Genetic Profiling in Soft Tissue Sarcoma

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Genetic Profiling in Soft Tissue Sarcoma

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Abstract

Soft tissue sarcomas (STS) are a heterogeneous group of highly malignant mesenchymal tumors that account for ~1% of all malignancies. Frequent heterogeneity and pleomorphism along with suboptimal diagnostic reproducibility and insufficient prognostic markers make clinical management of these tumors difficult. This thesis has applied microarray-based gene expression and copy-number profiling to STS. The studies provide clues to the genetic pathways involved in STS development and identify profiles linked to diagnosis and prognosis. The results from Study I that concerns intratumor versus intertumor heterogeneity of gene expression profiles in malignant fibrous histiocytoma (MFH) and leiomyosarcoma (LMS), suggest that intratumor heterogeneity may be particularly relevant in small tumor series and thus serve as a reminder to run larger sample sets for increased reliability. Study II established expression patterns related to the SS18-SSX fusion variants and metastatic potential in synovial sarcoma (SS). The differential expression of various developmental genes, transcription factors, histones, and metallothioneins suggests that the gene fusion variants have distinct downstream effects. In Study III, 177 STS of mixed histopathological subtypes were profiled using cDNA microarrays. Distinct gene expression patterns were identified in subtypes with specific translocations or mutations. Herein, frequent upregulation of developmental genes, from e.g. the Wingless and Hedgehog signaling pathways, was demonstrated. The more pleomorphic STS showed overexpression of genes involved in proliferation, adhesion, motility and protein degradation. Moreover, a prognostic signature partly characterized by hypoxia-related genes was identified within the pleomorphic STS. Study IV applied array-based comparative genomic hybridization in MFH and LMS, and demonstrated extensive genetic complexity with multiple recurrent gains and losses, novel amplifications and homozygous deletions. Losses in chromosomal regions 6q14 and 7q36 provided prognostic information independent of previously established risk factors. In summary, these studies demonstrate the potential of genetic profiling in STS and herein, define intratumor heterogeneity, demonstrate that gene fusion variants in SS yield different downstream effects, identify diagnostic and prognostic subsets within STS, and in the pleomorphic tumors, discern prognostically important alterations within the plethora of genetic aberrations that characterize many STS.

Key words: Soft tissue sarcoma, cDNA, microarray, gene expression analysis, array comparative genomic hybridization, aCGH, intratumor heterogeneity, synovial sarcoma, SS18-SSX, prognosis.

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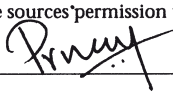
Signature  Date April 16, 2007

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Genetic Profiling in Soft Tissue Sarcoma

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Abstract

Soft tissue sarcoma (STS) is a heterogeneous group of highly malignant mesenchymal tumors that account for ~1% of all malignancies. Frequent heterogeneity and pleomorphism along with suboptimal diagnostic reproducibility and insufficient prognostic markers make clinical management of these tumors rather difficult. STS comprise more than 30 histological subtypes, with malignant fibrous histiocytoma (MFH), leiomyosarcoma (LMS), liposarcoma, and synovial sarcoma (SS) being the most common. Some subtypes are characterized by specific genetic alterations, e.g. *SS18-SSX* in SS and activating *KIT* gene mutations in gastrointestinal stromal tumors (GIST), but a large fraction of STS including MFH and LMS lack specific genetic changes and are characterized by numerous alterations such as mutations in *TP53*, deletions of *RB1* and *CDKN2A*, and amplifications of *MDM2* and *CDK4*.

This thesis has applied microarray-based gene expression and copy-number profiling to STS. The studies provide clues to the genetic pathways involved in STS development and identify profiles linked to diagnosis and prognosis. *Study I* concerns intratumor *versus* intertumor heterogeneity of gene expression profiles in MFH/myxofibrosarcoma and LMS. Multiple samples from the same tumors formed distinct clusters and average intratumor heterogeneity was lower than average intertumor heterogeneity, although the maximum intratumor heterogeneity was greater than the minimum intertumor heterogeneity. These results suggest that intratumor heterogeneity may be particularly relevant in small tumor series and thus serve as a reminder to run larger sample sets for increased reliability. *Study II* established expression patterns related to the *SS18-SSX* fusion variants and metastatic potential in SS. The differential expression of various developmental genes, transcription factors, histones, and metallothioneins suggests that the gene fusion variants have distinct downstream effects. In *Study III*, 177 STS of mixed histopathological subtypes were profiled using cDNA microarrays. Distinct gene expression profiles were identified in subtypes with specific translocations or mutations, e.g. GIST, SS, myxoid/round-cell liposarcomas, and malignant peripheral nerve sheath tumors. Herein, frequent upregulation of developmental genes from e.g. the Wingless (Wnt), Hedgehog and retinoic acid receptor signaling pathways was demonstrated. The more pleomorphic STS showed overexpression of genes involved in proliferation, adhesion, motility and protein degradation. Moreover, a prognostic signature partly characterized by hypoxia-related genes was identified within the pleomorphic STS. *Study IV* applied array-based comparative genomic hybridization in MFH and LMS, and demonstrated extensive genetic complexity with multiple recurrent gains and losses, novel amplifications and homozygous deletions. Losses in chromosomal regions 6q14 and 7q36 provided prognostic information independent of previously established risk factors.

In summary, these studies demonstrate the potential of genetic profiling in STS and herein define intratumor heterogeneity, demonstrate that gene fusion variants in SS yield different downstream effects, identify diagnostic and prognostic subsets within STS, and in the pleomorphic tumors, discern prognostically important alterations within the plethora of genetic aberrations that characterize many STS.

Abbreviations

aCGH	Array-based comparative genomic hybridization
AKT	Protein kinase B or PKB
BAC	Bacterial artificial chromosome
BCL2	B-cell CLL/lymphoma 2
BIRC5	Baculoviral IAP repeat-containing 5
BMP	Bone morphogenetic protein
CA9	Carbonic anhydrase IX
CCND1	Cyclin D1
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CT	Computed tomography
DFSP	Dermatofibrosarcoma protuberans
EASE	Expression Analysis Systematic Explorer
EGFR	Epidermal growth factor receptor,
FDR	False-discovery rate
FISH	Fluorescence in situ hybridization
GIST	Gastrointestinal stromal tumor
GLUT1	Glucose transporter 1
GO	Gene Ontology
HDAC	Histone deacetylase
HIF1A	Hypoxia inducible factor 1 alpha
HSP	Heat shock protein
HR	Hazard ratio
IGF1	Insulin-like growth factor 1
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LMS	Leiomyosarcoma
LOH	Loss of heterozygosity
LOWESS	Locally weighted scatterplot smoothing
MAPK	Mitogen activated kinase
MDM2	Mouse double minute 2 homolog
MDS	Multidimensional scaling
MFH	Malignant fibrous histiocytoma
MMP	Matrix metalloproteinases
MPNST	Malignant peripheral nerve sheath tumor
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
PCR	Polymerase chain reaction
PDGFB	Platelet derived growth factor beta
PDGFRA	Platelet-derived growth factor receptor alpha
PET	Positron emission tomography
PI3K	Phosphatidylinositol-3-kinase
PTCH	Patched
RB1	Retinoblastoma
SAM	Significance analysis of microarrays
SIN	Size-Necrosis-Invasion staging system
SMARC	SWI/SNF related matrix associated actin dependent regulator of chromatin
SMO	Smoothened
SNP	Single nucleotide polymorphism
SNR	Signal-to-noise ratio
SS	Synovial sarcoma
SS18	Synovial sarcoma translocation, chromosome 18
SSX1/2/4	Synovial sarcoma, X breakpoint 1/2/4
STS	Soft tissue sarcoma
SUFU	Suppressor of fused
TGFβ	Transforming growth factor beta
TGFBR2	TGFβ receptor type-2
TMA	Tissue microarray
TOP2A	DNA topoisomerase II alpha
TP53	Tumor protein p53
VEGF	Vascular endothelial growth factor
UPS	Undifferentiated pleomorphic sarcoma
Wnt	Wingless

Background

Epidemiology and etiology

Soft tissue sarcoma (STS) is a heterogeneous group of highly malignant mesenchymal tumors with over 30 different histological subtypes many of which are diagnostically, prognostically, and therapeutically challenging [81]. STS occur in the extraskeletal soft tissues of the body like fat, muscles, nerves, tendons, blood vessels and lymphatic vessels, and account for ~1% of all malignancies and ~7% of all childhood tumors [197]. There is a slight male predominance and age of onset varies within the different subtypes; some STS types, e.g. synovial sarcoma (SS) and fibrosarcoma, occur more often in adolescents and young adults, while others, e.g. malignant fibrous histiocytoma (MFH)/undifferentiated pleomorphic sarcoma (UPS) and leiomyosarcoma (LMS) are more frequent in elderly patients [104]. Differences in incidence rates have also been suggested in relation to ethnicity [159]. STS can occur at any anatomic site, but about two-thirds of the tumors arise in an extremity and the remaining in the trunkwall (~10%), head and neck (~9%), retroperitoneum (~15%) and in other locations. STS are thought to arise from mutations in pluripotent mesenchymal stem cells and many subtypes exhibit features resembling various normal connective tissue types [81]. Some subtypes, e.g. clear cell sarcoma, epithelioid sarcoma, and UPS, however, lack resemblance to other tissues.

The vast majority of STS are sporadic with unknown etiology, but like most malignancies STS are multifactorial with genetic as well as environmental determinants playing a causative role. Several genetic factors including cytogenetic aberrations and mutations in oncogenes and tumor suppressors have been associated with STS and are crucial in the genesis and progression of these tumors [103]. Several inherited cancer syndromes such as retinoblastoma, Li-Fraumeni, neurofibromatosis type 1 (von Recklinghausen's disease) and type 2, Gardner syndrome, Gorlin syndrome (naevoid basal cell carcinoma syndrome), Werner syndrome, Beckwith-Wiedemann syndrome, Carney triad have an STS component. The STS linked to hereditary syndromes account for only a very small minority of the tumors, but may provide clues to STS pathogenesis [273]. Many STS, if not all, have alterations in the retinoblastoma (RB1) and/or p53 pathways, which explains why patients with hereditary retinoblastoma (with germline mutations in the *RB1* gene) and Li-Fraumeni syndrome (with germline *TP53* mutations) are predisposed to developing sarcomas [69, 74, 153, 154, 162, 174, 203]. The Patched/Hedgehog/Smoothened signaling pathway has been implicated in the development of Gorlin syndrome and associated tumors such as rhabdomyosarcoma are known to have alterations of the same pathway [217, 250]. More recently, a gastrointestinal stromal tumor (GIST) syndrome has also been described in a family with germline *KIT* mutations [181]. 50% of malignant peripheral nerve sheath tumors (MPNST) occur in neurofibromatosis type 1 patients with germline deletions of the *NF1* gene [137]. The most common environmental risk factor for STS development is ionizing radiation, with sarcomas occurring in a dose-dependent manner after mean 10 years in patients treated with radiotherapy [164]. Other risk factors include lymphedema, exposure to chemicals such as vinyl chloride, phenoxyherbicides, arsenical pesticides, chlorophenols, dioxins, Thorotrast, alkylating agents and androgen-anabolic steroids, viral infections by Epstein-Barr virus, human immunodeficiency virus type 1 and human herpes virus type 8 (or Kaposi's sarcoma-associated herpes virus) and immunosuppression [73, 78, 273].

Clinical features and diagnostics

STS most often present as painless gradually

expanding masses that are often large with a mean size of 9 cm for deep-seated tumors and 5 cm for superficial tumors of the extremities (retroperitoneal sarcomas are often larger). STS grow in a spherical fashion compressing surrounding structures and infiltrating the tumor pseudocapsule and, rarely, adjacent structures. Imaging technologies applied in STS diagnostics include X-rays, computed tomography (CT), magnetic resonance imaging (MRI) and in some cases positron emission tomography (PET) [16, 57, 97]. Imaging is crucial for defining the local extent of the tumor, may aid in diagnostics, guide biopsies, and detect tumor spread, most commonly as lung metastases. CT is usually performed to evaluate retroperitoneal sarcomas while MRI with its multiplanar imaging and better anatomical definition is the preferred technique for extremity STS [57]. Biopsy techniques such as fine-needle aspiration, core-needle, incisional or excisional biopsies are almost always required for histological examination before initiating treatment. Fine-needle and core-needle biopsies are safe, accurate and effective with diagnostic accuracies of 95–99% depending on the skill of the cytopathologist [105, 107]. Incisional and excisional biopsies are performed less commonly in cases where fine-needle and core-needle biopsies fail to provide reliable results, and when definitive preoperative histopathological diagnosis is required in order to initiate neoadjuvant therapies [163].

Histopathology

The histopathological classification of STS is based mostly on similarity to particular differentiation lineages, e.g. fat, smooth muscle, skeletal muscle etc. but for many subtypes, including most of the pleomorphic subtypes and SS, the tissue of origin is still unknown. Some 30 different subtypes exist and the lineages are determined using both haematoxylin and eosin (H&E) and immunohistochemical staining with lineage-specific antibodies against e.g. muscle-specific actin, smooth muscle actin, desmin, S-100, epithelial membrane antigen, cytokeratins, CD45, CD30, CD20 and melanoma antibody HMB45 [Figure 1]. The most common subtypes in adults include MFH (28%), LMS (12%), liposarcoma (15%), SS (10%), and MPNST (6%), whereas rhabdomyosarcoma is the most common STS of childhood [81]. The histopathological classification sometimes suffers from poor reproducibility, which has, however, greatly improved with the aid of ancillary diagnostic tools such as electron microscopy, cytogenetics and molecular genetic techniques, including fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR)-based detection of fusion genes or specific mutations [8]. About 1/3rd of all STS harbor tumor-specific genetic aberrations like the recurrent reciprocal translocations in SS, myxoid/round-cell liposarcoma and clear cell sarcoma that result in novel fusion products and *KIT* gene point mutations in GIST that have successfully been used to refine diagnosis in these subtypes [6, 17, 172]. Due to complex nonspecific genetic alterations, such analyses add little to the diagnosis of the more pleomorphic subtypes, e.g. MFH/UPS, LMS and pleomorphic liposarcoma. Herein, any recognized line of differentiation will result in a specific diagnosis, whereas lack of the same will classify the tumor as MFH/UPS. The ancillary techniques have demonstrated similar chromosomal rearrangements in previously distinct subtypes, e.g. myxoid and round-cell liposarcomas, as well as different genetic changes or separate lines of differentiation within what used to be a single histopathological entity [81]. MFH was introduced as a distinct STS entity in the 1960's and used to be a predominant histopathological subtype in the 1980's, but is now regarded as a diagnosis of exclusion synonymous with UPS, and some authors claim it represents merely 5% of STS [79, 80, 81]. But even with the use of all the above mentioned techniques, STS diagnostics is challenging and there is a need for novel markers in order to improve the diagnostic accuracy. Herein, newer technologies such as genetic and proteomic profiling may play a role and are increasingly being applied in studies aiming at identification of novel diagnostic and prognostic markers [178].

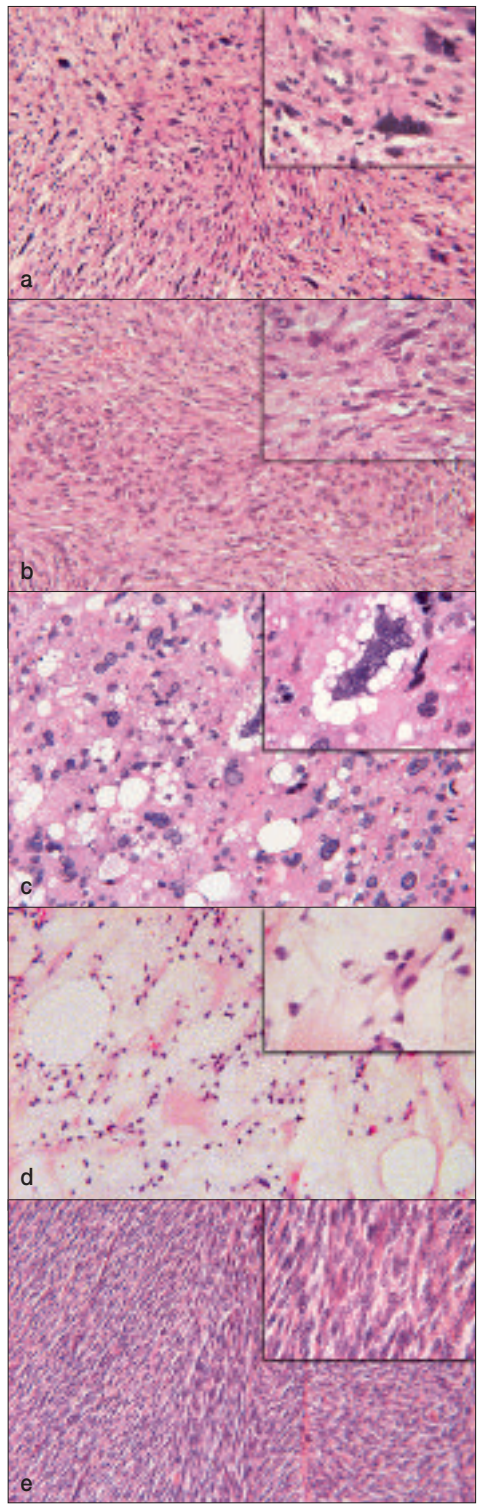

Figure 1. a. A MFH displaying variable cellularity and cytological pleomorphism.
b. A LMS with typical intersecting groups of spindle cells.
c. A pleomorphic liposarcoma displaying pleomorphic spindle cells, giant cells and lipoblasts.
d. A myxoid liposarcoma with signet-ring lipoblasts with multivacuolated cytoplasm.
e. A monophasic synovial sarcoma displaying the spindle cell component with fascicles and sheets of uniform neoplastic cells.

Prognostic factors

Staging is essential for prognostication and selection of appropriate therapies. The most commonly used of the current staging systems is the one devised by the American Joint Committee on Cancer (AJCC) and the International Union against Cancer (UICC), which is based on grade, size, depth and the presence of distant or nodal metastasis [215, 265]. All tumors with distant metastasis are classified as stage IV. The system used by the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) also takes tumor differentiation, which is linked to the histotype, into account [42, 45]. Within the Size-Necrosis-Invasion (SIN) prognostic system applied in Scandinavia, tumors with 2 of the 3 factors, size >8 cm, presence of tumor necrosis, and intratumor vascular invasion, are classified as high-risk tumors [95]. The microscopically determined invasive peripheral tumor growth pattern, which has recently been demonstrated to represent a strong adverse prognostic marker, will now be added to these high-risk factors [70]. 5-year survival rates for STS by stage are approximately 90, 70, 50 and 10–20% for stages I, II, III and IV, respectively [96, 242].

Histological grade, which is one of the most significant prognostic factors, is based on degree of cellularity, differentiation, pleomorphism, necrosis and the number of mitoses. Certain histotypes have fixed grades, e.g. well-differentiated liposarcomas are assigned grade I while rhabdomyosarcomas are assigned grade III, in 3-tiered systems. In the 4-tiered grading system used in Scandinavia, grades I and II are considered low while III and IV are high [211]. Metastatic rate for high grade tumors is

50–60% compared to <10% for low-grade tumors [45, 96]. Tumor size, often dichotomized at 5 cm, is another important prognostic variable in STS. Additional factors of potential prognostic value but not considered in the current staging systems include site of primary tumor, further stratification of size beyond 5 cm, the margin status of the resected tumor, and local recurrence [138].

A number of molecular markers have also been linked to prognosis in STS. Among these, proliferation has repeatedly been demonstrated to be associated with an adverse prognosis. Different methods for its determination – immunostaining for Ki-67, PCNA or cyclins, mitotic counts, and determination of S-phase fraction – have been applied but there is a lack of standardized recommendations for their use as well as a lack of consensus on which method to use and what cut-off levels to apply. Multiple other markers have also been linked to prognosis, including p53, RB1, VEGF, PDGFRA, IGFR1, KIT, CTNNB1, CD44, MDM2, p16INK4A, p19ARF, BCL2, VIL2 (ezrin) and WT1 [138, 195], although their clinical applicability has not been convincingly demonstrated since many of the studies were performed in small subsets not taking other markers and/or the currently applied clinicopathological factors into account.

Treatment

STS have a high rate of distant metastasis, mostly to the lungs through the hematogenous route. Metastasis at diagnosis is found in 10% of the patients, but an additional 30% of the patients will develop metastases within 5 years, of which 80% will develop within the first 2 years of follow-up. Local recurrence appears in 10–20% even after optimal treatment and overall 5-year survival is 50–60% [96, 242]. Treatment for STS should optimally be carried out in multidisciplinary settings at specialized centers. Surgery is central and several factors such as tumor location, size, depth of invasion, involvement of nearby structures etc. determine the type of surgery. The choice of surgical method, i.e. amputation or resection, and type of surgical margin, i.e. intralesional, marginal or wide, constitutes a balance between preserving function and reducing the risk of local tumor recurrence. For localized STS the main treatment strategy is wide surgical excision. Surgery is often combined with radiotherapy most commonly in the form of external beam therapy to reduce the risk of local tumor recurrence [200, 204, 233, 267]. Doxorubicin and ifosfamide are the only two active cytotoxic agents used in STS, but are associated with limited response rates. There is also a lack of data supporting the use of neoadjuvant chemotherapy. However most centers offer adjuvant chemotherapy based on doxorubicin, sometimes combined with ifosfamide in high-risk patients. Several new cytotoxic compounds are being tested in STS and include minor groove binders, topoisomerase I inhibitors (e.g. topotecan and irinotecan), agents that restore drug sensitivity, and cyclin-dependent kinase inhibitors [170].

