficulty to identify targets for treatment. We here show that *MED12* mutations are restricted to uterine smooth muscle tumours, and describe the occurrence of non-recurrent translocations in two cases of soft tissue leiomyosarcomas. How these alterations relate to the three molecular subtypes that have recently been proposed (7) remains to be investigated. In addition, we show high expression of the Bcl family members in leiomyosarcomas, and using cell lines we could demonstrate that these proteins play a role in chemoresistance. Also here it would be interesting to see how the expression of other Bcl family members is related to the molecular subtype. Moreover, we show that treatment with a Bcl-2 inhibitor overcomes the chemoresistance of leiomyosarcoma cells. This combination strategy should be further explored *in vivo* to investigate whether it might be a promising therapeutic approach for leiomyosarcoma patients. Next, the ongoing research of Next Generation Sequencing in combination with functional studies as metabolomics and proteomics might help in the identification of cellular processes in leiomyosarcomas which

might be used in diagnostics or for therapy. The identification of new, and potential immunogenic, proteins might provide new candidate targets for immunotherapy. A recent study demonstrates that concurrent targeting of the PI3K/AKT/mTOR pathway in combination with doxorubicin treatment results in reduced tumour growth *in vivo* in leiomyosarcoma xenografts (13).

Myxoid liposarcoma, genetically belonging to the group of tumours with a recurrent translocation, is generally sensitive to conventional chemotherapy and radiation. Trabectedin is initially quite effective in a substantial part of the patients, but this effect might cease after some time. We show expression of the cancer-testis antigen NY-ESO-1 in a large proportion of tumours, providing options for immunotherapy. The use of a dendritic cell vaccine with the NY-ESO-1 protein may teach the patient's immune system to target and kill tumour cells expressing the NY-ESO-1 protein. Another promising option is the adoptive transfer of autologous T-lymphocytes transduced with a NY-ESO-1-reactive T-cell receptor. As the first myxoid liposarcoma patients have entered clinical trials exploiting NY-ESO-1, soon we will learn whether this is a promising strategy for patients with advanced or metastatic myxoid liposarcoma. So far, preclinical studies on myxoid liposarcoma were hampered by the lack of representative preclinical models. In this thesis we present a novel, spontaneously immortalized myxoid liposarcoma cell line, DL-221, which was used together with the other two available cell lines for a large-scale drug screen. We identified survivin as a promising novel target, which is expressed in 100% of myxoid liposarcomas, and future *in vivo* studies should reveal whether the surviving inhibitor YM155 is an effective therapeutic option for patients with advanced myxoid liposarcoma.

References

1. Makinen N, Mehine M, Tolvanen J, et al. MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. Science. 2011;334(6053):252-5.
2. Schwetye KE, Pfeifer JD, Duncavage EJ. MED12 exon 2 mutations in uterine and extrauterine smooth muscle tumors. Human pathology. 2014;45(1):65-70.
3. Tan J, Ong CK, Lim WK, et al. Genomic landscapes of breast fibroepithelial tumors. Nature genetics. 2015;47(11):1341-5.
4. Croce S, Chibon F. MED12 and uterine smooth muscle oncogenesis: State of the art and perspectives. Eur J Cancer. 2015;51(12):1603-10.
5. de Andrea CE, Reijnders CM, Kroon HM, et al. Secondary peripheral chondrosarcoma evolving from osteochondroma as a result of outgrowth of cells with functional EXT. Oncogene. 2012;31(9):1095-104.
6. Hoekstra AS, de Graaff MA, Briaire-de Bruijn IH, et al. Inactivation of SDH and FH cause loss of 5hmC and increased H3K9me3 in paraganglioma/pheochromocytoma and smooth muscle tumors. Oncotarget. 2015;6(36):38777-88.
7. Guo X, Jo VY, Mills AM, et al. Clinically Relevant Molecular Subtypes in Leiomyosarcoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(15):3501-11.
8. Rudin CM, Hann CL, Garon EB, et al. Phase II study of single-agent navitoclax (ABT-263) and biomarker correlates in patients with relapsed small cell lung cancer. Clin Cancer Res. 2012;18(11):3163-9.
9. Cleary JM, Lima CM, Hurwitz HI, et al. A phase I clinical trial of navitoclax, a targeted high-affinity Bcl-2 family inhibitor, in combination with gemcitabine in patients with solid tumors. Invest New Drugs. 2014;32(5):937-45.
10. Venkata JK, An N, Stuart R, et al. Inhibition of sphingosine kinase 2 downregulates the expression of c-Myc and Mcl-1 and induces apoptosis in multiple myeloma. Blood. 2014;124(12):1915-25.
11. Harazono Y, Nakajima K, Raz A. Why anti-Bcl-2 clinical trials fail: a solution. Cancer metastasis reviews. 2014;33(1):285-94.
12. Robbins PF, Kassim SH, Tran TL, et al. A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. Clin Cancer Res. 2015;21(5):1019-27.
13. Babichev Y, Kabaroff L, Datti A, et al. PI3K/AKT/mTOR inhibition in combination with doxorubicin is an effective therapy for leiomyosarcoma. J Transl Med. 2016;14:67.

Chapter 9

Nederlandse samenvatting

Nederlandse samenvatting

Sarcomen vormen een heterogene groep maligne tumoren die uit gaan van mesenchymale of neuroectodermale weefsels, zoals bot, kraakbeen, glad en dwarsgestreept spierweefsel, vet, endotheel en zenuwweefsel. Ondanks dat sarcomen verantwoordelijk zijn voor minder dan 1% van alle maligniteiten, zijn er inmiddels meer dan 50 verschillende histologische subtypes beschreven. De verschillende sarcomen worden gekenmerkt door hun verschillen in klinische presentatie, hun sterk uiteenlopende biologische gedrag en prognose. Een correcte diagnose is dan ook uiterst belangrijk voor het kiezen van de optimale behandelstrategie. Het stellen van de juiste diagnose wordt door veel pathologen als lastig ervaren omdat sarcomen zeldzaam zijn en door het grote aantal verschillende subtypes. Daarnaast hebben sommige tumoren meerdere overlappende histopathologische kenmerken. De World Health Organisation (WHO) heeft een internationale consensus richtlijn opgesteld voor de classificatie van bot- en wekedelentumoren die handvaten biedt bij het stellen van de juiste diagnose. Geadviseerd wordt om patiënten met (verdenking op) een mesenchymale tumor te verwijzen naar een gespecialiseerd centrum waar een multidisciplinair team betrokken is bij de diagnostiek en behandeling van deze patiënten. Hier werken gespecialiseerde oncologisch chirurgen, orthopeden, radiologen, oncologen, radiotherapeuten en pathologen nauw samen. Sarcomen zijn bij uitstek tumoren waarbij niet alleen de histologie en moleculaire diagnostiek van belang zijn, maar ook het klinisch beeld, zoals de leeftijd van presentatie en het radiologisch beeld van de verschillende tumoren dienen bij het maken van de differentiaal diagnose meegenomen te worden in de overweging.

De groep van bot- en wekedelentumoren kan op basis van hun biologisch gedrag worden onderverdeeld in vier subgroepen, namelijk benigne, lokaal agressief, zelden metastaserend en maligne. Een bekend voorbeeld van een benigne tumor is een lipoom, een goedaardige vettumor, histologisch opgebouwd uit mature adipocyten. Dermatofibrosarcoma protuberans (DFSP) is een wekedelentumor die op de plaats waar deze ontstaat vooral schade veroorzaakt door zijn lokaal destructieve groei waardoor chirurgische resectie met tumorvrije marges moeilijk te bereiken kan zijn. Tijdige herkenning, diagnose en instellen van de goede behandeling is van groot belang bij sarcomen, omdat sommige subtypen een slechte prognose hebben. Bij de

diagnostiek is de histomorfologie van de tumor van belang, daarnaast kan immuunhistochemisch onderzoek bij met name de wekedelentumoren bijdragend zijn, omdat hiermee de differentiatielijn van de tumor vaak kan worden aangetoond. Zo zijn er bijvoorbeeld markers voor zenuw-, vaat- en spierdifferentiatie. Daarnaast is moleculair genetisch onderzoek in sommige gevallen van groot belang omdat van de 117 verschillende benigne en maligne wekedelentumoren die zijn beschreven in de WHO er 49% een unieke/ specifieke genetische of moleculaire afwijking heeft.

Op basis van de genetische achtergrond kunnen sarcomen ingedeeld worden in drie hoofdgroepen, te weten tumoren met een specifieke genetische verandering op het niveau van één gen, zoals *KIT* mutaties in gastro-intestinale stromaceltumoren, tumoren met een karakteristieke chromosomale translocatie en een verder normaal karyotype, zoals de FUS-DDIT3 translocatie in myxoid liposarcomen en de laatste groep bestaat uit tumoren met een complex karyotype zonder een terugkerende specifieke afwijking, zoals leiomyosarcomen. De ontdekking van specifieke afwijkingen in tumoren op niveau van het DNA heeft in sommige gevallen bijgedragen tot een begrip van het pathologische mechanisme wat leidt tot ongeremde celdeling en dus tumorgroei. Ook draagt het bij aan de diagnostiek van deze tumoren omdat met verscheidene moleculaire technieken de pathognomonische DNA afwijking vastgesteld kan worden. Zo kunnen mutaties in hot-spot gebieden met verschillende sequencetechnieken snel in kaart gebracht worden en fusieproducten van translocaties kunnen aangetoond worden door middel van fluorescentie *in situ* hybridisatie (FISH) of q-PCR van het fusieproduct. Ook is er onderzoek gaande naar het ontwikkelen van medicijnen die speciaal gericht zijn tegen de genetische afwijking in sommige tumoren en op die wijze mogelijk kunnen leiden tot een nieuwe therapeutische optie die bij de behandeling van patiënten toegepast kan worden.