Targeted therapies

The lack of highly effective treatments in STS underscores the need for novel therapeutic strategies. Emerging new technologies and increased understanding of the molecular mechanisms underlying STS pathogenesis provide important clues for the development of molecularly targeted therapies. Several such strategies, alone or in combination with chemotherapy, are currently being evaluated in clinical trials [128, 170, 173, 253]. A prime example is provided by the GIST where identification of the pathogenic activating mutations in the *KIT* receptor tyrosine kinase not only resulted in a novel diagnostic subgroup but also lead to use of an effective new targeted therapy using the tyrosine kinase inhibitor STI-571 also known as imatinib mesylate (Gleevec®) [58, 106, 209]. Some GIST subtypes that lack *KIT* gene mutations respond to imatinib due to inhibition of the platelet-derived growth factor receptor alpha (PDGFRA) which is also found mutated in GIST [101, 102]. Also dermatofibrosarcoma protuberans (DFSP) may respond to imatinib since they are driven by a translocation

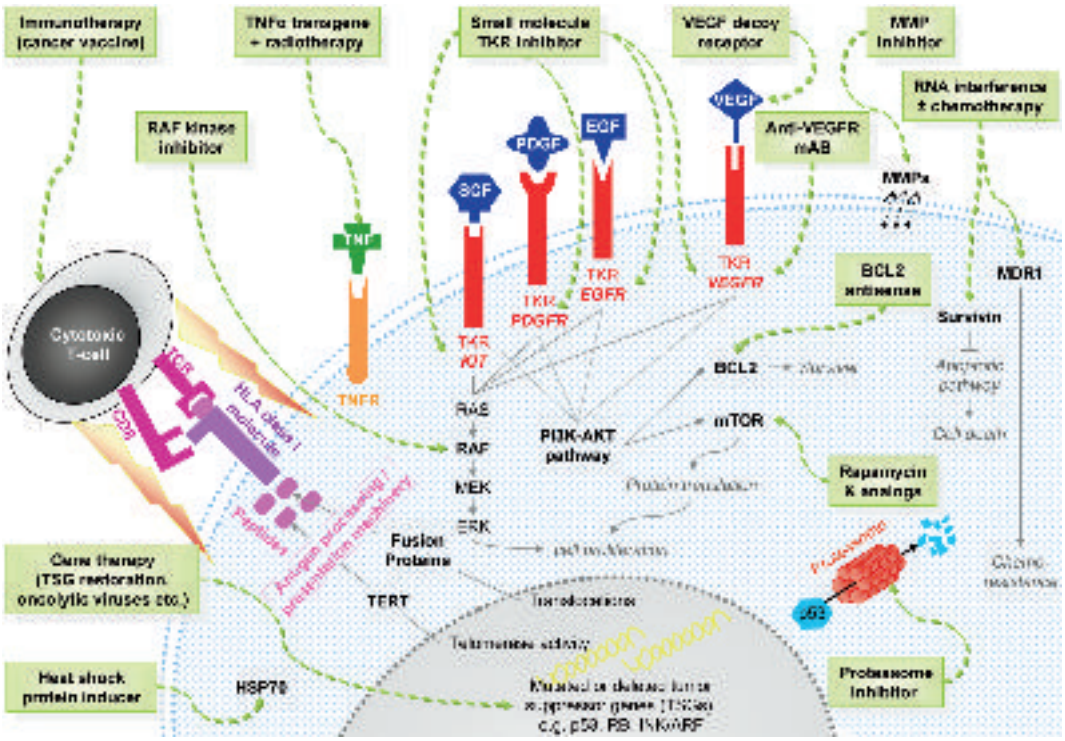

Figure 2. Molecular targets and biological therapies in STS.

that leads to overexpression of the platelet derived growth factor beta (PDGFB) whose receptor tyrosine kinase is also inhibited by imatinib [1].

Angiogenesis is a potential target for therapy in STS since the majority overexpress vascular endothelial growth factor (VEGF) which is the principle angiogenic growth factor that can be targeted by several drugs [128, 173]. These include the humanized anti-VEGF antibody bevacizumab (Avastin®) and the soluble decoy-receptor (VEGF-Trap®) that prevent VEGF from binding to its cell-surface receptor which are being tested in ongoing clinical trails in STS patients [15, 108]. Also sorafenib (Nexavar®), which is a multityrosine kinase inhibitor with activity against e.g. RAF1, VEGFR, PDGFR, and FLT3 is being evaluated in clinical trials in advanced STS [262].

Components of growth-factor signaling pathways such as the ERBB tyrosine kinase receptor family including ERBB1 (also known as epidermal growth factor receptor, EGFR or HER1) and ERBB2 (or HER2/neu) are promising targets for therapy [98]. The SS are known to overexpress EGFR and the efficacy of the small molecule tyrosine-kinase inhibitor gefitinib (Iressa®) that targets the catalytic domain of EGFR is currently under evaluation in a phase II trial in locally advanced SS expressing EGFR. The other kind of EGFR inhibitor is the monoclonal antibody cetuximab (Erbitux®) that targets the extracellular domain of EGFR. ERBB2 or HER2/neu overexpression has also been identified in STS, and could theoretically indicate a potential benefit from treatment with agents such as trastuzumab (Herceptin®) or lapatinib (Tykerb®) [128, 173].

Signaling through growth factor receptors is mediated by downstream activation of the phosphatidylinositol-3-kinase (PI3K)-AKT (also known as protein kinase B, PKB) pathway which results in the activation of the mammalian target of rapamycin (mTOR) pathway [Figure 2] [99, 175]. mTOR is the target of rapamycin and its analogues

which have undergone a phase II evaluation in STS with promising results [253]. Another consequence of the PI3K-AKT signaling is the anti-apoptotic effects through BCL2 [128, 170]. Preclinical studies have indicated a chemo-senitizing effect from the use of BCL2 antisense in STS [128]. Preclinical data in uterine LMS using a PI3K inhibitor indicate a benefit of developing novel therapies that target the PI3K-AKT pathway in LMS [170].

Matrix metalloproteinases (MMP) that promote cell invasion and metastasis through degradation of extracellular matrix may be targeted in STS by MMP inhibitors since MMP2 and MMP9 are known to be upregulated in STS and correlate with grade and poor prognosis [18, 160, 173, 208]. The heat shock proteins (HSPs) that are induced in response to stress and function as regulators of apoptosis are implicated in cancer. In a phase II study, patients with recurrent STS have been treated with the autologous tumor cell-derived HSP-peptide complex vaccine, HSPPC-96. A novel inducer of HSP70 is currently under investigation in a phase II study in combination with paclitaxel in STS patients. Bortezomib (Velcade®) is a reversible proteasome inhibitor investigated in STS patients in a multicenter phase II study and showed only minimal activity but further studies are warranted in combination with synergistic agents [128, 173].

The SS18-SSX fusion product in SS is suggested to cause deregulation of downstream targets through interactions with members of the epigenetic chromatin remodeling/modification machineries and interestingly, *in vitro* studies in SS have demonstrated its sensitivity to chromatin remodeling agents such as the clinically applicable histone deacetylase (HDAC) inhibitor depsipeptide [53, 116].

STS also represent an interesting target for anticancer vaccination strategies since highly tumor-specific immunogenic epitopes can be generated from the chimeric fusion products of the specific chromosomal translocations identified in several STS subtypes. Pilot studies have successfully been performed in pediatric and adult patients [131, 166]. An ongoing phase I trial is testing the immune response in STS patients against a telomerase-derived peptide belonging to a new class of tumor-associated antigens required by cancer cells to survive (including survivin and telomerase) [173]. Based on encouraging preclinical results, a clinical phase II study is being planned to determine the role of immunotherapy in patients with advanced SS using the anti-CTLA4 (cytotoxic T lymphocyte-associated protein 4) antibody where the tumor itself serves as the vaccine trying to activate tumor-fighting T-cells with the anti-CTLA4 [128].

Preclinical sarcoma models with high response rates to gene therapy are providing promising results, e.g. from antisense therapy targeting proliferation and survival genes [84]. Transfection with wild-type p53 causes regression in sarcoma models and restores doxorubicin sensitivity by downregulating MDR1 [274]. Recently, tumor regression has been reported following RNA interference knockdown of oncogenes such as MET and survivin [230]. There have been initial attempts of gene therapy in STS patients using e.g. protein kinase A1 (PKA1) antisense oligonucleotides, intratumoral injections of interleukin 2 (IL2)-coding plasmids and oncolytic adenoviruses that selectively replicate in and lyse p53-deficient tumor cells, and transgenes that code for human tumor necrosis factor alpha (TNFA) coupled with radiotherapy but these therapeutic approaches are still in their early stages [Figure 2] [128, 170, 173, 253].

Genetic alterations

Sarcomas may be divided into two broad categories based on genetic complexity – one characterized by relatively simple karyotypic changes including type-specific chromosomal translocations resulting in distinct fusion genes, and another group with complex unstable karyotypes with numerous numerical and structural aberrations [103]. The cytogenetically simple group includes subtypes such as SS, myxoid/round-cell liposarcoma, clear cell sarcoma and GIST that possess subtype-specific chromosomal translocations resulting in chimeric fusion products that may act

Table 1. List of fusion genes in STS

Translocation	Fusion gene	Fusion protein
Synovial sarcoma		
t(X;18)(p11:q11)	*SS18–SSX*	Transcription factor
Myxoid liposarcoma		
t(12:16)(q13;p11)	*FUS–DDIT3*	Transcription factor
t(12;22)(q13;q12)	*EWSR1–DDIT3*	Transcription factor
Clear-cell sarcoma		
t(12;22)(q13;q12)	*EWSR1–ATF1*	Transcription factor
Dermatofibrosarcoma protuberans		
t(17:22)(q22:q13)	*COL1A1–PDGFB*	Growth factor
Desmoplastic small round-cell tumor		
t(11;22)(p13:q12)	*EWSR1–WT1*	Transcription factor
Alveolar rhabdomyosarcoma		
t(2:13)(q35:q14)	*PAX3–FOXO1A*	Transcription factor
t1:13)(p36:q14)	*PAX7–FOXO1A*	Transcription factor
Congenital fibrosarcoma		
t(12;15)(p13;q25)	*ETV6–NTRK3*	Transcription factor receptor
Inflammatory myofibroblastic tumor		
2p23 rearrangements	*TMP3–ALK*	Growth factor receptor
	TMP4–ALK	Growth factor receptor
Alveolar soft-part sarcoma		
t(X;17)(p11.2;q25)	*ASPL–TFE3*	Transcription factor

as aberrant transcription factors or be involved in dysregulated growth-factor signaling cascades [Table 1]. The fusion products are known to alter gene expression patterns leading to transformation, but much is left to be understood regarding their exact functions and the targets within the cirtical pathways involved. Recent high-throughput genomic approaches have aided in the identification of several candidate genes downstream of the fusion products, but further studies are required to validate these genes as direct or indirect targets and establish a functional link with tumorigenesis [3, 14, 135, 177, 222, 223, 236]. Heterogeneity in gene fusion breakpoints or partners of the involved fusion gene within a specific subtype, has been observed resulting in important clinical and biological differences between the fusion variants (like in the case of SS discussed later) probably due to minor structural and biochemical changes. The karyotypically simple subtypes are also characterized by secondary genetic alterations in, e.g. cell-cycle genes like *TP53* and *INK4A* that seem to play an important role in the malignant progression and abnormal activation of growth factor signaling pathways that lead to increased survival and proliferation.

The second category of karyotypically complex subtypes, including MFH/UPS, LMS, MPNST, pleomorphic and dedifferentiated liposarcomas, lack known specific alterations making their diagnosis rather challenging. These STS types display a relatively high frequency of mutations in the p53 and RB1 signaling pathways [264, 269]. Some mouse models of sarcoma suggest that additional alterations in DNA repair pathways, apart from the defects in cell-cycle checkpoint genes seen in human sarcomas, may also be required for tumor development [103, 122, 226]. Several conventional and array-based CGH (aCGH) studies have confirmed complex aberration patterns characteristic of highly unstable genomes, with numerous copy-number alterations including several chromosomal regions harboring candidate oncogenes and tumor suppressors. The sarcomas seen in mice with defects in DNA repair mechanisms also display similar copy-number profiles and lack specific fusions [226]. Taken together, the karyotypically complex sarcomas may be a result of chromosomal instability and selection of aggressive clones

with amplifications of oncogenes (e.g. *CDK4* and *MDM2*) and deletions of tumor suppressors (e.g. *RB1, INK4A* and *ARF*) involved in cell-cycle regulation, mitogen signaling and other related functions [46, 85, 123, 224, 264, 269].

Signaling pathways

The tumor suppressors p53 and RB1 are dysfunctional in several cancer types including sarcomas in which >80–90% display inactivation of these genes and others within their pathways [46, 85, 123, 224, 264, 269]. RB1 prevents cells with damaged DNA from dividing or progressing through the G1 cell cycle phase into the S phase and is regulated by several kinases and their inhibitors. When dephosphorylated, RB1 inhibits cell cycle progression by binding and inhibiting transcription factors of the E2F family that drive cells into the S phase, whereas RB1 is functionally inactive and dissociates from E2F, when phosphorylated by complexes of cyclin-dependent kinases (CDK) and cyclins [Figure 3a]. RB1 loss is seen in >50–60% of STS and overexpression of the CDK4 and cyclin D1 (CCND1) and loss of p16 INK4A that regulate RB1 function are also commonly observed in sarcomas and other cancers [46].

p53 referred to as 'the guardian of the genome' is one of the most frequently mutated genes in cancer [113]. In normal cells, p53 is usually inactive, bound to the protein MDM2, which inhibits p53 and promotes its degradation, and is in turn inhibited by p14ARF. On DNA damage, kinases such as ATM, CHK1 and CHK2 activate p53 by phosphorylating it at sites that are close to or within the MDM2 binding region thereby dissociating it from MDM2 and allowing the expression of several target genes including the one encoding p21CIP1 that binds and inhibits CDK-cyclin complexes important for G1/S transition [Figure 3a]. *TP53* is mutated in ~50–60% of sarcomas and amplifications of *MDM2* and loss of p14 ARF are also commonly seen in STS [46, 85, 224, 269].

Increased antiapoptotic/survival signaling through deregulated growth-factor signaling pathways is a usual observation in STS, e.g. the insulin-like growth factor 1 (IGF1) receptor signaling abnormally activated in rhabdomyosarcomas and LMS, the KIT receptor signaling in GIST, the PDGFR signaling in desmoplastic round-cell tumors, FGFR and EGFR signaling in SS, MET receptor signaling in rhabdomyosarcomas and SS etc. with all of these pathways converging downstream with the activation of the PI3K, which phosphorylates phosphatidylinositols of the plasma membrane, and thereby converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) [17, 50, 193]. PIP3 recruits protein kinase AKT to the cell membrane where PDK1 and PDK2 phosphorylates and activates AKT which in turn phosphorylates a wide range of cellular signaling molecules relevant for the regulation of cell cycle, cell growth and cell proliferation like FKHR, GSK3ß, apoptosis related proteins such as BAD, caspase 9, IKB, FKHR and MDM2. Furthermore, AKT activates the mTOR signaling pathway that regulates protein translation and also leads to activation of the mitogen-activated protein kinase (MAPK) pathway [Figure 3b]

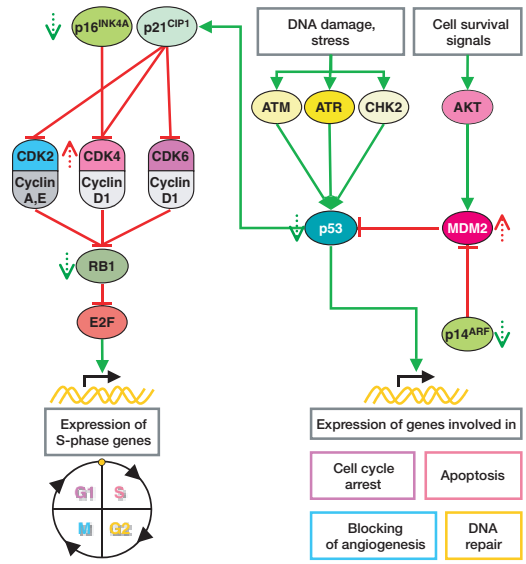

Figure 3a. The p53 and RB1 pathways.
Dotted arrows indicate the genes that are found defective in STS (red arrows denote gain of function whereas green arrows denote loss of function).

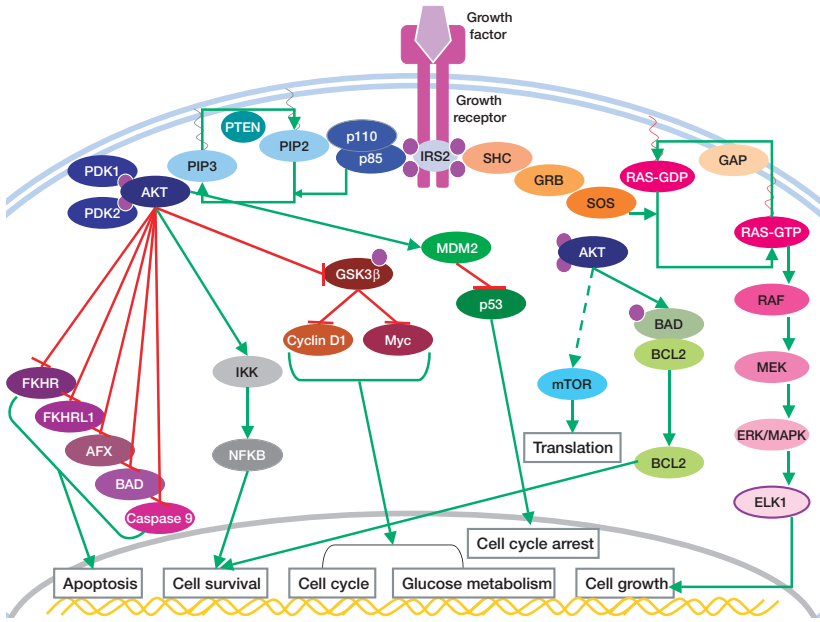

Figure 3b. Growth factor signaling pathway.

[175]. The tumor suppressor PTEN functions as an antagonist of PI3K and has been reported altered in a subset of STS [7].

Developmental pathways such as the Wnt, TGFβ, Notch and Hedgehog receptor signaling pathways play important roles in the pathogenesis of some STS subtypes [Figure 4]. In the Wnt signaling pathway, the secreted Wnt proteins bind to cell-surface receptors of the Frizzled family, causing the receptors to activate Dishevelled (DVL2), a key component of a membrane-associated Wnt receptor complex. This complex in turn inhibits the axin/GSK3β/APC complex that normally promotes the proteolytic degradation of the intracellular signaling molecule β-catenin (through phosphorylation by GSK3β). This eventually results in a change in the amount of β-catenin that reaches the nucleus where it is able to interact with TCF/LEF family transcription factors to promote expression of specific target genes [Figure 4a]. Alterations of Wnt, APC, axin, and TCFs are all associated with cancer development. This pathway has been intensely studied in colon cancer, but also plays a role in mesenchymal tumor development. Intracytoplasmic and nuclear β-catenin staining in STS has been reported to correlate with proliferative activity and poor prognosis [140]. Expression and mutation of β-catenin and *APC* have previously been described in aggressive fibromatosis, a subtype of mesenchymal tumors [5, 143]. Several microarray studies have consistently observed activation of the Wnt signaling pathway genes in SS which are known to accumulate nuclear β-catenin, indicating pathway activation [14, 177]. Recent work has shown that siRNA and polyclonal antibodies directed against FZD10, a gene identified in SS, expression profiles, is effective in suppressing growth in preclinical models [176].