Dit proefschrift richt zich op twee verschillende wekedelensarcomen, het myxoid liposarcoom en het leiomyosarcoom van de wekedelen. Wekedelen leiomyosarcomen tonen gladde spierceldifferentiatie en zijn verantwoordelijk voor ongeveer 10 tot 20% van de wekedelensarcomen. De tumoren komen het vaakst voor in de 5de en 6de decade en zijn iets frequenter bij vrouwen dan bij mannen. Op basis van de locatie kunnen deze tumoren verder worden verdeeld in vijf groepen, te weten gladde spierceltumoren van de diepe wekedelen, het retroperitoneum, de uterus, de bloedvaten en de superficiële

dermis. In dit proefschrift worden de leiomyosarcomen van de wekedelen en het retroperitoneum onderzocht. Aangezien deze tumoren in de diepe wekedelen ontstaan zijn ze vaak al groot op het moment dat ze ontdekt worden. De tumoren bestaan uit spoelvormige cellen met eosinofiel cytoplasma en langgerekte kernen die in elkaar kriskras kruisende bundels gelegen zijn. Hooggradige tumoren worden gekenmerkt door uitgesproken cytonucleaire atypie en necrose. De tumoren hebben een wisselende mate van gladde spierceldifferentiatie die kan worden aangetoond met immuunhistochemisch onderzoek waarbij er minimaal enige desmine of h-caldesmon expressie aanwezig moet zijn. Leiomyosarcomen hebben een instabiel, complex karyotype en tot op heden is er geen genetische afwijking bekend die in al deze tumoren voorkomt. Mutaties in *TP53*, verlies van de tumor suppressorgenen *PTEN* en *RB1* en veranderingen in *MYOCD*, *ATRX* en *ROR2* komen in een substantieel deel van de tumoren voor en zijn soms gecorreleerd met de prognose. Leiomyosarcomen worden behandeld met chirurgie en daarnaast met conventionele chemotherapie zoals doxorubicine en dacarbazine. Naast de maligne gladde spierceltumor bestaat er ook een benigne gladde spiercel tumor, het leiomyoom. Een veelvoorkomende variant van deze tumor is het leiomyoom van de uterus, welke bij ongeveer 70% van de vrouwen in de reproductieve leeftijd voorkomt. Benigne gladde spierceltumoren kunnen ook elders in het lichaam voorkomen, zo zijn er bijvoorbeeld de piloleiomyomen die in de huid ontstaan. De leiomyomen van de uterus kunnen genetisch in vier hoofdgroepen verdeeld worden, waarbij tussen de 50-70% van deze tumoren een mediator complex subunit 12 (*MED12*) mutatie heeft. In **hoofdstuk 2** wordt het voorkomen van *MED12* mutaties in leiomyomen van de uterus en wekedelen onderzocht. In overeenstemming met de literatuur wordt in 58% van de uterine leiomyomen een *MED12* mutatie aangetroffen. In de onderzochte angioleiomyomen, piloleiomyomen en abdominale leiomyomen wordt in slechts één abdominale tumor een *MED12* mutatie aangetroffen. Deze specifieke tumor toont ook oestrogeenreceptor expressie wat er op duidt dat deze laesie mogelijk afkomstig is van een leiomyoom van de uterus. *MED12* mutaties zijn meer recent ook aangetroffen in fibroadenomen van de borst en phyllodestumoren wat suggereert dat MED12 mogelijk vooral van belang is in hormoongevoelige tumoren. De afwezigheid van *MED12* mutaties in extra-uteriene leiomyomen duidt op een andere pathogenese van deze laesies. In deze studie was er één leiomyosarcoom van de uterus met een *MED12* mutatie,

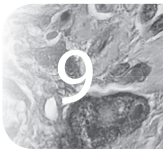
wat overeenkomt met het lage percentage (2 tot 30%) leiomyosarcomen met *MED12* mutaties wat is beschreven in de literatuur. Dit ondersteunt de hypothese dat het merendeel van de leiomyosarcomen van de uterus *de novo* ontstaat in plaats van dat ze zich ontwikkelen in een leiomyoom. Het is wel mogelijk dat leiomyomen een omgeving creëren waarin wild-type gladde spiercellen verschillende genetische veranderingen kunnen opbouwen die uiteindelijk resulteren in de ontwikkeling van een leiomyosarcoom, dit mechanisme is bekend van *EXT*-gemuteerde osteochondromen. De helft van de leiomyomen van de uterus met een *MED12* mutatie toont nucleaire β-catenine expressie. Normaal komt β-catenine in het cytoplasma voor, maar als de afbraak wordt voorkomen kan het naar de celkern gaan en daar binden aan MED12 wat resulteert in stimulatie van de Wnt-signaalpathway. In deze serie tumoren is ook gekeken naar fumaraathydratase (FH) deficiëntie, wat 'mutually exclusive' is met *MED12* mutaties. Een leiomyoom van de uterus en een cutaan leiomyoom van dezelfde patiënt, met mogelijk het HLRCC (hereditary leiomyomatosis and renal cell carcinoma) syndroom, waren sterk positief voor S-(2-succinyl) cysteïne (2SC) immuunhistochemie. 2SC is een robuuste biomarker voor FH mutaties omdat de afwezigheid van functioneel FH leidt tot hoge levels van eiwitsuccinatie. Daarnaast werden nog twee leiomyosarcomen met hoge 2SC expressie gevonden, deze tumoren vertonen een toegenomen H3K9me3 methylering in vergelijking met FH-wild type tumoren, dit is te verklaren doordat mutaties in genen van de citroenzuurcyclus een effect hebben op histon demethylasen.

Zoals hierboven beschreven worden leiomyosarcomen gekenmerkt door hun complexe genetische profiel en chromosomale instabiliteit. Om de pathogenese van leiomyosarcomen beter te proberen te begrijpen en om mogelijke kandidaat genen te identificeren zijn in **hoofdstuk 3** tumorcellen van enkele tumoren gekaryotypeerd met COBRA-FISH. Twee tumoren hebben een translocatie tussen de chromosomen 6 en 14 (t(6;14)). Interessant is dat één van de tumoren rearrangements toonde van de genen *HMGA1* en *RAD51L1*, die ook bij een deel van de leiomyomen betrokken zijn. De exacte breekpunten van de translocaties zijn in kaart gebracht met FISH en array-CGH. Hierbij bleek dat de twee tumoren met de t(6;14) verschillende breekpunten in de chromosomen hadden. Dit illustreert weer het complexe en heterogene genetische profiel van leiomyosarcomen. In deze studie werd ook een tumor aangetroffen met een relatief simpel karyotype en in de literatuur is beschreven

dat een minderheid van de tumoren een vrij simpel karyotype heeft. Recent onderzoek naar een grote groep leiomyosarcomen laat zien dat de tumoren op basis van hun differentiatie in verschillende subgroepen onderverdeeld kunnen worden. Zo is er een groep waarbij relatief veel genen tot expressie komen die betrokken zijn bij spieraanleg en –differentiatie. Een hoge expressie van LMOD1 in deze groep is geassocieerd met een betere ziekte specifieke overleving. Aanvullend onderzoek is nodig om te onderzoeken of de genen met een veranderde expressie als target voor therapie gebruikt kunnen gaan worden.

Leiomyosarcomen worden behandeld met sommige varianten van conventionele chemotherapie, helaas zijn sommige tumoren echter resistent voor deze medicijnen. Daarom wordt in **hoofdstuk 4** gefocusseerd op de mechanismen die betrokken zijn bij chemoresistentie. Bcl2 speelt een belangrijke rol in dit proces in, omdat dit eiwit een lid is van een familie van anti-apoptotische eiwitten, net als Bcl-xL en Bcl-w. Bcl2 komt hoog tot expressie in leiomyosarcomen wat er op duidt dat in de tumorcellen het mechanisme van cellen om in apoptose te gaan geremd wordt, waardoor de tumorcel dus kan overleven. Behandeling van tumorcellen met een BH3-mimeticum (een remmer van eiwitten van de Bcl2 familie) resulteerde slechts in een kleine reductie van de overleving van tumorcellen *in vitro*. Daarentegen resulteerde de combinatiebehandeling van de tumorcellen met een BH3-mimeticum en het conventionele chemotherapeuticum doxorubicine tot synergie waarbij er apoptose van de tumorcellen optreedt. De inhibitie van Bcl2 door een BH3-mimeticum maakt de tumorcellen gevoelig voor de chemotherapie. Dit zijn veelbelovende resultaten waarbij de volgende stap zou zijn om de combinatie van deze twee geneesmiddelen in *in vivo* modellen te testen. In de toekomst kan deze combinatie misschien gebruikt worden bij de behandeling van patiënten met Bcl2 positieve tumoren.