In the heavily regulated transforming growth factor beta (TGFβ) signaling pathway which is involved in many cellular functions such as cell growth and differentiation, apoptosis, and homeostasis, a TGFβ ligand binds to a type II receptor which recruits and phosphorylates a type I receptor which in turn phosphorylates a cytosolic receptor regulated SMAD2 or SMAD3 which then binds a SMAD4 and enters the nucleus where they form complexes with other transcription factors to drive

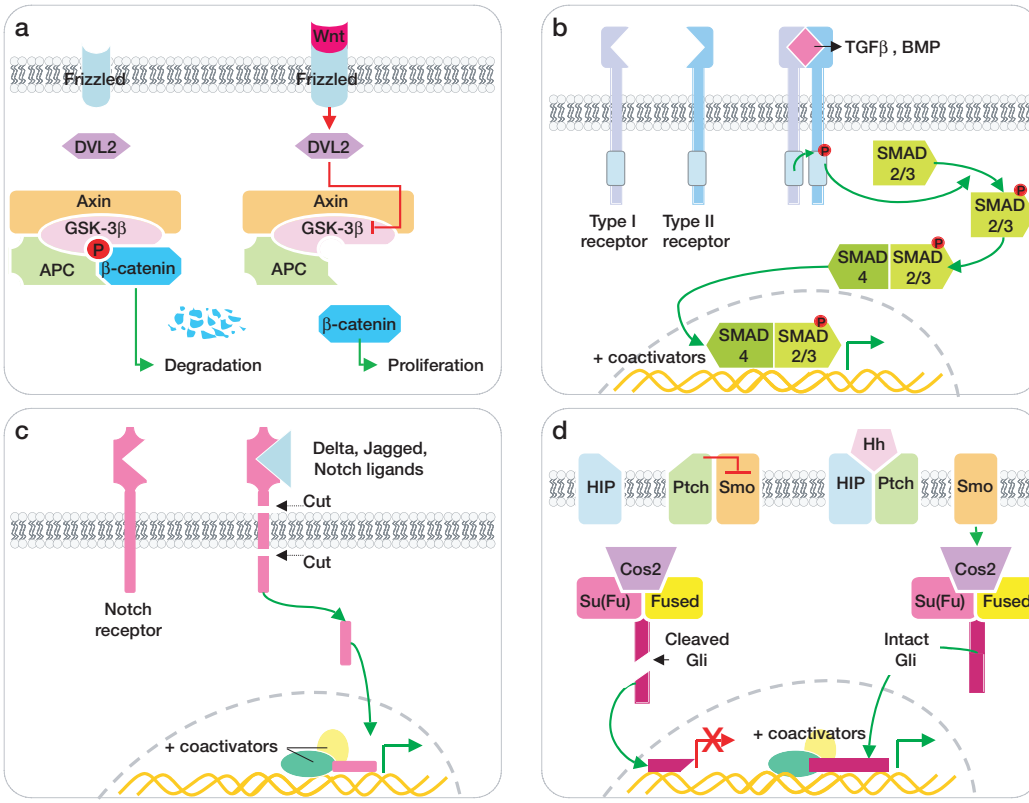

Figure 4. a. Wnt receptor signaling.
b. TGFβ receptor signaling.
c. Notch receptor signaling.
d. Hedgehog (Hh) signaling.

gene expression [Figure 4b]. The TGFβ superfamily of ligands consists of several ligands with specific receptors, e.g. the bone morphogenetic proteins (BMPs) that bind to the BMP receptor type-2 (BMPR2) and the TGFβ ligands that bind to the TGFβ receptor type-2 (TGFBR2) [152].

In the Notch receptor signaling pathway, the interaction of ligands NotchL, Delta and Jagged with the extracellular domain of the receptor causes β-secretase mediated cleavage and release of the cytoplasmic fragment, which then makes its way to the nucleus where it alters gene expression [Figure 4c]. There are 4 different Notch receptors, referred to as Notch1 to Notch4 and since both Notch and its ligands are transmembrane proteins, the cells expressing the ligands need to be adjacent to the Notch expressing cell. Faulty Notch signaling has been implicated in many diseases including cancers in which deregulation is mainly due to elevated expression of the Notch ligands [24].

The Hedgehog signaling is crucial for embryonic development controlling proliferation and/or cell fate determination and adult stem cell regulation and tissue regeneration. In the absence of the ligand proteins of the Hedgehog class, the cell surface transmembrane protein Patched (PTCH) inhibits high expression and activity of the 7-membrane-spanning surface receptor Smoothened (SMO) causing cleavage of the GLI transcription factor into a protein that moves into the nucleus and functions as a transcriptional repressor, whereas in the presence of the Hedgehog ligand, which binds and inhibits PTCH, SMO is allowed to accumulate and inhibit the proteolytic cleavage of GLI which

translocates to the nucleus and activates the target genes of Hedgehog [Figure 4d]. Activation of the Hedgehog pathway has been implicated in the development of cancers of the brain, lung, mammary gland, prostate and skin [72]. Loss-of-function mutations in PTCH and activating mutations in SMO have been identified in rhabdomyosarcomas [248]. Recently, mutant alleles of another downstream component SUFU (suppressor of fused) have also been described in rhabdomyosarcoma [250]. It is hypothesized that abnormal activation of the pathway leads to transformation of adult stem cells into cancer stem cells and Hedgehog signaling pathway inhibitors are being developed.

Synovial sarcoma genetics and epigenetics

The fusion of the *SS18* (previously known as *SYT*) gene on 18q11.2 to either one of three highly homologous *SSX* genes (*SSX1*, *SSX2* or *SSX4*) on Xp11.2 is a hallmark of SS [48, 238]. The majority carry the *SS18-SSX1* and *SS18-SSX2* fusions with the former variant being nearly twice as common as the latter. The lower frequency of the *SS18-SSX4* fusion type and absence of fusions involving the other *SSX* genes may be due to these genes being located in genomic regions less prone to rearrangement (depending on chromatin structure and repeat content) and/or due to lower transforming ability of the resultant fusion products. The higher frequency of *SSX1* fusions compared to *SSX2* may be attributed to a slightly higher oncogenic advantage leading to *in vivo* selection or due to differences in chromatin structure around *SSX1* and *SSX2* [142, 218].

The *SS18* and *SSX* genes encode nuclear proteins with opposite transcriptional activities that lack obvious DNA binding domains and probably exert their respective coactivator or corepressor functions through interactions with other sequence-specific DNA-binding proteins that may serve to bring SS18 or SSX in contact with their targets. The anomalous gene expression in SS could result from genes normally activated by SS18 being downregulated by the SSX repression domain and/or genes normally repressed by SSX being upregulated by the SS18 activation domain and/or from the effects of the fusion product on a novel set of targets [142, 218] [Figure 6].

The SS18 protein which is ubiquitously expressed in a wide range of human embryonic and adult tissues has two functional domains; a conserved 54-amino acid SS18 NH_2-terminal homology (SNH) domain and a C-terminal transcriptional activator domain rich in glycine, proline, glutamine and tyrosine (QPGY domain) [Figure 5]. Moreover, several regions serving as candidate ligands for binding to Src homology 2 and 3 (SH3 and SH2) domains implicated in protein-protein interactions have been found in SS18, which functions as a transcriptional coactivator and associates with components of the epigenetic chromatin remodeling machinery like the SWI/SNF complex. SS18 has been found to interact directly and colocalize with the AF10 (or the MLLT10 transcription factor which fuses with MLL in acute myeloid leukemias), brahma (BRM or SMARCA2) protein, the brahma related gene 1 (BRG1 or SMARCA4) protein, the histone acetyltransferase p300 and the histone deacetylase associated corepressor SIN3A in a mutually exclusive manner through the SNH protein-protein interaction domain. BRM, BRG1, and INI1 (SMARCB1 that interacts with AF10) proteins that associate with SS18 are components of the ATP-dependent SWI/SNF chromatin remodeling complex that alters the position of nucleosomes at promoter sites of genes and makes them more accessible to the transcription machinery or other transcription factors, whereas p300 and SIN3A are involved in epigenetic regulation through covalent chromatin modifications, e.g. deacetylation of histone tails [Figures 5 and 6]. A deletion of the SNH domain results in an even more potent activator suggesting that the binding of the SWI/SNF proteins negatively regulates the transcriptional activation by SS18 [52, 53, 129, 142, 199, 218, 240].

The *SSX* genes, on the other hand, function as transcriptional corepressors and contain a Krüppel-associated box (KRAB) domain at the N-terminus and an SSX repression domain (SSXRD) at the C-terminus [Figure 5]. The *SSX* genes are

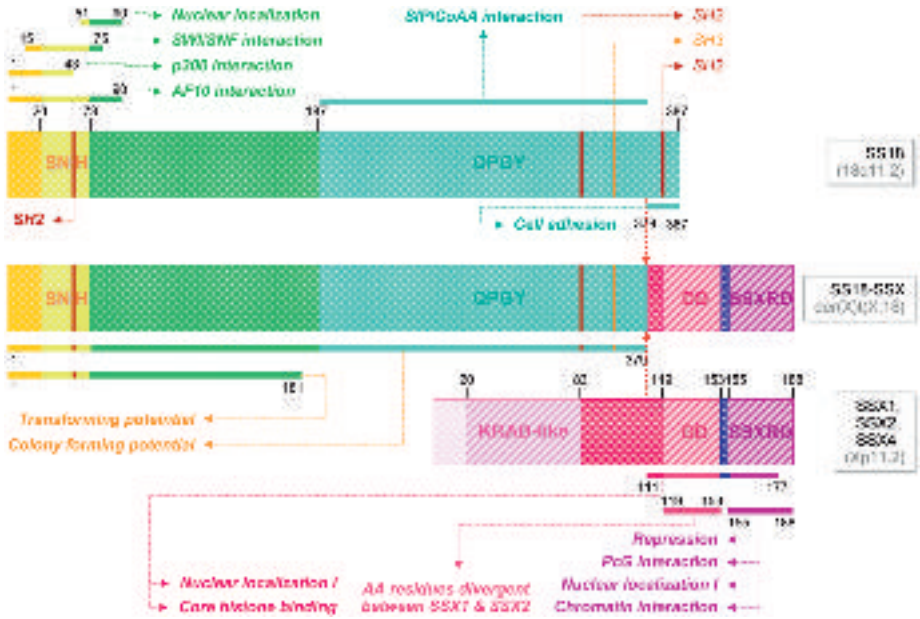

Figure 5. Schematic diagram of the structural and functional domains of the SS18, SSX and SS18-SSX proteins. Amino acid residues representing the boundaries of the domains are indicated along with functions and interacting molecules of the domains. The scale is approximate.

members of a family of 9 highly related genes that have a restricted expression in normal tissue confined mainly to the testis and to a lesser extent in the thyroid but are expressed in a variety of cancers (cancer-testis antigens). In the nucleus, they are found diffused or colocalized in nuclear speckles with many polycomb group (PcG) proteins (e.g. HPH1, ENX, EED, RING1, HPC2, BMI1) that function as epigenetic gene silencers that induce repression of target genes by modification of chromatin structures. The SSXRD domain which is retained in the pathogenic SS18-SSX fusion [Figure 5 and 6] plays a crucial role in the nuclear localization of SSX and it's interactions with the PcG proteins, core histones and mitotic chromosomes as well as association with the transcription factor LIM homeobox protein LHX4 involved in antiapoptotic and proliferative functions and found translocated and overexpressed in some cancers [52, 53, 129, 142, 199, 218, 240].

All of the available data point towards the probability that both the normal SS18 and SSX proteins function epigenetically as transcriptional coregulators in association with DNA binding proteins (like AF10 or LHX4) through the recruitment of chromatin remodeling/modification complexes such as SWI/SNF and PcG proteins, respectively. In the chimeric SS18-SSX fusion product, the C-terminal 8 amino acids of the SS18 protein are replaced in frame by the last 78 C-terminal amino acids of the *SSX* gene, thus retaining both the activating and repressing domains from the parent proteins and making it quite likely that the aberrant fusion product also uses epigenetic mechanisms to cause deregulation and malignant transformation. A recent study evaluating the direct transcriptional consequences of the SS18–SSX fusion protein in an experimental model, using gene expression profiling, chromatin immunoprecipitation and histone modification and DNA methylation assays showed exactly that, with deregulation of several genes among which the *IGF2* (known to be overexpressed in SS) was found highly upregulated as an effect of enhanced acetylation and methylation of specific histones in the promoter region [52, 53]. Another gene found prominently down-regulated after

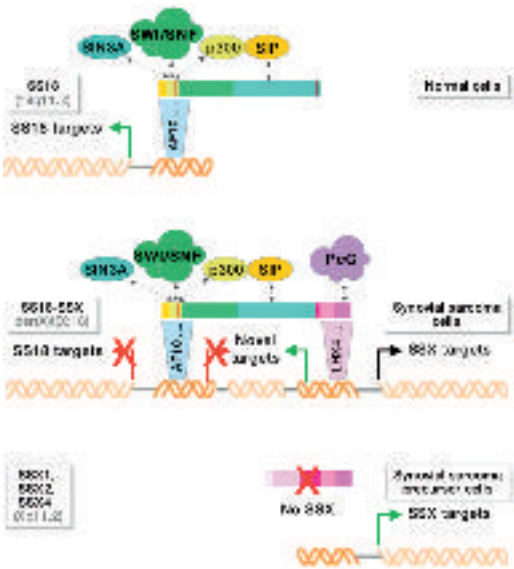

Figure 6. Hypothetical model depicting the mechanism of action of the SS18, SSX and SS18-SSX proteins.
The SS18 activates transcription by binding to the ATP-dependent SWI/SNF remodeling complex via the BRM/BRG, recruiting histone acetylases and methylases via the SIN3A, p300 and SIP proteins, and additional interaction with DNA binding proteins such as AF10. The SSX represses transcription in the spermatogonia and thyroid by recruitment of the PcG complex and interacting with DNA binding proteins such as LHX4, but is absent in other normal cells. The fusion protein SS18-SSX may result in abnormal regulation of the SS18 and SSX targets or result in deregulation of novel targets.

induction was the *CD44* known to be regulated by SWI/SNF and DNA methylation indicating that the SS18–SSX fusion proteins contribute to abnormal transcription of target genes by possible epigenetic mechanisms. This notion is further supported by reports of SS growth inhibition achieved through targeting using HDAC inhibitors in experimental systems [116].

The first ~181 amino acids (which are also present in the normal full-length SS18) of the fusion product have been implicated in the transforming potential. Since these same amino acids do not render any such potential to the normal SS18, the fused C-terminal SSX amino acids (including the SSXRD domain) are in some way essential for the oncogenicity. Moreover, it has been shown that the SSXRD is dominant over the SS18 moiety in the nuclear-localization signaling of the fusion protein. However, the HS-SY-3 cell line harboring an SS18-SSX1 fusion variant lacking the SSXRD domain, grows slowly compared to other SS cell lines, and is not tumorigenic in nude mice, suggesting that the SSXRD domain is dispensable for SS tumor genesis but may contribute to aggressiveness. Also, the last 8 amino acids of the normal SS18 absent in the fusion product are involved in cell adhesion suggesting that the loss of this region and consequent disruption of the p300/SS18 mediated adhesion may be another oncogenic mechanism [Figure 5 and 6] [142, 218].

Clinical correlates of the *SS18-SSX* fusion types

That *SSX1* and *SSX2* fusion variants differ by 13 amino acid residues, 12 of which lie in the DD domain [Figure 5]. The exact molecular consequences of this minor difference in structure are not clearly understood but could result in changes of potential phophorylation and N-linked glycosylation sites and differences in protein-protein interactions which have an important and detectable impact on the cellular, histological and clinical behavior of SS confirming that gene fusion type is crucial to their biology [53, 142, 218].

The most striking correlation is that with histopathology where most biphasic types (showing glandular epithelial differentiation in addition to spindle cells) contain the *SS18-SSX1* fusion whereas almost all tumors with the *SS18-SSX2* fusion variant are monophasic (with only the spindle cell component) suggesting that the two fusion types have different influences on differentiation patterns [94, 132, 143]. A correlation with primary site has also been observed with *SS18-SSX1* primary tumors arising more frequently in the limbs suggesting a divergence in susceptibilities of different cells to the two fusion variants [94, 143]. Many studies have confirmed the association of fusion type with sex with a 1:1 male:female ratio for *SS18-SSX1* and a 1:2 ratio for *SS18-SSX2* [94, 143]. Fusion type has also been suggested to correlate with outcome. A large multi-institutional study and 3 smaller stud-

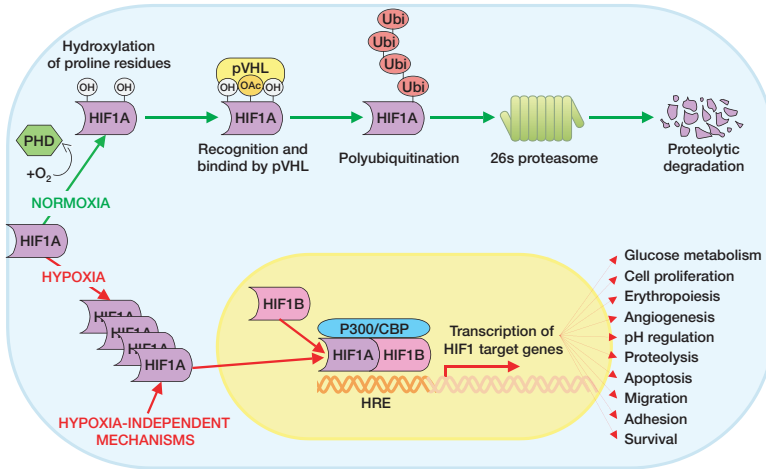

Figure 7. HIF1A regulation by oxygen-dependent proline hydroxylation.
HIF1 functions as a heterodimer consisting of two basic-helix-loop-helix proteins, HIF1A and HIF1B. In normoxic conditions, the cytoplasmic alpha subunit, HIF1A is hydroxylated at specific proline residues by oxygen-dependent proline hydrolase (PHD), which leads to interaction with the product of the von Hippel-Lindau tumor suppressor (pVHL). pVHL as part of a multisubunit E3 ligase-ubiquitination complex, tags HIF1A with polyubiquitin which allows recognition by the proteasome and subsequent degradation. Under hypoxic conditions, the PHD is inactive and ubiquitination is inhibited and HIF1A is no longer degraded resulting in an extended life time and accumulation in the cytoplasm from where it is translocated to the nucleus where it interacts with the constitutively expressed oxygen independent beta subunit HIF1B (also known as ARNT) to form the active HIF1 complex that functions as a transcription factor which then specifically binds to hypoxia-response elements (HREs) located in the promoters of target genes. For full transcriptional activity, HIF1 recruits the transcriptional coactivator p300/CREB-binding protein (CBP) and switches on a wide range of genes controlling processes such as angiogenesis, increased oxygen transport, glucose uptake, glycolysis, pH regulation and cell migration as an adaptive response to restore oxygen homeostasis and meet the metabolic needs of the rapidly growing tumor.

ies demonstrated that the fusion type in SS was a significant prognostic factor with a worse metastasis-free survival in patients with tumors carrying the SSX1 fusion type though another large study failed to confirm this association [94, 132, 143, 180].

Hypoxia and the HIF1 pathway

Hypoxia (low oxygen concentration and its consequences) is a well documented characteristic of solid tumors resulting from poor or impaired vascular supply and associated with treatment resistance and malignant progression [258]. In STS, hypoxia was significantly associated with poorer outcome either due to local recurrence or increased rate of metastasis [27, 182]. The effects of hypoxia on increased proliferation, local invasion and distant metastasis are mediated through several mechanisms including genomic instability and heterogeneity contributing to the selection and expansion of aggressive clones like those resistant to apoptosis and hypoxia-induced genomic and proteomic changes triggering processes such as angiogenesis, anaerobic glycolysis, and cell migration that enable tumor cells to survive or escape their oxygen deficient environment [11, 258]. The hypoxia-induced alterations in gene expression are mediated through a transcriptional response pathway involving the hypoxia inducible factor 1 (HIF1) [Figure 7] [133]. The HIF1 transcription factor, with over 100 target genes involved in a variety of cellular processes, is recognized as the predominant regulator of oxygen homeostasis and its regulatory oxygen-labile alpha subunit, HIF1A, which is expressed in many cancers and correlates with tumor progression and metastatic potential is a promising therapeutic target in hypoxic tumors

[133, 225, 275]. Two isoforms of the alpha subunit, HIF2A and HIF3A, have been identified albeit lesser characterized. HIF2A with a high degree of similarity in structure and function to HIF1A has a restricted distribution in the endothelium, heart, lung, kidney, and gastrointestinal epithelium and in some tumor types is more strongly associated with tumor development and poor outcome than HIF1A. The transcriptional effects of HIF3A are unclear but it is believed to act as a negative regulator of HIF1 [90, 133]. Although hypoxia is the most potent regulator of the HIF alpha subunits, recent data suggest that they can also be activated by hypoxia-independent mechanisms like direct phosphorylation and activation by cytokines, growth factors and environmental stimuli that may signal through the MAPK and PI3K pathways and hence subject to deregulation by various oncogenes and tumor suppressors. With an increasing number of target genes and diverse mechanisms of activation, the HIF1 pathway plays an important role in tumor biology and oncogenic progression [90, 133, 258]. Also, a vast majority of HIF1A targets are overexpressed in tumors and associated with poor outcome [31, 38, 63, 141, 165, 196, 231, 244, 272, 275].

HIF1 plays important roles in several central cellular functions, e.g. angiogenesis, erythropoiesis, iron metabolism, anaerobic glycolysis, proliferation, survival, apoptosis, matrix remodeling and metastatic progression [Figure 7]. VEGF is the principle angiogenic growth factor activated by HIF1 [30]. Several studies have shown VEGF expression to be an independent prognostic factor [38, 92, 141, 272]. In addition to VEGF, HIF1 also regulates multiple angiogenesis-promoting genes and receptors, *VEGFR1*, *PDGFB*, angiopoietin 1 (*AGP1*), endothelin 1 (*EDN1*), heme oxygenase (*HMOX1*), inducible nitric oxide synthase (*INOS*), monocyte chemotactic protein 1 (*MCP1*), adrenomedullin (*ADM*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), ephrins and their receptors, many of which are involved in controlling vascular tone and blood flow [90, 133, 258]. In order to enhance oxygen delivery, HIF1 upregulates the expression of genes such as erythropoietin (*EPO*) and its receptor (*EPOR*), and iron-metabolizing genes such as ceruloplasmin (CP), transferrin (*TF*) and its receptor (*TFR*) [90, 133, 258]. To meet energy requirements of the rapidly expanding tumor mass with high rates of glycolysis/glucose consumption and lack of oxygen, HIF1 triggers a metabolic shift to anaerobic glycolysis by upregulating enzymes such as lactate dehydrogenase A (LDHA), aldolase A (ALDA), hexokinase 1 and 2 (HK1, HK2), pyruvate kinase M (PKM), enolase (ENO), phosphofructokinase L (PFKL) and phosphoglycerate kinase 1 (PGK1). Increased glucose uptake by HIF1-mediated overexpression of glucose transporters 1 and 3 (GLUT1, GLUT3) further strengthen the glycolytic response [51, 90, 133, 258]. Cytokines and growth factors such as IGF2, TGFA and EGF that activate signal transduction pathways like the MAPK and PI3K pathways that promote cell proliferation and survival are induced by hypoxia and HIF1. Paradoxically, HIF1 also induces cell death by modulating various pro-apoptotic factors of the Bcl-2 family like NOXA, BCL2/adenovirus E1B 19kDa interacting protein 3 and its homologue (BNIP3 and BNIP3L) and cell cycle regulators such as p53 and p21 [90, 133, 258].

Hypoxia is also known to alter the expression of urokinase-type plasminogen activator (uPA), its receptor (uPAR) and the plasminogen inhibitor PAI1 that function in proteolytic processes, cell adhesion and migration. Matrix metalloproteinases (MMPs) such as MMP2, MMP9 and MMP13 involved in the breakdown of extracellular matrix are upregulated under hypoxic conditions and deregulation of their inhibitor tissue inhibitor of metalloproteinases 1 (TIMP1) has also been observed. Other mediators of metastasis upregulated by hypoxia include prolyl 4-hydroxylases involved in collagen synthesis, lysyl oxidase (LOX) involved in formation and repair of the extracellular matrix, chemokines and their receptors like CXCR4, osteopontin (OPN) etc [90, 133, 258].

Hypoxia is a heterogeneous characteristic in high-grade STS while low-grade STS are most often well-oxygenated [27, 75, 165, 182]. EF5 binding studies have demonstrated increasing levels of hypoxia with increasing necrosis in STS, with

the highest levels adjacent to necrotic regions [75]. However, the association between tumor size and hypoxia remain inconclusive, though larger STS tend to have higher levels of hypoxia [76]. In a study by Detwiller *et al.*, hierarchical clustering on 107 hypoxia-related genes clearly distinguished sarcoma samples from normal tissue samples suggesting an important role for hypoxia in sarcomas. [62]. Several studies in STS have shown correlations of both hypoxia and enhanced tumor glucose metabolism to high tumor-cell proliferation rate and grade [16, 75, 82, 184]. The fist study identifying a correlation between distant metastasis and hypoxia or pre-treatment tumor oxygenation measurement using the Eppendorf needle electrode was in 22 STS patients reported by Brizel *et al.*, who showed that patients with hypoxic STS had a significantly lower disease-free survival and higher risk of lung metastasis, which was later confirmed by Nordsmark *et al.* in 28 STS patients [27, 182]. More recently, in a study by Evans *et al*., hypoxia measured in terms of EF5-binding was significantly associated with tumor grade, presence of mitoses and development of metastasis in STS [75]. Studies using immunohistochemical staining of endogenous hypoxia-associated molecular markers have also confirmed the poor prognostic effects of HIF1 transcriptional response in STS. In GIST, HIF1A expression has been shown to correlate with aggressive behavior, angiogenesis and poor outcome [31, 244]. A recent study evaluated HIF1A expression levels in 49 spindle cell STS including MFH, SS, LMS and MPNST and found HIF1A overexpression to be an independent prognostic factor [231]. Unlike previous studies in other tumor types that showed intense HIF1A staining in perinecrotic areas, HIF1A expression was seen diffused all over the tumor section indicating that it not only is a consequence of hypoxia but also of other oxygen-independent mechanisms like abnormal activation by oncogenes or tumor suppressors and/or signaling by cytokines and growth factors through the MAPK and PI3K pathways [133]. STS express high levels of serum and tumor VEGF which appear to correlate with prognosis [91, 272]. PDGFB protein and mRNA levels in STS correlate with tumor grade and proliferation while overexpression of the receptors PDGFRA and PDGFRB conferred worse prognosis in pediatric rhabdomyosarcoma [22, 256]. Studies evaluating serum levels of other proangiogenic and antiangiogenic factors including basic fibroblast growth factor (bFGF), AGP2 and endostatin (or collagen, type XVIII, alpha 1, COL18A1) showed higher levels in STS patients correlating with grade and size but conflicting results regarding association to outcome [77, 91, 270, 271]. Increased expression of osteopontin (OPN), involved in cell migration stimulated by hypoxia, has also been associated with adverse prognosis in STS patients [26]. Carbonic anhydrases are transmembrane glycoproteins activated by HIF1 and carbonic anhydrase IX (CA9) has been suggested as a good prognostic marker in STS patients with deep, large, high-grade tumors, with CA9-positive tumors showing significantly lower disease-specific and overall survival than CA9-negative ones [165]. Expression of GLUT1 also studied as a marker for hypoxia, was evaluated in STS and showed positive correlation with tumor glucose metabolism, proliferative activity and tumor grade [245]. Also, increased expression of UPA, MMP2 and MMP9 correlated with poor outcome, while increased TIMP2 expression predicted correlate better prognosis [18, 19, 39, 160, 208].

Microarrays in STS

Microarrays comprise ordered arrays of thousands of microspots of gene-specific DNA elements immobilized on a surface (commonly a glass slide) that can be used for simultaneous measurements of mRNA levels of thousands of genes in order to generate gene expression profiles. Ever since the very first gene expression profiling study, in the early 90's, which concurrently measured the transcriptional activity of a number of genes using radioactive samples hybridized onto filter-immobilized cDNA probes [64, 150], the technology has steadily and vastly improved, with microarrays being increasingly utilized in numerous fields of research to answer a wide variety of biological

questions. Microarray-based gene expression profiling was first used to study cancer in the mid 90's but began to be applied in STS only around the year 2000 [60, 135, 144, 179]. Microarray analysis of STS have helped identify critical pathogenic pathway signatures in various STS subtypes and in screening for diagnostic markers and therapeutic targets, but its clinical application, however, is limited due to difficulties and complexities concerning specimen handling, cost, experimental design, data management and analysis. Recent developments of other microarray-based high-throughput technologies, including aCGH, which measures genome-wide copy-number profiles and tissue microarrays (TMAs) that can be used to measure DNA, RNA or protein in tumor sections, are also proving fruitful in the study of cancers including STS [111]. Other more recent array-based technologies are being used to analyze microRNA expression profiles, single nucleotide polymorphism (SNP) profiles to determine loss of heterozygosity (LOH) or gene copy-number changes, and whole-genome profiles of epigenetic changes using chromatin immunoprecipitation ChIP-chip analysis [157, 252, 263].

Several recent studies have successfully applied gene expression profiling to STS [3, 14, 135, 148, 177-179, 222, 223, 251], corroborating the findings from previous studies using conventional methods as well as obtaining new important information regarding underlying genetic mechanisms, downstream targets of the specific fusion proteins and mutations, novel markers for diagnosis and deregulated pathways and genes that may be therapeutically targeted. For instance, the identification of the KIT receptor and its downstream targets within the distinct discriminatory expression profiles of GIST, and the identification of PDGFB in DFSP expression signatures confirm the reliability of microarray analysis in identifying diagnostically and therapeutically relevant genes and pathways [4, 155]. The well-appreciated distinction of STS subtypes into two broad groups depending on karyotypic complexity was also evident from the profiling studies, with specific subtypes harboring fusion genes and mutations forming tight diagnosis-specific clusters, while the pleomorphic subtypes clustered separately on a different branch of the dendrogram and did not form consistent subtype-specific clusters on unsupervised analysis, except for a subset of LMS [148, 178, 179, 222, 251].

One of the first microarray analyses identified a distinct signature in desmoplastic small blue round cell tumors which are otherwise difficult to diagnose using histopathology alone [136]. MPNST when compared with their precursor Schwann cells display downregulation of several genes involved in differentiation and upregulation of neural crest markers [171]. A comparison of expression profiles from sporadic MPNST and neurofibromatosis-associated MPNST has been reported [109, 110, 127, 257]. The dedifferentiated liposarcomas have been distinguished from the myxoid/round-cell liposarcomas with overexpression of *MDM2*, *CDK4* and *SAS* from the 12q amplicon involved in ring chromosome formations observed in well-differentiated and dedifferentiated liposarcomas, whereas the myxoid/round-cell liposarcomas displayed upregulation of developmental genes [83, 179, 221, 229, 235]. A gene expression study of uterine LMS was able to distinguish benign from malignant tumors and myometrium from leiomyoma while another study evaluated the differences in expression profiles of uterine LMS, LMS and leiomyomas [205, 237].

Despite the differences in array platforms, tumor subsets, reference RNA and statistical methods, DNA microarray studies have identified consistent expression profiles for many STS subtypes with specific genetic alterations. In the SS, expression profiling has identified alternative therapeutic targets and overexpression of developmental genes involved in embryonic mesenchymal development, retinoic acid and Wnt receptor signaling, as well as several genes coding receptor tyrosine kinases including *FGFR3*, *EGFR*, *KIT*, and *ERBB2*, many of which have been validated at the protein level and have specific inhibitors, being evaluated in *in vitro* studies as well as clinical trials [3, 148, 177-179, 222, 236]. In addition to GIST and SS, highly informative expression profiles have been identified in other specific subtypes such as alveolar rhabdomyosarcoma, clear cell sarcoma and myxoid/

round-cell liposarcoma [155, 222, 223]. Expression profiles from clear cell sarcoma suggest close relationship with melanoma with overexpression of several genes involved in melanocytic differentiation. Combined gene expression and gene copy-number profiles in DFSP resulted in the identification of *APOD* as a useful diagnostic marker [261]. Screening through the lists of highly expressed genes in specific subtypes, for biomarkers that can be tested using immunohistochemical staining has identified several potential diagnostic markers, e.g. protein kinase C-θ which was identified from discriminatory profiles of GIST and has been independently validated as a useful diagnostic marker [23]. Microarrays have also been used for the purpose of generating prognostic signatures in sarcomas by comparing expression profiles of primary tumors from patients with known outcome. Such profiles have been reported in LMS with upregulation of cell cycle and signal transduction genes in the metastasizing tumors [149, 205].

Microarray expression data from sarcomas may be screened not only for novel therapeutic targets against which new therapies may be designed but also, for more practical reasons, for molecular targets that already have clinically established drugs used for treating other cancer types. Only a relatively small number of preexisting drugs and treatment strategies have been tested in sarcoma and even in cases where this has been done, the various subtypes were grouped together making it impossible to identify useful subtype-specific treatments. But now with expression data from the various subtypes, drugable targets specific for one or more of the subtypes have been identified which may be inhibited using already available agents, e.g. the increased expression of the VEGFR in SS and hemangiopericytoma and the increased *HER2* and *EGFR* expression in SS.

Comparative genomic hybridization

In the conventional CGH method developed by Kallionemi *et al.* in 1992, differentially fluorescently-labeled tumor and normal DNA are cohybridized to metaphase chromosomes and fluorescence ratios used as the measure of copy-number alterations [121]. In aCGH, the samples, are instead hybridized onto array platforms and copy-number profiles of thousands of chromosomal regions/genes are determined in a similar manner as expression profiling. These platforms may be composed of large genomic BAC or PAC clones, cDNA or oligonucleotides and have proven highly useful in the detection of cancer-related chromosomal changes, but balanced translocations and ploidy shifts cannot be detected [2, 158, 201]. The resolution of aCGH slides depends on the number and size of the spotted clones and their spacing and distribution throughout the genome. An important advantage of aCGH is that DNA from tumors preserved in formalin-fixed paraffin-embedded blocks may be utilized, thus overcoming the problem of scarcity of fresh frozen material that hampers STS research.

CGH is particularly useful in STS which are often characterized by numerous and complex copy-number imbalances. Several cCGH studies have identified many chromosomal aberrations in STS, but only a few aCGH studies of STS have been reported till date [34, 37, 55, 83, 100, 130, 139, 145, 155, 169, 189, 260]. The first study combined cDNA microarray analysis with aCGH in 16 dedifferentiated and pleomorphic liposarcomas and demonstrated that copy number gains of numerous oncogenes including *CCND1*, *MDM2*, *GLI* and *CDK4* correlated with high-level mRNA expression [83]. A study by Linn *et al.* reported gene expression profiles in 9 and array-based copy number profiles in 4 DFSP showing that genes characteristically overexpressed were also found amplified [155], whereas another more recent study reported aCGH profiles in 10 DFSP [130]. Chibon *et al.* characterized the 6q23 amplicon seen in majority of the MFH/UPS with high-level amplifications in 12q14-12q15 using aCGH and demonstrated that amplification and overexpression of target gene *ASK1* could be responsible for inhibition of adipocytic differentiation in these MFH/UPS [34], whereas Kresse *et al*. characterized the 1q23 amplicon in sarcomas using aCGH suggesting cell growth related genes *ATF6* and

DUSP12 to be the likely targets of the amplification [139]. Larramendy *et al*. performed genome-wide aCGH in 14 LMS and reported ~2,000 genes in 25 altered chromosomal regions. They also showed that the frequency of copy-number gains in high-grade LMS was higher than low-grade LMS [145]. Another high-resolution aCGH study in uterine LMS reported target genes of recurrent copy-number aberrations including frequent gains of *PDGFRB, VAV2, FGF4, WISP1* and *HER2*, and frequent losses of *LEU1*, *DCC*, *SCCA1*, *FVT1*, and *ETS2/E2* [37]. Heidenblad *et al.* reported genomic profiles using tiling BAC arrays in STS with supernumerary ring chromosomes demonstrating that >40% of all amplicons in both STS and bone sarcomas were mapped to chromosome 12 with amplified clones predominantly from the 12q13-12q15 region, including the *SAS*, *CDK4* and *MDM2* genes, which was seen in 85% of the STS analyzed. They also report frequent high-level amplifications of the 1p32 region harboring the *JUN* oncogene [100]. An aCGH study of myxoid liposarcoma and myxofibrosarcoma demonstrated that the two subtypes were genetically distinct, with the latter subtype showing frequent and diverse copy-number alterations whereas only 3/10 myxoid liposarcomas showed any copy-number alteration [189]. A recent study by Meza-Zepeda *et al*. generated aCGH profiles for 7 GIST and 12 LMS showing numerous but distinct copy-number changes in both tumor types and identified likely targets of the recurrent high-level 17p13-17p11 amplicon seen in LMS [169]. Majority of these studies have been performed in small sample sets, hence further studies in larger tumor material are warranted in STS to obtain a more complete picture of the copy-number alterations and the genes they affect.

Materials

Ethical permission for the studies was obtained from the Lund University research ethics committee (LU302-02). Fresh tumor material was obtained from surgical excisions and was stored at –80°C until use. All tumors were reviewed by the Scandinavian Sarcoma Group (SSG) review board of pathologists and many tumors had in addition been re-evaluated by the reference pathologists. The diagnoses were based on the combined information from histopathology, cytogenetic and/or molecular genetic analyses, and immunohistochemical stainings to exclude melanoma, lymphoma and carcinoma. Tumor necrosis and vascular invasion were evaluated as part of the prognostic system used for clinical decisions. Malignancy grading was based on a IV-tiered grading system and majority of the tumors were classified as high-grade (grades III and IV). Most tumors were located in the extremities or the trunk wall and only a small minority of the patients received preoperative treatment with radiotherapy or chemotherapy. The patients were followed according to a standardized follow-up protocol with clinical examinations and chest X-rays and/or CT scans every 3rd month for the first 3 years and biannually thereafter for at least 5 years.

Study I

Multiple pieces were obtained from 2 deep-seated, high-grade pleomorphic STS operated at the Lund University Hospital, Lund; a myxoid MFH (17x15x10 cm) that developed in the thigh of an 87-year-old man and a LMS (11x8x7 cm) that developed in the groin of an 82-year-old man. The 8 different tumor pieces from the MFH and the 10 pieces from the LMS were separately histopathologically characterized and showed variable degrees of myxoid degeneration, vascular invasion, necrosis and cellular pleomorphism (*Study I*, Table 1). Moreover, single samples from 20 MFH and 16 LMS were used for comparison. (*Study I*, Table 2).

Study II

26 tumor samples including 19 primary tumors, 3 local recurrences, and 4 metastases were obtained from 24 patients operated at the Lund University Hospital (n=16) and the Karolinska Hospital, Stockholm (n=8) between 1986 and 1999 (*Study II*, Table 1). 3 of these patients received preoperative treatment, chemotherapy in 2 cases and radiotherapy in 1 case. Among the 19 primary tumors used for the evaluation of a gene expression signature associated with metastasis, 12 were from patients that developed metastasis. A gene expression profile related to gene-fusion type was studied in 21 samples, of which 12 had the *SS18-SSX1* fusion type and the remaining had the *SS18-SSX2*. Tumors were also classified as monophasic or biphasic and as carrying simple alterations including the t(X;18) or a more complex karyotype.

Study III

Tumor samples were obtained from 177 patients operated between 1972 and 2003 at the Lund University Hospital, Lund (n=122), the Norwegian Radium Hospital, Oslo (n=47) and the Karolinska Hospital, Stockholm (n=8) and included 154 primary tumors, 16 local recurrences and 7 metastases (*Study III*, Table 1). The 177 samples represented 13 histopathological subtypes, among which MFH/UPS, LMS and SS together constituted 75%. 17 xenografts including 6 MFH, 4 MPNST, 3 SS, 2 pleomorphic liposarcomas, 1 myxoid/round-cell liposarcoma and 1 GIST were also included. Only a small minority of the patients received preoperative treatment with radiotherapy (n = 1) or chemotherapy (n = 5). A prognostic expression profile was evaluated in 89 primary pleomorphic tumors, mostly including MFH/UPS and LMS that had undergone primary surgery without preoperative radio- or chemotherapy.

Study IV

The 60 primary MFH/UPS and LMS which represents a subset of the tumors included in *Study III* were operated at the Lund University Hospital between 1987 and 2003 and were chosen to equally represent two groups of tumors that did (n=30) and did not (n=30) metastasize (*Study IV*, Table 1). All tumors were localized to the extremities or the trunk wall and none of the patients had received preoperative radio- and/or chemotherapy.

Reference RNA (Studies I–III)

The Universal Human Reference RNA was used as the common reference for the hybridizations and was prepared as per the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The reference RNA was composed of total RNA from 10 different cell-lines representing different human tissues and approximating the expression profile of majority of the genes thereby ensuring maximum coverage of the genes on the cDNA microarrays.

Reference DNA (Study IV)

Commercial genomic DNA derived from a pool of healthy male individuals was used as the reference (Promega, Madison, WI, USA) for the aCGH.

cDNA microarrays (Studies I–III)

The spotted cDNA microarray slides used were produced at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University, Sweden (http://swegene.onk.lu.se) as described elsewhere [93] and contained ~27,600 sequence-verified IMAGE clones from the Research Genetics 47k IMAGE clone library (http://www.resgen.com/). Clone information was linked to gene names using the current build of the UniGene database (http://www.ncbi.nim.nih.gov/unigene) at the time of analysis hence representing ~17,500 (UniGene build 164; studies I & II) and ~16,000 (UniGene build 180; *Study III*) unique UniGene clusters.

Bacterial artificial chromosome (BAC) arrays (Study IV)

The high-resolution BAC arrays used in *Study IV* containing ~32,400 BAC clones were produced at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University, Sweden (http://swegene.onk.lu.se) as previously described using the 32K BAC clone library (CHORI BACPAC Resources, http://bacpac.chori.org/genomicrearrays.php) [118]. The tiling BAC arrays provide >99% coverage of the human genome with an average resolution of 50–80 Kbp.

Methods

RNA isolation (Studies I–III)

80–120 mg of grossly dissected tumor tissue was powdered under frozen conditions (liquid nitrogen) and total RNA was extracted using TRIzol® (Invitrogen, Carlsbad, CA, USA) and further isolation and cleanup was performed using the RNeasy® Midi Kit (QIAGEN, Valencia, CA, USA) as per the manufacturers' recommendations. In brief, the TRIzol homogenate was treated with chloroform to obtain a colorless upper aqueous phase containing the RNA, which was mixed with 70% ethanol before running it through the RNeasy columns and finally eluting in RNase-free water. RNA quality was checked using the Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and concentration using the NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), after which it was stored at –80ºC till further use.