Het myxoid liposarcoom is verantwoordelijk voor ongeveer 5% van alle wekedelensarcomen. De tumor gaat uit van het vetweefsel en komt het vaakst voor bij patiënten in het 4e of 5e decade. Deze tumor is in tweederde van de gevallen in de wekedelen van het bovenbeen gelokaliseerd. Histologisch zijn het tumoren met een myxoide matrix met korte spoelvormige cellen met beperkte cytonucleaire atypie en tussen de tumorcellen een delicaat patroon van kleine bloedvaatjes. Als de tumor voor meer dan 5% bestaat uit een component van monomorfe, kleine cellen met weinig cytoplasma en



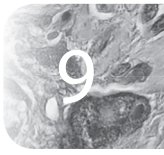
donker chromatinepatroon die dicht opeen gepakt liggen, de zogenaamde 'small blue round cells' dan wordt de tumor als hooggradig beschouwd. Het myxoid liposarcoom heeft een translocatie t(12;16)(q13;p11), wat betekent dat de lange arm van chromosoom 12 fuseert met de korte arm van chromosoom 16, resulterend in een fusieproduct van de genen *FUS* en *DDIT3*. Het exacte mechanisme waarbij het fusieproduct resulteert in ongeremde tumorgroei is niet compleet opgehelderd, waarschijnlijk werkt het fusieproduct als een aberrante transcriptiefactor en zet het andere genen aan tot proliferatie terwijl tegelijkertijd de lipogene differentiatie wordt geremd.

In **hoofdstuk 5** wordt de expressie van NY-ESO-1 onderzocht in verschillende benigne en maligne wekedelentumoren. NY-ESO-1 is een eiwit wat normaal alleen in de testis tot expressie wordt gebracht en behoort tot de groep van 'cancer testis antigenen'. Het immuunsysteem van de patiënt kan tegen een dergelijk eiwit een immuunreactie opwekken waardoor de cellen die dit eiwit tot expressie brengen worden vernietigd. In deze studie blijkt dat NY-ESO-1 in 88% van de myxoid liposarcomen tot expressie komt. Ook in sommige andere maligne tumoren, zoals synoviosarcomen en myxofibrosarcomen, komt dit eiwit in een deel van de gevallen voor. NY-ESO-1 expressie was niet aanwezig in de benigne mesenchymale tumoren. Aangezien het eiwit immunogeen is, is het mogelijk om een immuunrespons tegen de tumorcellen uit te lokken. Dit kan door patiënten een vaccin te geven tegen NY-ESO-1, waarbij het immuunsysteem van de patiënt wordt getraind om dit eiwit te herkennen en om de tumorcellen op te ruimen. Ook kan geselecteerd worden op T-cellen van de patiënt die gericht zijn tegen NY-ESO-1 of dendritische celtherapie waarbij monocyten uit het bloed van de patiënt worden gehaald en worden opgewerkt tot dendritische cellen die bijdragen aan een effectieve immuunrespons van het lichaam. Deze vormen van immuuntherapie worden in trial verband onderzocht. Een nieuwe therapie tegen myxoid liposarcomen is van groot belang omdat ondanks dat patiënten in eerste instantie vaak goed reageren op conventionele chemo- en/of radiotherapie, patiënten in een later stadium moeilijk te behandelen zijn mede omdat deze tumor zich diffuus kan uitbreiden naar andere wekedelen verspreid door het hele lichaam.

Om meer onderzoek naar myxoid liposarcomen mogelijk te maken is het van belang om voldoende tumormodellen te hebben. Myxoid liposarcomen zijn bij uitstek tumoren die moeilijk te kweken zijn in tweedimensionale kweekflesjes met medium. Daarom zijn er wereldwijd slechts twee cellijn

modellen beschikbaar. In **hoofdstuk 6** wordt de ontwikkeling van een nieuwe myxoid liposarcoomcellijn (DL-221) beschreven. De tumorcellen zijn geïsoleerd van de tumor van een patiënt en daarna in kweekmedium opgegroeid. Op veel momenten in het traject zijn de tumorcellen getest voor de aanwezigheid van het FUS-DDIT3 fusieproduct om te controleren of het daadwerkelijk tumorcellen zijn en geen andere celtypen zoals bindweefselcellen. De nieuwe cellijn is spontaan geïmmortaliseerd waarmee deze zich onderscheid van de andere twee cellijnen (402-91 en 1765-92) die door middel van SV40 virus transformatie onsterfelijk zijn gemaakt. De DL-221 is uitgebreid gekarakteriseerd waarbij gekeken is naar de mutatie status van *PIK3CA*, *TERT* en *TP53* en de eiwitexpressie van NY-ESO-1. Ook is van zowel de cellijn als van het ontwikkelde xenograft model Next Generation Sequencing (NGS) verricht om de biologie en mogelijke kandidaat targets voor de behandeling van myxoid liposarcomen te bestuderen.

In **hoofdstuk 7** is een uitgebreide 'drug screen' gedaan, waarbij in alle drie de beschikbare myxoid liposarcoomcellijnen is onderzocht hoe gevoelig de tumorcellen zijn voor de behandeling met verschillende medicijnen. Er zijn in totaal 273 medicijnen getest, waarbij zowel reeds geregistreerde medicijnen alsook nieuwe stoffen en specifieke (tyrosine kinase) inhibitors zijn geïncludeerd. Hieruit bleek dat de tumorcellen, zoals verwacht, gevoelig zijn voor behandeling met conventionele chemotherapeutica zoals anthracyclines en taxanen. Daarnaast werd survivin (BIRC5) geïdentificeerd als nieuwe kandidaat target omdat de tumorcellen van twee cellijnen al bij lage concentratie een sterke afname van de viabiliteit liet zien. Intracellulair heeft survivin twee belangrijke functies, in de kern is het betrokken bij de regulering van de celcyclus en in het cytoplasma remt het de apoptose. Een hoge nucleaire survivin expressie werd gevonden in myxoid liposarcomen wat er op duidt dat dit eiwit een rol heeft in de regulatie van de celcyclus in myxoid liposarcomen. Verder onderzoek naar het effect van survivin inhibitie *in vivo* is nodig en dit kan in de toekomst mogelijk leiden tot een nieuwe behandelmogelijkheid voor myxoid liposarcomen.



Chapter 10

Curriculum vitae

Curriculum vitae

Marieke Anna de Graaff was born on February 12th, 1986 in Haarlem, the Netherlands. She graduated from the Athenaeum College Hageveld in Heemstede in 2004. As she wanted to gain a thorough understanding of human biology, physiology and pathophysiology, she started her studies Biomedical Sciences at the Leiden University Medical Center in Leiden. After she successfully finished the first year she studied Medicine from 2005 to 2011 at the University of Leiden and also obtained during this period the bachelor degree of Biomedical Sciences. During the study she was invited (based on study results) to follow master classes at the Department of Pathology of the Leiden University Medical Center. In 2011, she started a research project at the Molecular Cell Biology and Pathology departments to study soft tissue sarcomas. After obtaining her medical degree in 2011, she started as a PhD student in the Bone and Soft Tissue Tumour Group at the department of Pathology in the LUMC to study the pathology and molecular genetics of various soft tissue tumours (J.V.M.G. Bovée). During her research period she received the "René Vogels" travel grant for young cancer investigators to visit the MD Anderson Cancer Center in Houston, Texas. In June 2014 she started her clinical training as a resident in Pathology at the Leiden University Medical Center.

List of publications

Mediator complex subunit 12 exon 2 mutation analysis in different subtypes of smooth muscle tumors confirms genetic heterogeneity. de Graaff MA, Cleton-Jansen AM, Szuhai K, Bovée JVMG. *Human Pathology. 2013; 44(8): 1597-1604.*

A translocation t(6;14) in two cases of leiomyosarcoma: molecular cytogenetic and array-based comparative genomic hybridization characterization. de Graaff MA, de Jong D, Briaire-de Bruijn I, Hogendoorn PCW, Bovée JVMG, Szuhai K. *Cancer Genetics. 2015; 208(11): 537-544.*

NY-ESO-1 (CTAG1B) expression in mesenchymal tumors. Endo M*, de Graaff MA*, Ingram DR, Lim S, Lev DC, Briaire-de Bruijn I, Somaiah N, Bovée JVMG, Lazar AJ, Nielsen TO. *Modern Pathology. 2015; 28(4): 587-595. *Shared first authorship*

Inactivation of SDH and FH cause loss of 5hmC and increased H3K9me3 in paraganglioma/pheochromocytoma and smoothmuscle tumors. Hoekstra AS, de Graaff MA, Briaire-de Bruijn I, Ras C, Seifar RM, van Minderhout I, Cornelisse CJ, Hogendoorn PCW, Breuning MH, Suijker J, Korpershoek E, Kunst HP, Frizzell N, Devilee P, Bayley JP, Bovée JVMG. *Oncotarget. 2015; 6(36): 38777-38788.*

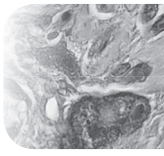
Inhibition of Bcl-2 family members sensitizes soft tissue leiomyosarcomas to chemotherapy. de Graaff MA*, de Rooij MAJ*, van den Akker BEWM, Gelderblom H, Chibon F, Coindre JM, Marino-Enriquez A, Fletcher JA, Cleton-Jansen AM, Bovée JVMG. *British Journal of Cancer. 2016; 114(11): 1219-1226. *Shared first authorship*

Establishment and characterization of a new human myxoid liposarcoma cell line (DL-221) with the FUS-DDIT3 translocation. de Graaff MA, Yu JS, Beird HC, Ingram DR, Nguyen T, Juehui Liu J, Bolshakov S, Szuhai K, Åman P, Torres KE, Lev D, Nielsen TO, Bovée JVMG, Lazar AJ, Somaiah N. *Laboratory Investigation. 2016; 96(8): 885-894.*