DNA isolation (Study IV)

DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) followed by a phenol-chloroform purification. In short, freshly frozen tumor tissue was digested overnight at 55°C in a mixture of EDTA, Nuclei Lysis Solution and Proteinase K (20 mg/ml, ICN Biomedicals Inc, #193504). After treatment of the lysate with RNase Solution and Protein Precipitation Solution, DNA was precipitated using isopropanol. Phenol-chloroform purification of the DNA was performed in 2 ml Phase Lock Gel Light tubes (Eppendorf, Hamburg, Germany) and the purified DNA was precipitated using 100% ethanol. Quality and concentration were checked using the NanoDrop.

Labeling and hybridization

Studies I–III

The CyScribe cDNA Post labelling kit (Amersham Biosciences, Little Chalfont, UK) was used to synthesize amino allyl-modified cDNA from 25–35 µg of tumor RNA and 20–25 µg of reference RNA. The tumor and the reference were indirectly labeled using Cy3 and Cy5 respectively, purified using the Cyscribe GFX purification kit (Amersham Biosciences), and pooled before vacuum drying into a pellet. The blocking reagents Poly d(A) (Amersham Biosciences), Yeast tRNA (Sigma, St. Louis, MO, USA), and Human Cot-1 DNA® (Invitrogen) were added to the pooled sample in order to reduce non-specific hybridization. The pre and post hybridization steps were carried out manually with the help of the Pronto!™Universal Hybridization Kit (Corning Life Sciences, Corning Inc., NY, USA) according to the manufacturer's instructions. The labeled pellet was dissolved in the Pronto!™ Hybridization Solution and applied to the slide, which was then sealed into a humidified Corning® Hybridization Chamber at 42°C for 18–20 hours.

Study IV

2 µg each of tumor and reference DNA were labeled by random priming with Cy3 and Cy5, respectively, using the BioPrime Array CGH Genomic Labeling Module (Invitrogen). Unincorporated nucleotides were removed using the CyScribe GFX Purification Kit (Amersham Biosciences). The labeled tumor and reference DNA were combined with 100 mg Cot-1 DNA, dried by speed vacuum centrifugation, and redissolved in 40–50 ml of hybridization solution (50% formamide, 10% dextran sulfate, 2xSSC, 2% sodium dodecyl sulfate (SDS), and 10 µg/ml yeast tRNA) and applied to the slides. Hybridization was performed for 72 hours at 37°C in a humidified chamber. The post-hybridization washes were carried out in 2xSSC, 0.1% SDS for

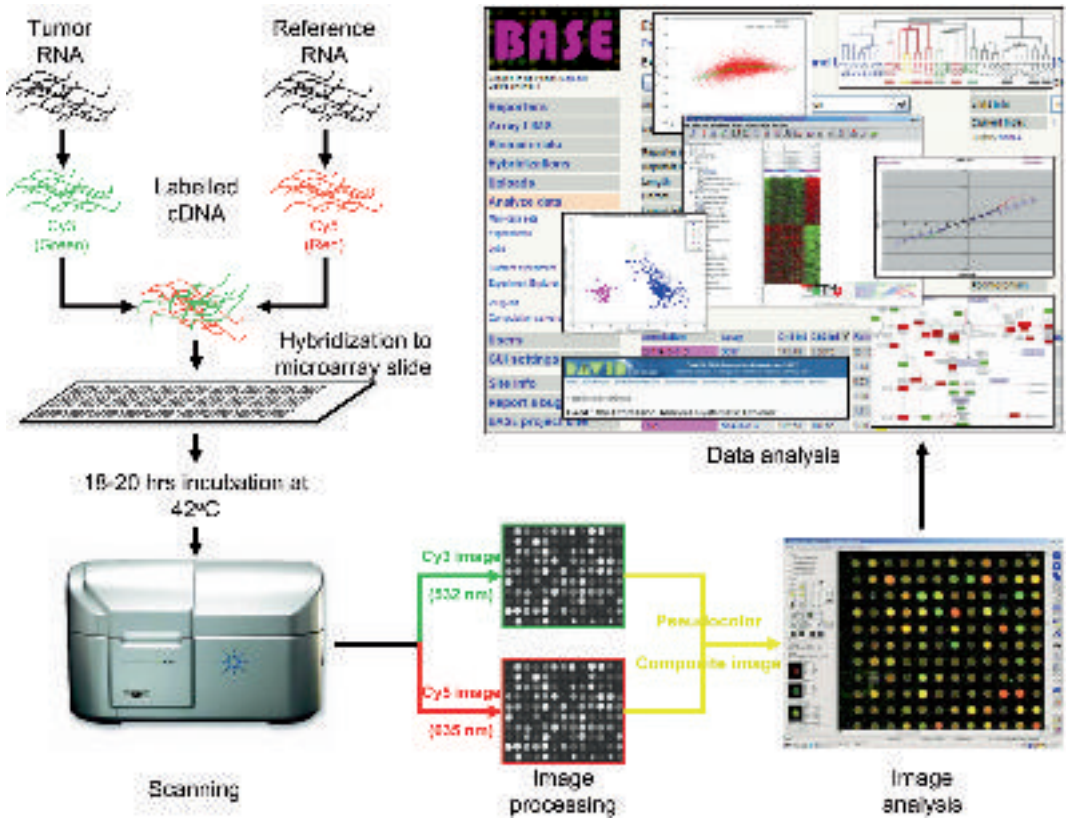

Figure 8. A brief overview of the cDNA microarray technology. The extracted mRNA from the tumor tissue and the reference RNA were converted to cDNA and labeled using different fluorescent dyes (green for tumor and red for reference) after which they were pooled together with blocking reagents and hybridized to the microarray slide. After washing to remove nonspecific hybridization, the slides were scanned in a microarray scanner, which works like a laser confocal microscope. The obtained grey-scale images for both fluorescent dyes were then overlaid to generate a composite pseudocolored image where the color of a spot represents the relative abundance of the respective gene transcript in both the samples (green spots represent upregulation in the tumor compared to the reference whereas red spots represent downregulation, and yellow spots are genes with equal expression in both samples). The image was then analyzed to extract intensity values in both channels for all spots. These data along with spot IDs and gene names were uploaded into BASE and other data analysis software for further management, analyses and interpretation of results.

15 min, followed by 2xSSC, 50% formamide (pH 7.0) for 15 min at 45ºC, 2xSSC, 0.1% SDS for 30 min at 45ºC, and in 0.2xSSC for 15 min at room temperature.

Image acquisition and analysis

The cDNA and aCGH slides were scanned using the Agilent DNA microarray scanner (Agilent Technologies) to obtain images of 5 µm to 10 µm resolution setting the photo multiplier tube in both the red (635nm) and green (532nm) channels to 100%. Using the image analysis and data extraction software GenePix™ Pro 4.1.1.4 version (Axon Instruments Inc., Foster City, CA, USA), the grey-scale images obtained from the two scanned channels were converted into a composite pseudocolored image depending on intensity-ratios [Figure 8]. The software automatically detected spots and aligned grids associating the spots with their reporter IDs and gene names. It also marked spots

as 'found' or 'not found' depending on spot intensity and quality. The automatic spot detection and gridding were manually verified for inaccuracies thereby eliminating artifacts and bad spots. The quantified foreground and local background pixel intensities for each spot along with IDs and gene names were extracted into a GenePix Pro result file which was uploaded and stored in BASE, the web-based BioArray Software Environment for management and analysis of microarray data (http://base.onk.lu.se/int) [213].

Computational analysis of gene expression data (Studies I–III)

Data filtrations and transformations

The result files from GenePix Pro for all hybridizations were imported into BASE and grouped together into an experiment. Background correction, filtering, transformations and analyses were performed uniformly on the group of array data. Preliminary quality filters excluded spots flagged as 'not found' in GenePix Pro, spots with diameter less than or equal to 55–60 μm, spots with signal-to-noise ratio (SNR) less than 1.5 in either channel, spots with more than 10% pixel saturation etc. Filters for presence and variation of expression across hybridizations were also used. The median of the local background pixel intensities was subtracted from the median of the foreground/spot pixel intensities in both channels for all spots. The background corrected intensity values were then rescaled or normalized to adjust for differences in quality of initial RNA from the two samples and differences in labeling and detection efficiencies of the fluorescent dyes. By using a pin-based locally weighted scatterplot smoothing (LOWESS) method of normalization [268], intensity-dependent adjustments or LOWESS fits were performed within groups of 8 print-tip blocks to compensate for spatial bias due to local background effects. The expression values, defined as the 2-logarithm of the expression ratios, were transformed using the error model [32, 207], which moved the expression value of a poor quality spot (with low SNR) towards the mean expression of that particular spot across all hybridizations thereby reducing its importance in later analysis steps. Data from replicate hybridizations were merged in a weighted fashion as were data from spots representing the same reporter ID or gene symbol on an array.

Hierarchical clustering

The expression ratios were analyzed mathematically for relationships among the samples with the help of an unsupervised agglomerative (or bottom-up) hierarchical clustering algorithm using the average-linkage method and the Pearson correlation distance metric. Here, pair-wise distances based on similarities in expression patterns were calculated (distance = 1 – Pearson correlation coefficient) between samples followed by successive clustering of the nearest most similar samples or subclusters till eventually all samples were fused into a single cluster or dendrogram, in which the height of a node represented the distance between two samples or subclusters. A two-dimensional (2D) heat-map accompanying the dendrogram with a red-back-green color-scale was used to represent up or down regulation of individual genes across the samples. Moreover, supervised hierarchical clustering based on genes discriminating various sample groups was performed for visualization purposes. The TMeV application from the TM4 microarray software suite (http://www.tm4.org/mev.html) was used to perform the cluster analyses in *Studies II–IV* [216]. In *Study I*, clustering of samples was performed in BASE using the Pearson correlation distance metric.

Multidimensional scaling (MDS) (Study I)

The similarities or distances between the samples were visualized using 2D-MDS in *Study I* where samples were represented as points and arranged in a two-dimensional Euclidean space such that the distances between pairs of points represented the similarities among the pairs of samples. In other words, two similar samples were represented by two points close together while two dissimilar samples were represented by two points far apart.

Discriminatory signatures (Studies II & III)

The differential expression between two classes of samples was tested using the Golub score (also known as the SNR method), named after the widely referenced paper by Golub *et al.* (88) that calculates the difference in expression between classes relative to the standard deviation within the classes. Genes were ranked based on their Golub scores defined as –

$$\left| \frac{m_1 - m_2}{s_1 + s_2} \right|$$

where, m_1 and m_2 are the mean expressions for class 1 and class 2 respectively, and s_1 and s_2 are the standard deviations. A high Golub-score implicates minor variation in gene expression within the class, but large variation between the classes. A random permutation test with 1,000 permutations was performed to determine whether the number of ranked genes associated with a class was significantly higher than expected by chance. For each rank, the average number of genes in a permutation list above that rank was divided by the number of genes in the true list to get the false-discovery rate (FDR) which was used to assess the strength and robustness of the discriminatory profiles.

Gene ontology and functional classification (Studies II & III)

Identification of functional correlations between the differentially expressed genes aided in biological interpretation of the discriminatory signatures. Over-represented functional groups and pathways were identified using the freely available EASE (Expression Analysis Systematic Explorer) software (http://david.abcc.ncifcrf.gov/ease/ease.jsp) [112]. The top ranking genes were classified into groups within the categorical systems of the Gene Ontology (GO) Consortium (GO Biological Process and GO Molecular Function), the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway, biochemical process, cellular role and chromosome.

Computational analysis of aCGH data (Study IV)

Data filtrations and transformations

Spots flagged as 'not found' in GenePix Pro and with SNR less than 3 in both channels were excluded in addition to all spots representing clones on the sex chromosomes. The median of the local background pixel intensities was subtracted from the mean of the foreground/spot pixel intensities in both channels for all spots. The background corrected intensity values were then normalized using global LOWESS (268). A 150-Kb weighted moving average fitting each clone to the local mean in the window was then applied to smooth out and reduce experimental noise over the chromosome profiles, thus highlighting the actual gains and losses [Figure 9].

Identification of gains and losses

A BASE-adapted MATLAB toolbox, CGH-plotter, was used for visualization of CGH data and to identify gains and losses (10). Copy number data was plotted as a function of position of the BAC clones along the human genome and copy number changes were identified using k-means clustering (k = 3, denoting amplified, deleted and baseline clusters) and dynamic programming. Regions of gains and losses were defined as two or more consecutive clones showing a $\log_2$ratio ≥0.2 and ≤–0.2, respectively and high-level amplifications were defined as at least two clones with a $\log_2$ratio ≥1.5, and homozygous deletions as $\log_2$ratio ≤–1.5. Hierarchical clustering of samples was performed in the same manner as described earlier but based on copy-number profiles of BAC clones instead of gene expression profiles.

Discriminatory copy-number profiles

A Student's T-test was used to identify BAC clones with significant ($P \leq 0.05$) differences in $\log_2$ratio of copy-number between two classes of samples like the MFH *versus* the LMS or the primary tumors that metastasized *versus* the ones that remained metastasis-free. The clones were then used to manually create regions which were further tested using the

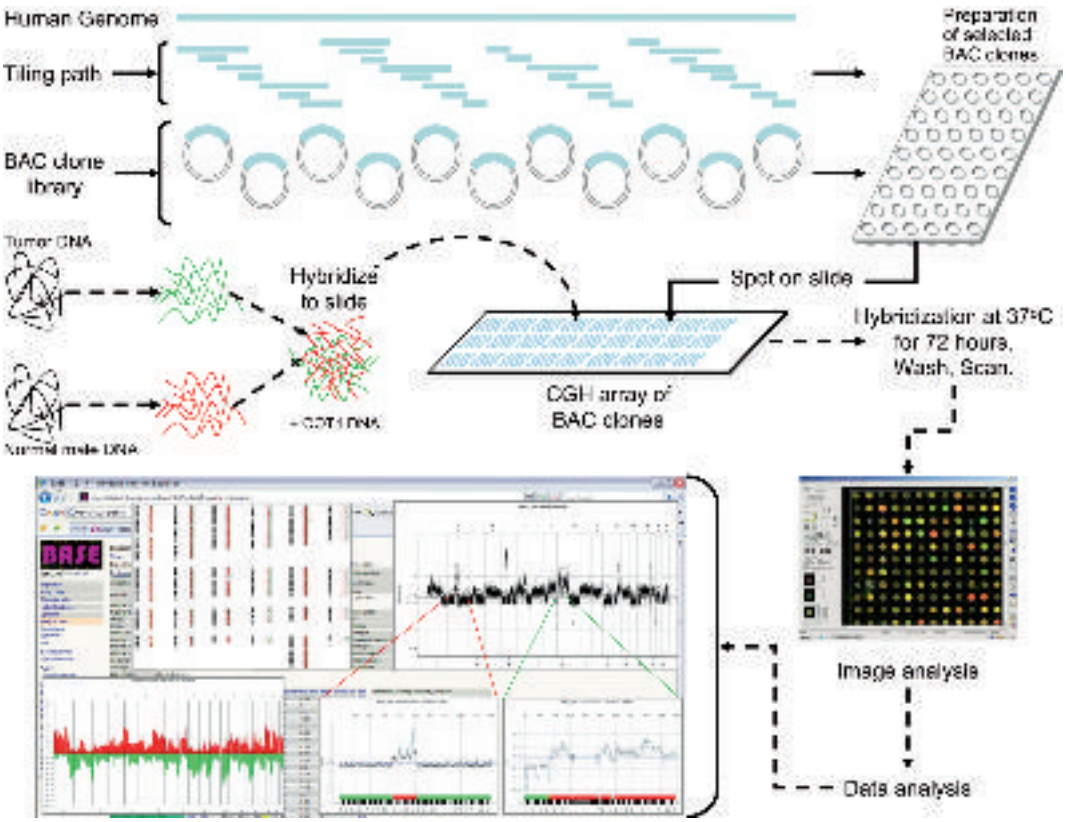

Figure 9. A brief overview of the aCGH technology.
A 32K BAC clone library with tiling coverage of the human genome was used to construct the aCGH slides. Tumor and reference DNA were differentially labeled using florescent dyes, pooled together with Cot-1 DNA and hybridized to the microarray slide. Thereafter, the slides were washed and scanned using the Agilent DNA microarray scanner to generate a pseudocolored image. The image was then analyzed to quantify foreground and background spot intensities in both channels, which were extracted and uploaded along with spot/clone IDs into BASE for further data analyses, visualization and identification of gains and losses.

significance analysis of microarrays (SAM) algorithm (with a delta value of 0.5 giving 0 fasle-positives) The SAM analysis assigns a score to each region depending on the change in copy-number relative to the standard deviation within the group. For the regions with scores greater than an adjustable threshold, SAM uses random permutations to estimate the FDR [147]. The regions found to be significant in the test were used for further statistical analysis like the univariate and multivariate analyses.

Statistical analysis (Studies III & IV)

The $\chi 2$ test for association, the Mann-Whitney's U test, and the Kruskal-Wallis test were used to assess associations of tumor size (as a continuous variable), necrosis (present *versus* absent), vascular invasion (present *versus* absent), and the SVM cross-validated classification with the development of metastasis in *Study III*. Follow-up time was computed from the time of primary diagnosis/surgery to the date of an event or to the date of the most recent follow-up. Metastasis-free survival curves were constructed by the Kaplan-Meier method [124] and compared by the log-rank test. Univari-

ate and multivariate Cox-regression analyses were performed to estimate hazard ratios (HRs) and to assess the independence of the cross-validated classification *(Study III)* or chromosomal regions (*Study IV*) from the prognostic factors [47]. Proportional hazards assumptions were checked using Schoenfeld's test [220]. Areas under ROC curves were compared using an algorithm suggested by DeLong *et al.* [56]. A two-tailed P-value of less than 0.05 was considered significant for all tests. Stata 9.2 was used for the statistical analyses (Stata Corporation, 2003, College Station, TX, USA).

Results and discussion

Study I

Genetically distinct clonal subpopulations differing in karyotype, growth rate, metastatic potential, expression of markers, and sensitivity to drugs give rise to intratumor heterogeneity seen in cancers [89, 241]. STS frequently display extensive intratumor heterogeneity in macroscopic as well as microscopic features [71, 183, 191, 192]. Örndal *et al.* demonstrated that cytogenetic heterogeneity and clonal evolution commonly occur in STS [192]. Moreover, extracellular matrix components and stromal cell populations like fibroblasts, immune cells and inflammatory cells add to the heterogeneity identified.

Gene expression studies in STS use total RNA from single pieces, thus obtaining profiles reflecting the different cell populations present in the tumor pieces, and raising concerns that sampling of single pieces from STS with significant intratumor heterogeneity and stromal cell contamination could introduce bias precluding the use of gene expression profiling for diagnostic and prognostic purposes. Some studies have shown that tumor heterogeneity can affect the accuracy of microarray analysis [186]. One way to overcome this problem would be the use of laser capture microdissection to isolate cancer cells from heterogeneous tumors but this is a rather expensive and laborious technique [25, 68]. Moreover, the RNA obtained from microdissected samples carry a risk of degradation due to the lengthiness of this procedure and is usually insufficient requiring additional amplification rounds which are not only difficult to perform starting from such small amounts but also introduces another level of bias and reduces reproducibility. Hence, total RNA extraction is the method of choice with its relative ease of performance and high yield. Furthermore, tumor stroma plays an important role in invasion and metastasis and its inclusion may provide important information regarding tumor microenviroment [21, 156].

In *Study I*, we evaluated how the genetic intratumor heterogeneity within multiple pieces from a myxoid MFH (8 pieces from M1) and a LMS (10 pieces from L1), displaying varying degrees of necrosis, vascular invasion, and cellular pleomorphism, could affect the sensitivity and tumor specificity of gene expression profiles. Additional single random pieces from 36 different MFH and LMS were used to measure the degree of intertumor heterogeneity within the subtypes. Hierarchical clustering, 2D-MDS and pair-wise Pearson correlation distance measurements all revealed much lower intratumor heterogeneity compared to intertumor heterogeneity.

Unsupervised cluster analysis after merging data from replicate assays, revealed tight subclustering of the multiple pieces from M1 and L1 [Figure 10] indicating that the multiple pieces from the same tumor displayed a higher similarity in expression profiles when compared to the single pieces from distinct tumors, suggesting a much lower intra- than inter-tumor heterogeneity. Subclustering of the multiple pieces based on their original location within the tumor was also observed, with the peripheral tumor pieces G, E, and H from M1 forming one subcluster, whereas the more central pieces, A–D and F, formed another. Also, in the LMS L1, which was exceptionally macroscopically heterogeneous with several macroscopic tumor nodules, subclustering of pieces next to each other was evident; pieces A and B (which formed one nodule) clustered together and were most closely related to nearby pieces C and D. Pieces E–H, which formed the largest nodule, clustered together, and finally, I and J from the last nodule, formed a separate subcluster [Figure 10].

M1 displayed a greater degree of intratumor variability with ~3,000 reporters showing variable expression levels (SD≥3) in the multiple pieces as

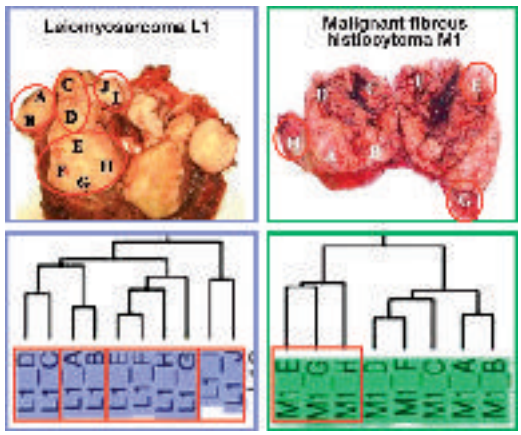

Figure 10. Unsupervised clustering showing tight clustering of the multiple pieces from the same tumors and subclustering of pieces depending on their original location within the tumor. Pieces from the same nodules in L1 clustered together while the peripheral pieces from M1 clustered separately from the more central pieces.

compared to only ~450 reporters in L1. This was reflected in the 2D-MDS analysis where pieces within M1 clustered farther apart from each other in comparison to the L1 pieces. Grouping of the intratumor pieces separate from the intertumor pieces was also observed in the 2D-MDS plot (*Study I*, Figure 2).

The Pearson correlation (centered, unsquared) coefficient, *r*, was measured for all pairs of samples within the groups M1 (M1A–H), MFH (M2–21), L1 (L1A–J), and LMS (L2–17), and corresponding pair-wise distances were calculated as $d = 1 - r$. The average pair-wise distances calculated within the groups showed that this value was close to 1 for the LMS and MFH groups and close to 0.5 for the L1 and M1 groups. Thus, the average intratumor distance in both tumor types was only about half of the average intertumor distance. Here again L1 displayed lower intratumor variability with an average intratumor distance smaller than the minimum intertumor distance, whereas the average intratumor distance in M1 was slightly higher but comparable to the minimum intertumor distance. The maximum intratumor distances in L1 and M1, however, were higher than the corresponding minimal intertumor distances (*Study I*, Table 3). Although some pieces in L1 and M1 were quite distant from each other in expression profiles, (represented by the heights of the nodes in the dendrogram and the maximum intratumor distance measures) the pieces were still closer to each other than they were to the single samples from the different tumors (*Study 1*, Figure 1). This information is taken into account by the clustering algorithm resulting in the multiple pieces forming tight clusters separate from the single pieces, despite the maximum pair-wise intratumor distance being greater than the minimum pair-wise intertumor distance.

Three intratumor pieces from the LMS L1 and 15 single samples from the different MFH and LMS were hybridized in duplicate and 1 in triplicate albeit on different print batches. In an unsupervised cluster analysis including the replicates, all replicate assays except for one pair (M18-1 and M18-2) clustered next to each other [Figure 11] and the average pair-wise distance (distance, $d = 1 -$ Pearson correlation, *r*) between the replicate assays (including the outlier pair M18-1 and M18-2) was 0.26 (range 0.07–0.58) suggesting a minimal degree of experimental variation. Furthermore, the distances between the 3 duplicate assays from L1 were comparable to the distances between the closest intratumor pieces, L1A-L1B and L1E-L1F indicating that the difference in expression profiles between the closest pieces within L1 was comparable to the experimental variability [Figure 11]. In a previously published study, Shmulevich *et al*. evaluated intratumor heterogeneity in 3 LMS from which 3 peripheral tumor samples and 1 core sample were obtained and concluded that variability between the different tumor sections was within the experimental variability between replicate experiments [232]. Another study of gene expression signatures in a limited set of mesenchymal tumors including STS, evaluated intratumor heterogeneity using two or more pieces from 7 tumors confirming that with-in tumor variability had very little impact on the analysis [119]. Similar results have been reported also in other cancer types [12, 117, 134].

The results from *Study I* suggest that intratumor heterogeneity is not a dominant source of error in

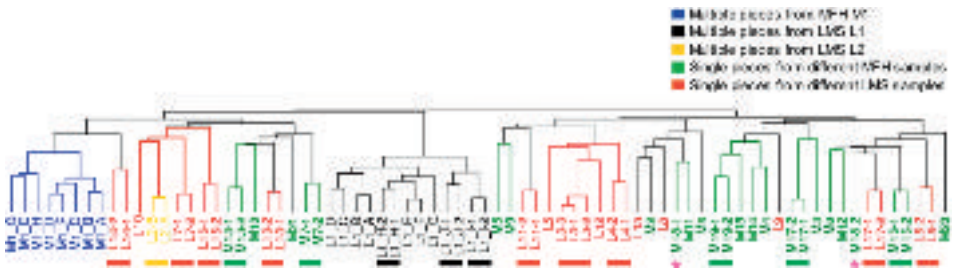

Figure 11. Unsupervised cluster of 74 MFH and LMS samples including replicate assays (denoted by the colored dashes), and multiple pieces from a MFH (M1) and two LMS (L1 and L2) based on ~5500 most variable reporters. Multiple pieces from the same tumors formed tight subclusters and all replicate hybridizations, except M18-1 and M18-2 (denoted by pink stars), clustered next to each other. The intratumor distances between the closest pieces within L1 (L1A-L1B and L1E-L1F) were comparable to the distances between the duplicate hybridizations (denoted by black dashes) with the height of a node in the dendrogram representing the distance between samples. Hence, suggesting that the intratumor variability was comparable to the experimental variability within L1.

microarray analysis. Thus, total RNA extraction from single pieces may be an effective strategy to obtain tumor representative profiles of diagnostic and prognostic utility in STS, despite the large size and heterogeneity. But intratumor variability may have an impact on the results from small sample sets and thereby limit the validity of the findings therein. It is therefore important to run large tumor series of pieces carefully selected avoiding necrotic tissue and normal cell infiltration in order to minimize the effects of heterogeneity, normal cell contamination and experimental variability and maximize chances of obtaining reliable and reproducible expression patterns in STS.

Study II

SS are aggressive spindle cell tumors that account for ~10% of all STS and are comprised of two major histopathological subtypes, biphasic and monophasic according to the presence or absence of a well-differentiated glandular epithelial component in addition to the spindle component. >95% of the tumors carry the t(X;18) (p11;q11) resulting in the *SS18-SSX* fusion gene product, which functions as an 'activator–repressor' transcription factor probably through epigenetic mechanisms [Figure 5 and 6] [48, 238].

The cDNA microarray analysis in *Study II* was performed in 26 SS and unsupervised analysis on all 26 samples did not reveal obvious clusters depending on any of the clinical parameters tested. But unsupervised clustering performed within the subsets used for further supervised analysis (i.e. the 23 samples included in the analysis of the fusion type discriminators and the 19 samples used for evaluation of the prognostic signature), a weak clustering based on the fusion type was seen [Figure 12 and 13].

Golub-score analysis identified gene expression profiles associated with gene fusion type (*SS18-SSX1 versus SS18-SSX2*) and development of metastasis (metastasis developed *versus* metastasis-free). 100 genes (~40% FDR) were found differentially expressed between the two gene fusion types and among the genes overexpressed in the *SS18-SSX1* variant were several developmental genes, e.g. *TCF7, DTX3, ZIC2* and *COL6A3,* groups of histones and metallothioneins (*Study II*, Figure 1 and Table 2). Metallothioneins, involved in metal-ion binding, protection against oxidative stress, cell proliferation and apoptosis, chemoresistance and radiotherapy resistance, are known to be expressed in majority of the SS and correlate with proliferation and grade in STS [65, 66, 247]. A high expression of histones in tumors with the *SS18-SSX1* fusion type indicates increased chromatin assembly activity and DNA replication (202). Moreover, the C-terminal end of SSX1 is known to bind histones and associate the SS18-SSX1 fusion product with the chromatin [129]. Hence,

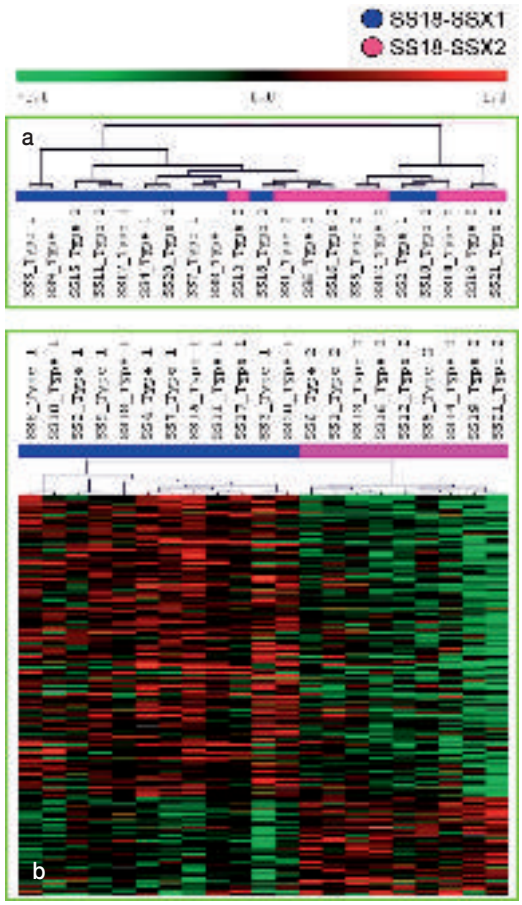

Figure 12. a. Unsupervised clustering of the 23 SS samples used to generate the fusion-type discriminating signature based on ~3,500 reporters.

b. Supervised clustering based on the top 300 genes discriminating the SS18-SSX1 fusion type tumors from the SS18-SSX2 type.

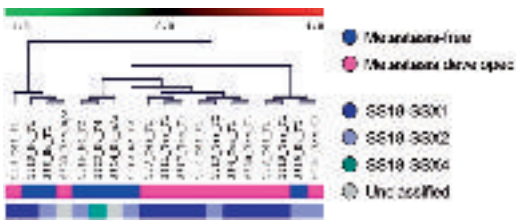

Figure 13. Unsupervised clustering of 19 SS samples used in the generation of the prognostic signature based on ~3,600 reporters.

the upregulation of metallothioneins and histones in SS with the *SS18-SSX1* fusion type may indicate increased proliferative activity as compared to those with the *SS18-SSX2* fusion. And interestingly, the *SS18-SSX1* fusion has previously been associated with a high proliferative rate and poor outcome [180]. Among the genes overexpressed in the *SS18-SSX2* fusion type were transcription factors and developmental genes including *FOXC1, GAS1, GATA6, NCAM1* and *NEDD4*. Figure 12b shows a supervised cluster based on the top 300 fusion type discriminating genes with ~50% FDR.

30 genes (~30% FDR) were found discriminating the primary tumors that metastasized from those that remained metastasis-free (*Study II*, Figure 2). Several of the top ranked genes overexpressed in the metastasizing tumors were those involved in cell cycle progression, e.g. *BIRC5, CCNB2, CENPF, PRC1, RRM2, TOP2A*, and *UBE2C*, many of which are proliferation markers highly expressed in cancers and associated with poor prognosis [185, 187, 190, 227, 228, 255]. The top gene overexpressed in the metastasizing tumors was the baculoviral IAP repeat-containing 5 (*BIRC5*) also known as survivin which is upregulated in cancers and inhibits apoptosis and regulates cell division. Survivin has previously been shown to correlate with proliferation and angiogenesis and predict poor prognosis and shorter survival in various cancer types including STS [125, 126]. Survivin is a useful diagnostic marker and a potential target for cancer treatment since it is not expressed in normal adult tissue and its inhibition reduces tumor growth by induction of apoptosis [36, 266]. Another interesting gene overexpressed in the metastasizing SS was the nuclear enzyme DNA topoisomerase II alpha (*TOP2A*) which is a well-known target for chemotherapeutic agents [87].

In previous gene expression studies, SS have revealed distinct homogenous expression profiles with increased expression of genes from the EGFR, FGFR, Wnt and retinoic acid receptor signaling pathways [3, 14, 148, 177, 222, 236, 251]. Many of the discriminating genes identified in our study, e.g. *AGRN, AXL, SPAG7, TNNT1* and *ZIC2*, have previously been shown to be upregulated in SS [222, 236]. Expression profiling of SS subtypes have

shown subclusters based on tumor morphology. Allander *et al.* identified 21 genes in 14 SS that distinguished monophasic tumors from biphasic, and included keratins which were overexpressed in the latter histotype, whereas Nagayama *et al.* identified ~1,400 genes that split 13 SS into 2 groups – one consisting of only monophasic tumors and the other of both monophasic and biphasic with the biphasic subtypes clustering together [3, 177]. Our study, however, did not identify expression profiles related to histotype (monophasic *versus* biphasic) or karyotypic complexity (simple with t(X;18) *versus* complex). This could be due to insufficient sample numbers to detect the small differences in gene expression between subsets within a homogenous tumor type. Only 6 samples including two pairs from the same patients were biphasic and only 5 samples had the simple karyotype with the t(X;18) translocation including a pair from the same patient. Hence, after excluding one of the two tumors from the same patients, we were left with only 4 biphasic and 4 karyotypically simple samples for the Golub-score analysis.

The *SS18-SSX* fusion variants have important effects on SS biology as is clear from its correlations with glandular epithelial differentiation, sex, primary site and survival [9, 94, 132, 143]. The SS18 nuclear protein associates with the SWI/SNF complex and functions as a transcriptional coactivator, whereas the SSX proteins associate with the polycomb complex and function as transcriptional corepressors, but not much is known about the functions and targets of the *SS18-SSX* fusion proteins [48, 199, 240, 246]. This is the only report of an expression profile related to gene fusion type in SS confirming that *SS18-SSX1* and *SS18-SSX2* have different downstream effects leading to differential expression of various developmental genes, transcription factors, histones and metallothioneins, which may provide important information regarding the histopathologic and prognostic differences seen in the SS subtypes. A recent study suggesting that the SS18-SSX2 fusion product causes deregulation of downstream targets through epigenetic mechanisms identified groups of genes responsive to SS18-SSX2 including genes involved in cholesterol synthesis (52). Some of the genes identified therein were also among our fusion type discriminators, e.g. *TCF7, INSIG1, ALDOC, SLC3A2, AGRN, RRM2, ERCC2, DCT, PLP2, STX4A, TUBG1* and *GAS1*.

Metastases develop in about 50% of the SS patients and development of a molecular prognosticator would be valuable for decisions on adjuvant chemotherapy. Our study identified a metastasis signature in 19 primary tumors, but with a relatively high FDR (*Study II*, Figure 2 and 3). However, with respect to biology and function, the top 30 differentially expressed genes included many cell cycle regulators previously associated with proliferation and poor outcome in other tumor types [36, 87, 185, 187, 190, 227, 228, 255, 266]. And although majority of the metastasizing tumors (9/12) in the current study had the *SS18-SSX1* fusion and the majority of the non-metastasizing tumors (4/7) had the *SS18-SSX2* fusion, the top 30 metastasis discriminators (including 9 false positives) had only 2 genes (*RRM2* and *STXBP6*) in common with the top-100 gene fusion discriminators and only 7 in common with the top 300 gene fusion discriminators, confirming that the two signatures were distinct with limited overlap between the gene lists. The genes identified in this study provide clues to SS biology, but need to be confirmed in larger sample sets.

Study III

Expression microarrays constitute one possible tool with potential to improve diagnostic accuracy and identify previously unrecognized STS entities. Distinct and homogenous gene expression profiles have been reported in SS, DFSP, clear cell sarcoma Ewing sarcoma, rhabdomyosarcoma, myxoid/round-cell liposarcoma and GIST [3, 4, 13, 14, 148, 155, 177, 222, 223, 236, 254]. Furthermore, the discriminating genetic profiles often contain genes located downstream of the type-specific pathogenetic gene fusions or mutations. The possibility to identify upregulated genes also constitutes an important basis for the identification of therapeutic targets. Gene expression profiles have also provided clues

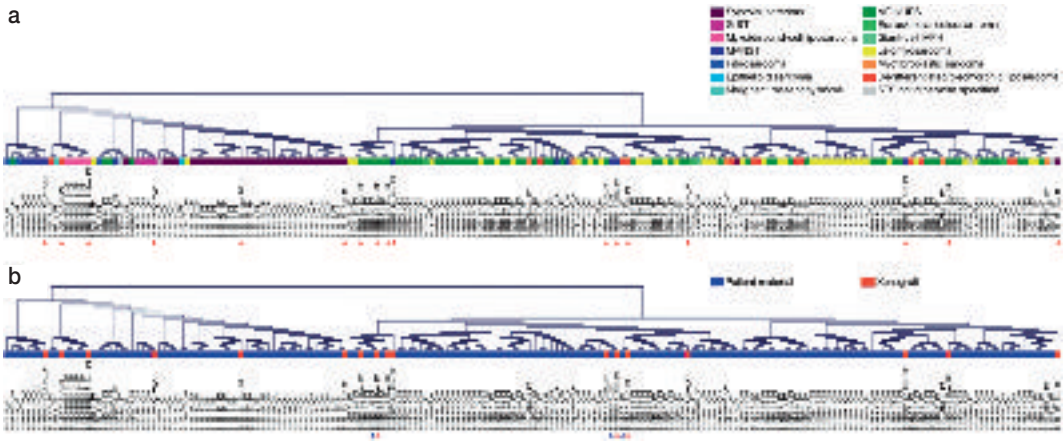

Figure 14. a. The unsupervised cluster analysis of the 194 STS including 17 xenografts samples, where 2/3 synovial sarcoma xenografts and the single GIST and myxoid/round-cell liposarcoma xenografts clustered with their respective tumor histotypes, whereas all 6 MFH xenografts were part of the pleomorphic STS subcluster (the xenografts are indicated by red arrows).

b. The same unsupervised cluster as above, this time showing the 3 xenografts derived from tumors included in the study (red arrows) that clustered next to their respective patient samples (blue arrows).

to the histogenesis of some STS subtypes and may facilitate the distinction between pathologically inseparable lesions, e.g. neural crest origin of SS, classification of clear cell sarcoma as a subtype of melanoma, and distinct expression profiles for well-differentiated lesions from dedifferentiated liposarcomas and well-differentiated liposarcomas [177, 222, 229]. Current data from the more pleomorphic subtypes, however, indicate that the expression profiles are indeed heterogeneous and in these tumor types the primary goal may rather be to identify novel, yet unidentified subgroups, and to clarify deregulated genes and pathways.

In *Study III*, we used 27K cDNA microarrays to characterize the expression patterns in a mixed series of 177 STS, with the aim to establish diagnostic expression profiles for STS, and to assess whether gene expression profiling provides prognostic information, with particular focus on high-grade pleomorphic STS. Unsupervised cluster analysis, based on the 6,140 spots that passed the filter criteria identified two major clusters (*Study III*, Figure 1). One cluster consisted mainly of STS with distinctive histopathology and type-specific genetic defects, e.g. SS, myxoid/round-cell liposarcoma and GIST. Herein, SS, GIST, myxoid/round-cell liposarcomas, and MPNST formed tight subclusters. The other subcluster contained STS subtypes that are more often characterized by complex genetic alterations, such as MFH/UPS, LMS, and dedifferentiated/pleomorphic liposarcomas. A subset of 11 LMS formed a separate subcluster, which was characterized by upregulation of muscle-specific genes. This kind of separation based on genetic complexity has previously been shown using genomic and proteomic profiling [179, 222, 243]. Developmental pathways and genes involved in differentiation and morphogenesis seemed to play a more important role in the STS subtypes from the former cluster, whereas the latter more pleomorphic cluster was characterized by overexpressed genes involved in cell-cycle, proliferation, adhesion, motility, protein degradation, homeostasis and immune-response.

In a separate unsupervised analysis including the 17 xenografts a similar pattern of clustering was observed with xenografts from SS, myxoid/round-cell liposarcoma and GIST clustering with the patient samples of the same histotype and the xenografts from MFH/UPS scattering among the pleomorphic samples [Figure 14a]. Moreover, 3 xenografts derived from tumors included in the

study clustered right next to their respective patient sample [Figure 14b], indicating that the xenografts show higher similarity to their patient samples than to other xenografts of the same histotype. These results suggest that xenografts reflect the expression patterns of patient tumor material and can be included with some caution in gene expression studies of STS to increase sample size for rare tumor types.

In a supervised analysis, genes discriminating the diagnostic subsets were identified except in the myofibroblastic sarcomas and the extraskeletal osteosarcomas. The SS, GIST and myxoid/round-cell liposarcomas were characterized by the strongest signatures including 4,000, 1,500 and 1,000 genes (11–15% FDR) out of the ~6,000 most variable genes used in the discrimination scoring, whereas the dedifferentiated/pleomorphic liposarcomas and fibrosarcomas were characterized by the weakest signatures with high FDR [Figure 15 and Appendix Table 1].