High-grade sarcoma diagnosis and prognosis: Biomarker discovery by mass spectrometry imaging. Lou S, Balluff B, de Graaff MA, Cleven AH, Briaire-de Bruijn I, Bovée JVMG, McDonnell LA. *Proteomics. 2016; 16(11-12): 1802-1813.*

High nuclear expression of proteasome activator complex subunit 1 predicts poor survival in soft tissue leiomyosarcomas. Lou S, Cleven AH, Balluff B, de Graaff MA, Kostine M, Briaire-de Bruijn I, McDonnell LA, Bovée JVMG. *Clinical Sarcoma Research. 2016; 6: eCollection 2016.*

High-throughput screening of myxoid liposarcoma cell lines reveals survivin as a potential novel druggable target. de Graaff MA, Malu S, Guardiola I, Kruisselbrink AB, de Jong Y, Corver WE, Gelderblom AJ, Hwu P, Nielsen TO, Lazar AJ, Somaiah N, Bovée JVMG. *Submitted*



Dankwoord

Veel mensen hebben bijgedragen aan de totstandkoming van dit proefschrift. Graag wil ik van de gelegenheid gebruik maken om hen te bedanken.

Prof. dr. J.V.M.G. Bovée, promotor. Beste Judith, in de eerste plaats dank voor je geweldige begeleiding, je kritische en stimulerende feedback en onuitputtelijke gedrevenheid om het maximale resultaat uit het onderzoek te halen. Jouw passie voor zowel de wetenschap als de diagnostiek zijn inspirerend. Je bent voor mij een lichtend voorbeeld.

Dr. K. Szuhai, copromotor. Beste Károly, jouw onuitputtelijke theoretische en praktische kennis zijn van onschatbare waarde en ik ben je erg dankbaar dat ik zoveel van je heb mogen leren op zowel het vakinhoudelijke als het persoonlijke vlak. Dank voor de fijne begeleiding en samenwerking en al het vertrouwen dat je mij hebt gegeven. Je hebt een belangrijke rol gespeeld in mijn ontwikkeling als wetenschapper en arts.

De samenwerking met de onderzoeksgroepen van Prof. dr. Alexander Lazar en Dr. Neeta Somaiah (MD Anderson Cancer Center) en Prof. dr. Torsten Nielsen, Jamie Lim en Makoto Endo (University of British Columbia) heb ik erg gewaardeerd en dit heeft geleid tot mooie resultaten en een aantal publicaties. Ik ben Alexander en Neeta erg dankbaar voor het warme welkom en de fantastische tijd in Houston.

De bot- en wekedelengroep wil ik bedanken voor de goede samenwerking van de afgelopen jaren. In het bijzonder dank aan Alwine, Inge en Pauline, jullie inzet en technische expertise hebben ervoor gezorgd dat het onderzoek goed afgerond kon worden. Ook wil ik mijn collega AIOS van de pathologie bedanken die altijd geïnteresseerd waren in het onderzoek en er begrip voor hadden als er een deadline van een manuscript naderde. Daarnaast ben ik de andere collega's van de afdeling pathologie dankbaar, die vaak geïnteresseerd waren naar de gang van zaken in het onderzoek, dit heb ik altijd zeer op prijs gesteld.

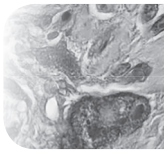
Dr. Arjen Cleven en Prof. dr. Vincent Smit, hartelijk dank voor de goede gesprekken en het vertrouwen dat jullie mij hebben geven. Jullie toonden er alle begrip voor dat de combinatie van onderzoek en opleiding soms lastig was en waren altijd bereid om mee te denken.

Daniëlle de Jong en Willem Sloos, wat een genoegen om jullie als collega's te mogen hebben. In de eerste plaats dank voor respectievelijk jullie ondersteuning bij experimenten en hulp bij computer gerelateerde problemen. Meer nog wil ik jullie danken voor de positieve energie, alle gezelligheid en jullie goede dosis relativeringsvermogen.

Graag wil ik ook mijn vrienden bedanken die altijd geïnteresseerd waren in het onderzoek en mij veel gezellige momenten en de soms nodige afleiding bezorgden. In het bijzonder hartelijk dank aan Marco en Jan voor de diepgaande gesprekken.

Lieve Korneel, jij wist dat ik dit goed zou afronden en dat vertelde je dan ook trots aan iedereen die je tegenkwam. Dank voor je steun en stimulans, dat heeft mij erg geholpen en ik kijk uit naar onze volgende plannen samen.

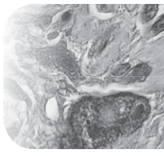
Mijn ouders, Brigitte en Floris-Jan, ik wil jullie graag bedanken voor jullie onvoorwaardelijke steun. Jullie staan altijd voor mij klaar en gaven mij vertrouwen en goede raad op momenten dat ik dat nodig had. Jullie vertrouwen, stimulans en nimmer aflatende interesse zijn van onbeschrijfelijke waarde geweest bij het voltooien van dit proefschrift.