Synovial sarcoma

SS has in several studies displayed homogenous and distinct expression profiles that enable clear distinction of these tumors from other STS [3, 148, 177, 179, 222, 236]. Our findings are in line with previous results, with 30 of the 32 SS forming a distinct cluster and characterized by the strongest expression signature of over 4,000 genes differentially expressed between the SS and other STS with a FDR of only 11% [Appendix Figure 1]. The SS chromosome X breakpoint genes, *SSX3* and *SSX1* were among the top upregulated genes. Multiple developmental pathways that interact to regulate embryonic development and organogenesis were upregulated in the SS as compared to the other STS subtypes, and included the FGF, EGF, TGFβ, Wnt, Notch, retinoic acid and Hedgehog receptor signaling pathways. *FGF18, FGFR1, FGFR3, FGFR4, FRAG1, ERBB2, TOB1, TOB2* and *SHC1* among others from the FGF and EGF receptor signaling pathways were highly expressed. EASE analysis identified upregulation of the Wnt receptor signaling pathway, including *FZD1, FZD7, FZD8, AXIN2, DKK3, LEF1, PPP2R1A, SENP2,* and *TCF7,* and the TGFβ signaling pathway including *BMP4, BMP5, BMP7, INHBA, RUNX3, SMAD6, TGFB1, TGFB2* and *TGFB1I4*. Several genes from the Notch receptor signaling pathway were differentially expressed in the SS, among which were *NOTCH1, JAG1, DTX3, TLE2, TLE3*, and *TLE4* and target genes like *CCND1, CDKN1B*, and *KRT14*. Members of the Hedgehog signaling pathway like *SMO, PTCH, BMP7*, and *CSNK1E* were overexpressed as were many retinoic acid receptor pathway genes including *RARG, RARA, RARB, RAI17, ENC1, MDK* and *MEIS1*. One of the largest groups of upregulated genes, also identified in the EASE analysis, was that involved in chromatin remodeling including several histones and SMARC genes, as well as *CBX1, ARID1A, HDAC1, SET, CHD2, CHD3, CHD6* and *CHD9*. A large number of neural differentiation genes like *APLP1, CPNE6, EFNA1, EFNA4, ENC1, MDK, NCAM1, NEDD5, NPDC1* and *OLFM1*, ribosomal proteins and many forkhead box transcription factors that regulate cell growth, proliferation, differentiation, and embryonic development were also highly expressed [Appendix Figure 1, 9–12]. In the supervised clustering based on the top 4,000 discriminating genes, the SS clustered closest to the single epithelioid sarcoma and then to the MPNST subcluster [Appendix Figure 1]. Epithelioid sarcoma and SS are believed to share a common histogenetic background owing to similarities in epithelioid morphology, ultrastructural features and expression of common epithelial and mesenchymal proteins, and recently the *SS18-SSX1* gene fusion was reported in an epithelioid sarcoma [54]. Previous studies have shown SS and MPNST to share similar patterns of gene expression with upregulation of neuroectodermal differentiation genes thereby suggesting a neural crest origin [3, 14, 177]. Several neural differentiation genes were also among the upregulated genes identified in the present study. The SS18-SSX fusion product is believed to control gene expression by association with chromatin remodeling complexes: SS18 is known to interact with SWI/SNF complexes

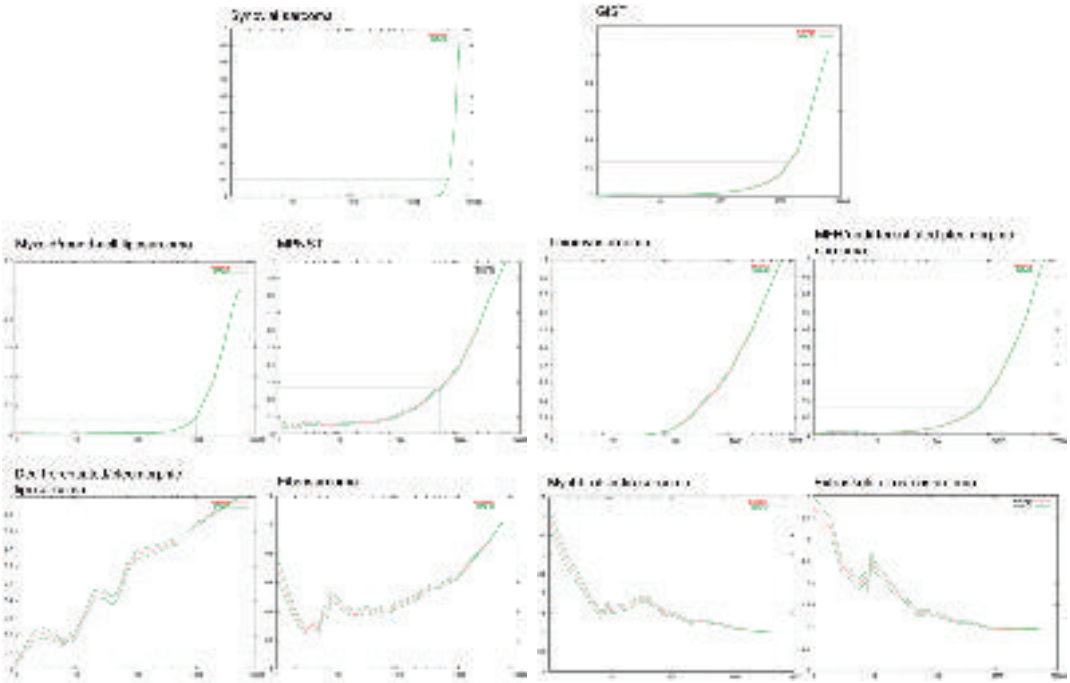

Figure 15. FDR plots for the diagnostic signatures with the number of ranked genes plotted along the x-axis and FDR along the y-axis. Random permutation tests with 1000 permutations were performed to assess the discriminating power of the Golub-score ranked genes. For each score, the average number of genes in a permutation list above that score was divided by the number of genes in the true list to get the FDR.

and SSX with histones [129, 199, 246]. Interestingly, chromatin remodeling genes, including several histones and SMARC genes, constituted one of the largest functional groups upregulated in the SS. And most importantly, the genes identified herein verified the significance of developmental pathways including the FGF, EGF, TGFβ, Wnt, Notch, retinoic acid and Hedgehog receptor signaling pathways, the targeted inhibition of which may have significant therapeutic implications [Appendix Figures 9–12]. Disruption of the retinoic acid receptor, FGFR, and Wnt signaling pathways have been shown to inhibit growth and proliferation in SS cell lines [29, 115, 176].

Gastrointestinal stromal tumor

GIST constitute a distinct STS subtype that is increasingly believed to originate from the interstitial pacemaker cells of Cajal that control gut motility [234]. Constitutive activation of the transmembrane tyrosine kinase KIT and its signaling pathway is fundamental in the development of GIST, with about 80% of the tumors carrying activating mutations in *KIT*, thus allowing the KIT-inhibitor imatinib mesylate to be used as a molecular target drug for this subtype [58, 106, 209]. The GIST, as previously demonstrated, were characterized by a distinct expression profile of 1,500 genes with a ~25% FDR, including several over-expressed genes involved in the KIT receptor signaling pathway, e.g. *KIT*, the KIT ligand *SCF, PIK3CG, PIK3R1, PIK3CB, PRKCQ, BAD, CREB3, JAK3* and *STAT3*. Other groups of upregulated genes identified in the EASE analysis were those involved in protein transport like *ARF1, ARF3, COG2, COG5, FXC1, LMAN2, SEC5L1, SRP68, VPS28*, and *WRB*, lipid metabolism genes like *ACSL3, ACSL5, PTGIS, AGPAT3, MIR16, SMPD1, ALDH1A2, ALDH2, MVD, MVK*, and *APOL1*, in addition to several genes with kinase activity like *ACK1, CSNK1D,*

CPNE3, CDK9, HIPK2, HIPK3, MADD, NME4, PFKM, PHKB, ROCK2, STK17A, TK1 and *TRRAP*. Developmental genes like *BMP4, DLK1, DTX3L, FGF2, FOXF1, HOXA4, HOXA9, IGF2, SFRP1*, and *TLE4* and several genes involved in neurogenesis and neural differentiation like *APBB1, APLP1, APP, DLG4, NCAM2, NEDD5, NPTX1, NTE, SIM2*, and *SRA1* were also upregulated in the GIST [Appendix Figure 2]. GIST are known to have features of myogenic and/or neural differentiation, both of which characterize the Cajal cells. Several developmental genes and genes involved in neurogenesis and neural differentiation were overexpressed, as were muscle-specific genes like smoothelin and myosin.

Malignant peripheral nerve sheath tumor

The MPNST may be hard to morphologically distinguish from SS [239] and in the unsupervised hierarchical clustering, 5/8 MPNST samples clustered with the STS with type-specific defects, 3 of them forming a tight subcluster close to the SS. There was considerable amount of overlap between the discriminating genes and pathways identified for the two subtypes, with several developmental genes from the Wnt, Hedgehog, and retinoic acid signaling pathways as well as neural differentiation genes and ribosomal proteins upregulated in the MPNST. Golub-score analysis identified a 500-gene signature (27% FDR) that grouped 5/8 MPNST samples tightly and was characterized by overexpression of several Wnt receptor signaling genes, including *FRAG1, PRKCB1, WISP2, CSNK2A2, CSNK1D, PRICKLE2, VANGL2, TLE2, VILL, ERBB2, IGFBP2, EFNA4*, and *DAAM1*. The sprouty homologues *SPRY1* and *SPRY2, SMO, RARA, RARB*, and *BMP7* from the Hedgehog and retinoic acid signaling pathways as well as neural development genes such as *NPDC1, GSTP1, KCNQ2, SEMA3A, PDE4A, PEX5*, and several ribosomal proteins were also upregulated. [Appendix Figure 4].

Fibrosarcoma

Like the MPNST, the fibrosarcomas have also been shown to cluster with the SS [14, 222]. Though the fibrosarcomas in this study did not form a tight cluster, 2/3 were part of the subcluster of STS with type-specific defects and a weak expression signature including many developmental genes distinguished these tumors from the remaining STS [Appendix Figure 8]. Golub-score analysis identified a relatively poor discriminatory signal of 200 genes for the fibrosarcomas, which regardless of high FDR (50% FDR) contained several of the upregulated genes previously associated with fibrosarcoma, e.g. *BMI1, H1F0, LEF1, RBM4, ITM2A, IGFBP2* and *PTGS2* [222]. Upregulation of developmental genes like *BMP7, SMO, VANGL2, SFRP1, PRRX1, MDK, OLFM¸ IGFBP3, IGFBP5* and *TGFBR3* in the fibrosarcoma samples suggests similarity to SS, GIST, myxoid/round-cell liposarcoma and MPNST explaining its classification within the subcluster with specific subtypes (*Study III*, Figure 1). This shows that FDR, though important, cannot be taken at face value without the risk of losing biologically relevant information, especially in the case of STS where high FDR may result not only from technical variability, heterogeneity and errors in diagnosis but also due to common pathogenic pathways resulting in similarities or overlap of expression profiles between different STS subtypes.

Myxoid/round-cell liposarcoma

Liposarcomas account for about 20% of STS and appears in distinct histopathological types; well-differentiated, round-cell, myxoid, dedifferentiated, and pleomorphic liposarcomas. The myxoid/round-cell liposarcomas, characterized by the *TLS-CHOP* or *EWS-CHOP* fusions, formed a distinct cluster closely related to the SS and were characterized by a distinct gene expression signature of about 1,000 genes (FDR of ~10%) [Appendix Figure 3] [49]. EASE analysis identified upregulation of several lipid metabolism genes, including *LPL, PPARG, FABP5, EBPL, MGLL*, and *DGKD*. A group of about 30 ribosomal subunit genes was also upregulated in the myxoid/round-cell liposarcomas, in addition to other genes involved in development, and amino acid and carboxylic acid metabolism. Furthermore, among the top overexpressed genes was lipid metabolism gene *PPARG*

that regulates adipocyte differentiation and the use of PPARG agonists for treatment of liposarcomas has been investigated [59, 249].

Dedifferentiated/pleomorphic liposarcoma

The dedifferentiated/pleomorphic liposarcomas, on the other hand, display complex genetic alterations and scattered among the pleomorphic samples on unsupervised clustering. However, Golub-score analysis identified a relatively weak discriminatory signature of 150 genes (~66% FDR) that grouped 3 dedifferentiated and 2 pleomorphic liposarcomas tightly with 2 MFH/UPS, 2 LMS and 1 fibrosarcoma. The remaining 2 dedifferentiated liposarcomas formed a separate tight cluster farther away on a different branch whereas 5 pleomorphic liposarcomas were scattered randomly within the pleomorphic cluster [Figure 16a]. Among the top overexpressed genes, were genes previously linked to 12q alterations, including *MDM2, SAS, CDK4, MDM1* and *OS4*. These genes along with several others upregulated, e.g. *YEATS4, SLC35E3, DKFZP586D0, SLC26A10, TSFM, NUP107* and *TMBIM4* are located in the amplicons on 12q14.1 and 12q15. Also, the MFH/UPS, the LMS and the fibrosarcoma that clustered tightly with the dedifferentiated/pleomorphic liposarcomas showed amplification of *CDK4* and *MDM2* when analyzed by aCGH [Figure 16b] or Southern blot analysis [20]. Lipid metabolism genes such as *PPARA, PDE3A, DHRS3, ACAA2* and *ARSA* were also overexpressed, as were several genes with receptor activity, e.g. *DCL1, RAMP3, KIT, GPR124, CHRNA4,* and *TYROBP*.

Myxoid/round-cell liposarcoma versus dedifferentiated/pleomorphic liposarcoma

When the myxoid/round-cell liposarcomas were directly compared to the dedifferentiated/pleomorphic liposarcomas in an independent analysis of the 19 liposarcomas (including 16 tumor and 3 xenograft samples), the 5 myxoid/round-cell liposarcomas clustered separately from the 14 dedifferentiated/pleomorphic liposarcomas in the unsupervised analysis and a 1,000-gene signature (15% FDR) distinguished the two groups [Figure 17]. Developmental genes, including many involved in neurogenesis e.g. *LHX2, CPNE6, MDK, EFNA5, FEZ2, NTNG1, GPM6B*, were upregulated in the myxoid/round-cell liposarcomas along with several ribosomal proteins and amino acid metabolism genes. Genes involved in adhesion, e.g. *CDH15, CDH16, CTNND1, BYSL, NCAM2, NID, NID2, LAMC1,* and *AM1* as well as genes within the Wnt receptor signaling pathway, e.g. *WISP2, FZD8, DAAM1, DAAM2, PRICKLE1, PRICKLE2, SFRP1, WSB1, MYC,* and *FRAG1* were upregulated in the myxoid/round-cell liposarcomas as compared to the dedifferentiated/pleomorphic liposarcomas. Genes overexpressed in the dedifferentiated/pleomorphic liposarcomas included cell-cycle genes, e.g. *CCNA2, CCNB2, CDC2, KIFC1, KIF23, KIF11,* and *PTTG1* as well as motility genes like *ANXA1, CNN2, FN1, AMFR, CKB* and homeostasis related genes including several metallothioneins, *NUCB1, NUCB2,* and *ATOX1*. A greater number of lipid metabolism genes were overexpressed in the dedifferentiated/pleomorphic liposarcomas compared to the myxoid/round-cell liposarcomas, with genes such as *ANXA1, ANXA4, GRN, CRYL1, PLA2G4A, PLA2G12A, PLTP, PLD1,* and *ADM* upregulated in the former group, while *FDFT1, DGKD LPL, PPARG, FABP5,* and *EBPL* were overexpressed in the latter. A previous study by Skubitz *et al.* has demonstrated separation of myxoid liposarcoma from non-myxoid liposarcoma using a set of ribosomal genes [235].

Leiomyosarcoma

About one third of the LMS formed a distinct tight cluster in the unsupervised analysis and herein supervised analysis identified a 500-gene signature (26% FDR) with an over-representation of muscle-specific genes like *ACTA2, ACTG2, ACTN3, CALD1, CNN3, GENX-3414, MBNL1, MLC1, MYH11, MYL4, MYL9, SLMAP, SMTN, SNTA1, TPM1, TPM2* and *TAGLN3*. This profile grouped together half of the LMS samples while the rest remained scattered among the pleomorphic samples [Appendix Figure 5]. Carbohydrate metabolism and energy pathway genes were also upregulated in the LMS cluster. This is in line with previous observations

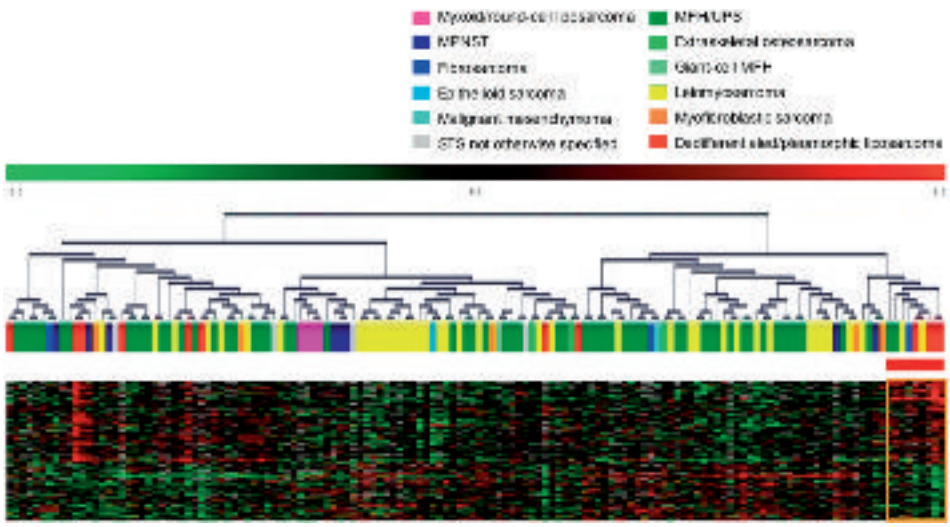

Figure 16a. Supervised clustering of 142 STS samples based on the top 150 dedifferentiated/pleomorphic liposarcoma discriminating genes showing 5/12 dedifferentiated/pleomorphic liposarcomas clustered tightly with 2 MFH/UPS, 2 LMS and 1 fibrosarcoma, all of which possess the 12q14-12q15 amplicons harboring the *CDK4-MDM2* amplification.

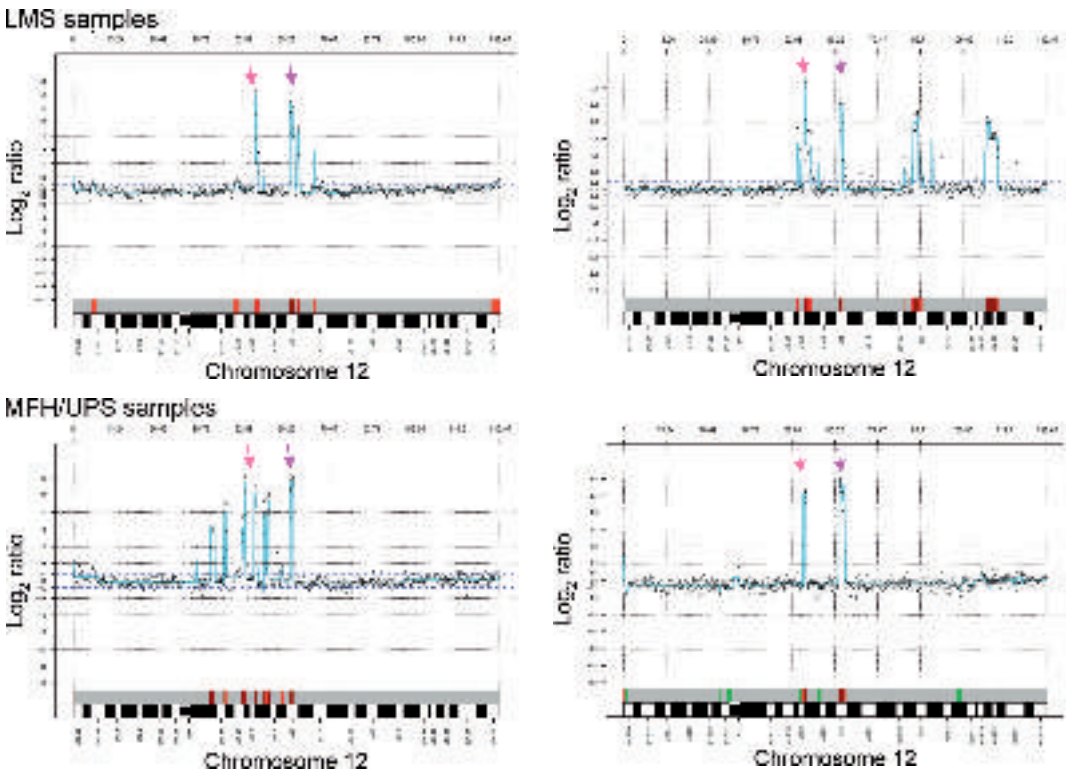

Figure 16b. Copy-number profiles over chromosome 12 showing high-level amplifications of *CDK4* (pink arrow) and *MDM2* (purple arrow) in the 2 LMS and 2 MFH/UPS samples that clustered with the dedifferentiated/pleomorphic liposarcomas.