List of abbreviations

2SC	S-(2-succino)-cysteine
ABC	Aneurysmal bone cyst
a-CGH	array-Comparative Genomic Hybridization
ACTN1	Alpha-actinin-1
ADCK1	AarF Domain Containing Kinase 1
AKT	AKT Serine/Threonine Kinase
ARL4C	ADP-Ribosylation Factor-Like 4C
ATRX	Alpha thalassemia/mental retardation syndrome X-linked
AXL	AXL receptor tyrosine kinase
BAC	Bacterial artificial chromosome
BCL	B-Cell CLL/Lymphoma 2
BH3	Bcl-2 homology (BH) domain 3
BIRC5	Baculoviral IAP Repeat Containing 5
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
CCR	Complex chromosomal rearrangement
CDH11	Cadherin 11, type 2, OB-cadherin
CDK4	Cyclin-dependent kinase 4
C/EBP	CCAAT-enhancer-binding protein
CHOP	C/EBP homologous protein
COBRA FISH	Combined binary ratio labeling fluorescence *in situ* hybridization
COL1A1	Collagen, type I, alpha 1
COL4A5	Collagen, type IV, alpha 5
COL4A6	Collagen, type IV, alpha 6
CT	Computed tomography
CTA	Cancer/testis antigen
CTAGB1	Cancer testis antigen 1B (NY-ESO-1)
CTNNB1	Beta-catenin
DAPK	Death associated protein kinase
DDIT3	DNA-damage-inducible transcript 3
DPF3	Double PHD Fingers 3
DSB	Double strand breaks
DSS	Disease specific survival
ER	Estrogen receptor

EXT	Exostosin glycosyltransferase
EWSR1	Ewing sarcoma RNA-binding protein 1
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
FH	Fumarate hydratase
FIGO	Federation of Gynaecology and Obstetrics
FISH	Fluorescence *in situ* hybridization
FNCLCC	Fédération Nationale des Centres de Lutte Contre le Cancer
FUS	Fused in sarcoma
GIST	Gastrointestinal stromal tumour
HDAC	Histone deacetylase
H&E	Haematoxylin and eosin
HHF35	Actin
HLRCC	Hereditary leiomyomatosis and renal cell cancer
HMGA1/2	High-mobility group AT-hook 1/2
HRR	Homologous recombination repair
IC_{50}	Half maximal inhibitory concentration
IGF1R	Insulin-like growth factor 1 receptor
KIT	proto-oncogene c-Kit
LM	Leiomyoma
LMOD1	Leiomodin 1
LMS	Leiomyosarcoma
MDM2	Mouse double minute 2 homolog/E3 ubiquitin-protein ligase Mdm2
MED12	Mediator complex subunit 12
MFS	Myxofibrosarcoma
MLS	Myxoid liposarcoma
MPNST	Malignant peripheral nerve sheath tumor
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
MYOCD	Myocardin
NFKBIZ	NF-kappa-B inhibitor zeta
NGS	Next Generation Sequencing
NK-cell	Natural killer cell
NY-ESO-1	New York esophageal squamous cell carcinoma 1 (a.k.a. CTAG1B)



OMD	Osteomodulin
OS	Overall survival
PCR	Polymerase chain reaction
PDGFRA/B	Platelet Derived Growth Factor Receptor A/B
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha
PPARγ	Peroxisome proliferator activated receptor gamma
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
q-PCR	quantitative Polymerase Chain Reaction
RAD51L1	RAD51 Paralog B
RB1	Retinoblastoma 1
REST	Repressor element 1 silencing transcription factor
RET	Rearranged during transfection proto-oncogene
RMS	Rhabdomyosarcoma
ROR2	Receptor tyrosine kinase-like orphan receptor 2
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMA	Smooth muscle actin
SNP	Single nucleotide polymorphism
SSX	Synovial sarcoma X
STR-typing	Short tandem repeat typing
STS	Soft tissue sarcoma
SUPT3H	Transcription initiation protein SPT3 homolog
SV40	Simian vacuolating virus 40
TAPBP	Transporter associated with antigen processing (TAP) binding protein
TC-NER	Transcription-coupled nucleotide-excision repair
TERT	Telomerase reverse transcriptase
TLS	Translocated in Sarcoma
TMA	Tissue microarray
TRAP150	Thyroid hormone receptor-associated protein complex 150 KDa component
UPS	Undifferentiated pleomorphic sarcoma
USP6	Ubiquitin specific peptidase 6 (Tre-2 oncogene)

WB	Western Blot
WD/DDLPS	Well-differentiated/dedifferentiated liposarcoma
WHO	World Health Organisation
ZNF9	Zinc finger protein 9
WNT4	Wingless-type MMTV integration site family, member 4
WT1	Wilms tumour protein