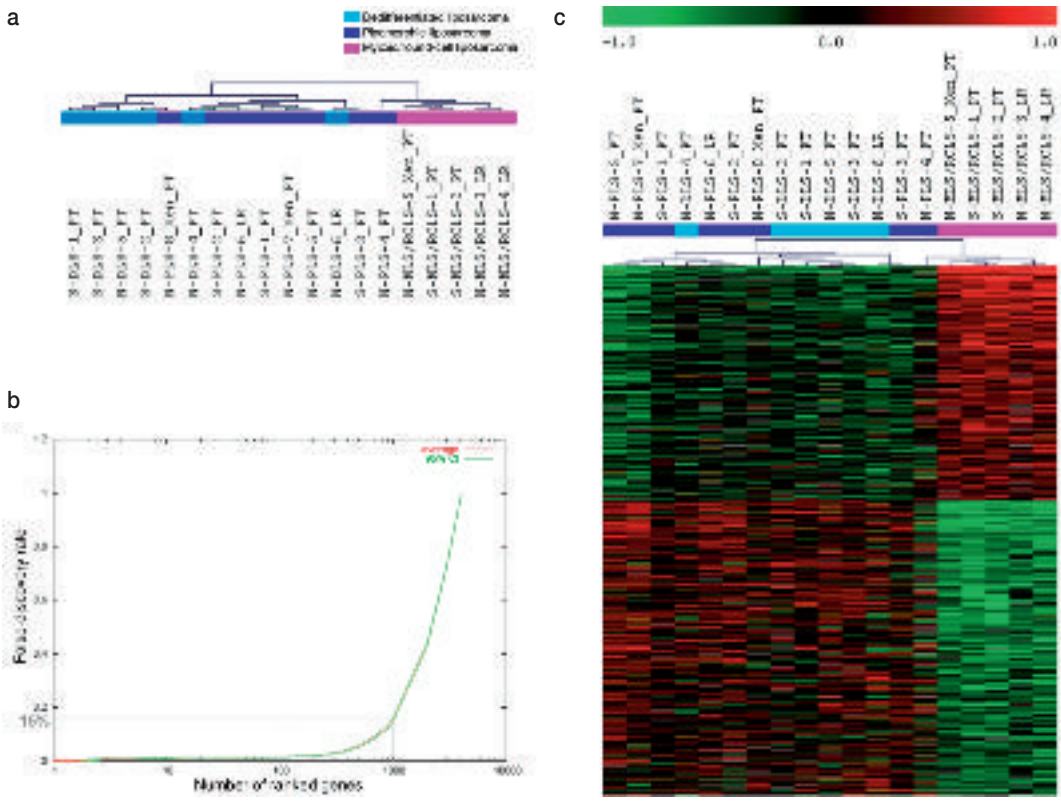

Figure 17. Independent analysis of the 19 liposarcoma samples including 16 patient samples and 3 xenografts.
a. The unsupervised cluster analysis of the 19 liposarcoma samples.
b. The plot b shows FDR within the Golub-score ranked genes distinguishing the myxoid/round-cell liposarcomas from the dedifferentiated/pleomorphic liposarcomas. The number of ranked genes is plotted along the x-axis and FDR along the y-axis.
c. Supervised clustering of the 19 liposarcoma samples based on the top 1,000 genes discriminating the myxoid/round-cell liposarcomas from the dedifferentiated/pleomorphic liposarcomas.

demonstrating that while the more pleomorphic LMS cluster with the MFH, another more distinct subset cluster separately [179]. Proteomic profiling of STS has also demonstrated a similar clustering pattern with the conventional LMS forming a distinct group from the pleomorphic ones that clustered with the MFH samples [243].

Malignant fibrous histiocytoma/undifferentiated pleomorphic sarcoma

Our series contained a large proportion, 60/177 (34%), of MFH/UPS. In the unsupervised analysis, all but one of the 60 MFH/UPS fell within the pleomorphic subcluster and were characterized by a 500-gene signature (16% FDR) that clustered half of the samples together [Appendix Figure 6]. The top upregulated functional groups included protein degradation genes like *SQSTM1, DAB2, CASP6, PPGB, MMP1, FAP* and cathepsins, genes involved in inflammatory response and cell motility like *ACTR3, ANXA1, CCL13, CCR1, CEBPB, CXCL1, IL8, LGALS3, NMI, PXN, RAC1,* and *RALA*, cell-proliferation and cell-cycle genes like *S100A11, TOPK, KLF4, BUB1, EMP3, KNTC2, MAD1L1, NOL1, TNFSF4, TNFSF7, TAL1,* and *PTTG1*, and genes from the intracellular signaling cascade like *ARL4A, ARL7, CIT, FYN, IRAK1, PEA15, RHOC, RIN1* and *SOCS3*.

Prognostic signature

Highly malignant STS with complex genetic alterations and with often pleomorphic appearance represent about half of the STS in clinical practice. The different STS subtypes are associated with variable outcome, e.g., a favorable prognosis for patients with myxoid liposarcomas and a high risk of metastases for patients with SS. Since 76% of the tumors in our series consisted of STS subtypes characterized by complex genetic defects without specific recurrent alterations and frequent pleomorphic histopathologic differentiation, we choose to assess whether a prognostic signature could be discerned within the unsupervised subcluster containing the more pleomorphic STS. After excluding the 11 LMS samples with a distinct expression profile, 16 liposarcomas, 8 MPNST, and one extraskeletal osteosarcoma treated with preoperative chemotherapy, 89 primary STS remained for analysis.

Gene expression profiles that correlate with poor outcome have been recognized in various tumor types, including Ewing sarcoma and LMS [149, 188, 205]. Ren *et al.* identified a 92-gene signature in 11 LMS that separated high-grade metastatic tumors from low-grade well-differentiated ones, whereas Lee *et al.* took a different approach and predicted metastasis in a set of 30 primary LMS and local recurrences using the expression profile of 335 genes that initially distinguished primary LMS from metastases [149, 205]. However, none of the reported genes were among our 244 discriminators, which may be due to different approaches and limited sample sets in the previous studies and inherent difficulties in identifying a prognostic signature as they are generally weaker that the diagnostic ones.

Golub-score analysis and random permutations were applied to generate a list of genes that distinguished the tumors that metastasized (n = 39) from those that did not (n = 50) and hereby about 200 genes (with a 35% FDR) found to be deregulated in the metastasizing tumors were identified. In order to obtain a more robust list of discriminators, a consensus gene list of 244 genes with median rank less than 700 was generated. Hierarchical clustering based on the consensus list split the 89 samples into two clusters with metastases developing in 6/40 (15%) STS in the first cluster (the metastasis-free cluster) compared to 33/49 (67%) in the second (the metastasis cluster) (*Study III*, Figure 2 and 3). Hence, the 244-gene signature correctly classified 75% of the samples.

Development of metastasis correlated with tumor size ($P=0.006$, Mann-Whitney's U and Kruskal-Wallis tests) and necrosis ($P=0.013$, Pearson χ^2 test), but not with vascular invasion ($P=0.166$, Pearson χ^2 test). The SVM leave-one-out cross-validation correctly classified 64% of the samples (area under receiver operating characteristic (ROC) curve = 0.64, $P=0.007$) into two groups with metastasis developing in 58% (25/43) of the patients in the high-risk group, compared to 30% (14/46) in the low-risk group ($P=0.008$, Pearson χ^2 test) and significantly predicted metastasis-free survival ($P=0.01$, log rank test, *Study III*, Figure 4, Table 2). The corresponding HR from a univariate Cox-regression analysis was 2.4 ($P=0.01$) and in a multivariate analysis including the established prognostic factors size, necrosis and vascular invasion, the profile predicted outcome with a HR of 2.2 ($P=0.04$).

The genes over-expressed in the metastasizing tumors clearly suggested an expression program triggered by hypoxia and included genes like *HYOU1, HIF1A, HIG2, ENO1, DDIT4, HK2, TFRC, FUT1, ERO1L, GLUT1, PLOD2,* and *ADM*. Hypoxia, which has previously been associated with poor disease-free survival and metastatic potential in STS, causes the stabilization of the HIF-1 transcription factor that mediates the induction of several genes including those that promote anaerobic glycolysis [86]. The most significant functional group identified in the EASE analysis was that involved in the process of glycolysis, further supporting the hypoxia-induced signature of the metastasizing tumors. This group included glycolytic enzymes and glucose transporters, many of which are known markers for hypoxia, like *ENO1, ENO2, PYGL, FUT1, HK2, GLUT1, GYS1, PDK1,* CA2, CA12, *PGK1* and *LDHB*. In addition, several proliferation genes, cell adhesion and motility genes e.g. *SYMPK,*

ACTN1, BYSL, VCL, NRCAM, YARS and *TLN1* were also among the discriminating genes.

An adverse prognostic impact of hypoxia has been demonstrated in several human malignancies including STS, and studies of tumor oxygenation has suggested that hypoxia may predispose for metastases in STS [27, 28, 75, 182]. More recently, hypoxia as measured by EF5-binding was suggested to be associated with tumor grade, presence of mitoses and metastatic development [75]. Hierarchical clustering on 107 hypoxia-related genes has shown that a hypoxic profile allows distinction of sarcoma samples from normal tissue samples suggesting an important role for hypoxia in sarcomas [62]. Several of the genes reported therein as highly upregulated in the sarcomas were also among our list of discriminators. A recent study demonstrated that HIF1A expression was an independent prognostic factor in STS [231]. *CA9*, an intrinsic cellular marker for hypoxia activated by HIF1, has been suggested as a prognostic marker in high-grade STS, with CA9-positive tumors showing significantly lower disease-specific and overall survival than CA9-negative ones [165]. Hypoxic tumors display high rates of glucose uptake and glycolysis regulated by HIF1 that induces expression of glucose transporters like GLUT1 and glycolytic enzymes like CA9, ENO1, HK2, LDHB and PGK1 [86]. Expression of GLUT1 also studied as a marker for hypoxia, was recently evaluated in STS and showed positive correlation with tumor glucose metabolism, proliferative activity and tumor grade [245]. Several studies in STS have shown significant correlations of both hypoxia and enhanced tumor glucose metabolism to high tumor-cell proliferation rate and grade [245].

In summary, diagnostic gene expression profiles could be generated for many STS subtypes. The pathways and genes identified herein provide important information about tumor origin and constitute potential therapeutic targets. One interesting aspect is that the tumors containing specific gene aberrations cluster closely and are characterized by upregulation of developmental genes and pathways, suggesting that these aberrations are different ways to obtain similar phenotypes. However, the diagnostically difficult pleomorphic sarcomas are challenging also when applying gene expression analysis and display relatively heterogeneous inconsistent expression profiles, but even so, supervised subclusters of the MPNST, LMS, MFH/UPS, dedifferentiated/pleomorphic liposarcomas, and fibrosarcomas could be discerned. Gene expression profiles allowed prognostication within a highly malignant subset suggesting that upregulation of hypoxia-related genes predict metastatic potential. Further evaluation of hypoxic markers, including *HIF1A* and its targets, is warranted but the present findings indicate that tumor hypoxia maybe useful in selecting patient groups with poor outcome and hypoxia-driven therapies may be of great value in STS.

Study IV

In *Study IV*, we applied 32K BAC-arrays with tiling coverage of the human genome to a series of 60 high-grade primary MFH/UPS and LMS, of which 30 tumors had developed metastases, in order to obtain high-resolution copy-number profiles with the aim of characterizing novel genetic subsets and assessing the prognostic value of aCGH. MFH/UPS and LMS frequently display complex copy-number profiles with numerous imbalances and previous CGH studies have revealed similarities between MFH/UPS and other poorly differentiated sarcomas like LMS and pleomorphic liposarcoma and have identified several recurrent imbalances in high-grade STS [35, 37, 44, 61, 114, 145, 151, 189, 214, 260]. Whole-genome copy-number profiling using the aCGH method has been performed in a limited number of MFH/UPS and LMS [37, 145, 189, 260]. The vast majority of the tumors in our study displayed complex profiles with numerous gains and losses. Alterations were found in all chromosomes with gains commonly occurring at 1p, 1q, 5p, 7p, 7q, 9q, 17p, 19p, 19q and 20q, and losses at 1qter, 2pter, 2qter, 8p, 9p, 10p, 10q, 11p, 11qter, 12p, 13q, 16q and 20p. Loss of chromosomal region 13q14 was found in ~65% of the samples and was the most frequent imbalance in this series (*Study IV*, Figure 2 and Table 2).

While majority of the tumors displayed complex genomic imbalances (*Study IV*, Figure 1a and 1b), 5 samples, including 3 MFH and 2 LMS, were characterized by relatively simple profiles with very few imbalances including high-level amplifications along 12q with or without additional high-level amplifications in other regions such as 1p, 1q, 3p, 3q or 6q (*Study IV*, Figure 1c). All 5 samples displayed the 12q15 amplification peak corresponding to *MDM2* whereas only 4/5 possessed the 12q14 amplification peak corresponding to *CDK4*. The sample lacking the *CDK4* peak displayed a high-level amplification in the 12q21.33-q22 region that was also seen in another sample with the *MDM2*, *CDK4* and 12q24.21-q24.23 amplification peaks. While two MFH with the simple profiles contained amplification peaks solely in the 12q region, one contained additional high-level amplifications in 3p12.2-p11.1. In one LMS the 12q14-q15 amplifications were associated with amplifications in 3 other chromosomes involving regions 1q23.1, 1q24.1, 1q24.2, 1q24.3, 3q22.3, 3q23, 6q22.1, 6q23.3 and 6q24.2-q25 while the other displayed several discontinuous high-level amplicons on chromosome 1 in regions 1p32.2-p31.3, 1q21.3, 1q23.3, 1q24.1, 1q24.1, 1q24.2, 1q25.1 and 1q41. These simple copy-number profiles are reminiscent of those described in de-differentiated and well-differentiated liposarcomas with supernumerary ring or giant rod-marker chromosomes with amplified sequences from the 12q14-15 region in association with amplified segments from other chromosomes [100, 198]. Moreover, it has been shown that MFH with the *CDK4-MDM2* amplification peaks developed in the retroperitoneum are most likely to be de-differentiated liposarcomas but all 4/5 of the cases here were localized in the extremity and one case in the trunk-wall [43, 44]. When re-evaluated by an experienced pathologist to assure that none were dedifferentiated liposarcomas, 2/5 of the diagnoses were confirmed but one MFH was diagnosed as a LMS and another as an unclassified spindle-cell sarcoma of intermediate grade and one LMS as a myofibroblastic sarcoma of low to intermediate grade. De-differentiated areas in de-differentiated liposarcomas usually comprise of high-grade poorly differentiated areas resembling MFH and less commonly low-grade spindle cell or myxoid sarcomas. These results demonstrate a subgroup of 5 MFH and LMS with simple profiles closely related to the de-differentiated/well-differentiated liposarcomas that could very well be de-differentiated liposarcomas which have lost their well-differentiated component.

Losses of DNA sequences were as common as gains. Minimal common regions for the most frequent losses were 13q14.2-q14.3 (~65%), 13q21.2 (~60%), 16q12.2 (~55%), 2q37.1-q37.3 (~50%), 9p21.3 (~50%), 10q22.3 (~50%) and 16q12.1 (~50%) (*Study IV*, Table 2). Homozygous deletions of 13q14.2-q14.3, including the *RB1* gene, were found in 11/60 samples and of 9p21.3, containing the *CDKN2A* (encoding the *INK4A, INK4B* and *ARF* genes) gene, in 6/60 tumors. Loss of chromosome 13q14-21 has been described as the most frequent copy-number alteration in MFH and LMS, with two separate clusters of deletions, one in 13q14 and the other in 13q21 [33, 161]. The tumor suppressor gene *RB1* located at 13q14.2 has been implicated in STS pathogenesis [33]. The 13q21.2 was deleted in ~60% of the samples suggesting the presence of an unidentified tumor suppressor. The 13q21 is a putative breast cancer susceptibility locus harboring the *BRCA3* tumor suppressor [120]. Other chromosomal regions lost in more than half of the samples included 16q12.2 harboring the retinoblastoma-like 2 (*RBL2*), a candidate tumor suppressor gene implicated in several cancers, 16q12.1 containing the tumor suppressor *CYLD*, 2q37.1-q37.3 harboring many candidate tumor suppressor genes like *HDAC4, ING5, STK25*, and *BOK* and 10q22.3 with the tumor suppressor *BMPR1A*. The *CDKN2A/CDKN2B/ARF* locus at 9p21.3 is frequently lost in STS and in line with previous results the 9p21.3 locus was lost in 50% of the samples and homozygous deletions were found in 6 (*Study IV*, Table 3) [214].

The most frequent gains affected 5p15.33 (~60%), 7p22.3 (~60%), 19p13.3 (~60%), 20qter (~60%), 21qter (~60%), 19p13.11 (~55%), 1q22 (~50%), 19p13.13 (~50%) and 19q13.42 (~50%)

(*Study IV*, Table 2). High levels of DNA amplification were detected in 28/60 recurrently involving bands 1p32.1-1p32.2 (3/60), 1q24.2 (2/60), 1q24.3-1q25.1 (2/60), 3p11.2-3p12.1 (2/60), 4q11-4q12 (3/60), 5q11.1-5q11.2 (2/60), 7p22.3 (2/60), 11q22.3 (2/60), 12q15 (5/60), 12q13.3-12q14.1 (4/60) and 12q21.33-12q22 (2/60) (*Study IV*, Table 3). The amplicons on chromosome 12q14-15 corresponding to *CDK4* and *MDM2* were observed only in the 5 samples with the simplest profiles. Several other candidate oncogenes such as *JUN, PDGFRA, KIT* and *PDGFA* were among the genes present in the recurrent amplicons (*Study IV*, Table 3). In order to identify likely targets of the recurrent high-level amplifications, expression data for 86 genes within these amplicons were retrieved from 55/60 samples. Centered expression values of the genes relative to the mean expression across the 55 samples showed good correlation between copy-number and gene-expression levels. Among the overexpressed ($\log_2$ratio>1) genes located in highly amplified regions ($\log_2$ratio>2) were *CYP2J2, FLJ10986, OMA1* and *JUN* in the two samples with the 1p32.1-1p32.2 amplicon, *FIP1L1* and *PDGFRA* in 3 samples with the 4q11-4q12 amplicon and *FAM119B, CDK4, CYP27B1, TSFM, MARCH9, TSPAN31* and *CTDSP2* in more than half the samples with the 12q15 amplicon (*Study IV*, Figure 3).

MFH and LMS display complex karyotypic changes with several numerical and structural abnormalities suggesting that many different oncogenes and tumor suppressor genes may be implicated in their biology [103]. Among the 60 samples analyzed homozygous deletions of the *RB1* and *INK4A/INK4B/ARF* loci were found in 11 (18%) and 6 (10%) cases, respectively. High-level *MDM2* amplification was seen in 5 cases (8%) with co-amplification of *CDK4* in 4 cases (7%). Hence, 22/60 (37%) of the samples had an obvious disruption of the *RB1/CDK4/INK4* and/or *TP53/ARF/MDM2* pathways. The alterations in *RB1, INK4/ARF* and *MDM2/CDK4* were mutually exclusive in the 22 samples. The most common (in up to 80% of the samples) copy-number alteration reported in MFH and LMS is the loss of 13q14-21 resulting in RB1 deletions in about 70–80% of the cases [33, 61, 194]. Hence, deregulation of the G1 to S cell-cycle checkpoint plays an important role in the pathogenesis of pleomorphic STS.

Although the MFH and LMS share similar genomic imbalances and unsupervised hierarchical clustering based on their whole-genome profiles did not separate these two histopathological subtypes, our study identified 924 clones in a Student's T-test ($P \leq 0.05$) encompassing ~70 regions that were differentially gained/lost between the two STS subtypes, with gains in 4p14, 6q27, 15q11-15q14 and 16p13 occurring more frequently in LMS and gains in 12q24.23-12q24.31, 5q and 4q28.3-4q34.3 in MFH/UPS. Hierarchical clustering based on these 70 regions grouped together 25 MFH/UPS with 2 LMS [Appendix Figure 13].

Current prognostic factors in MFH/UPS and LMS include clinicopathological factors, but although novel prognostic markers are needed data on the prognostic importance of genetic alterations are limited. Cytogenetic studies have approximately 18 MB resolution and have in high-grade STS suggested that specific chromosomal breakpoints correlate with prognosis [40, 167, 168, 210, 212]. Deletion of 19p has been found in recurring LMS in a single case [206] and an increase in copy number changes has been associated with tumor size of LMS [67]. Larramendy *et al.* reported that gain of 1p was significantly associated with a poor overall survival and gain of 7q32 with a less favorable metastasis-free/overall survival and an increased risk for local recurrences in MFH [146]. It has also been suggested that a 19p+ marker may serve as an indicator for high risk of local recurrence and aggressive behavior in MFH [40, 212]. Gains of 12q in adult fibrosarcoma have also been suggested to correlate with a poor outcome [219]. Another study identified gains of 17q in MFH to be strongly associated with better outcome [259]. The largest study identified 5 independent cytogenetic predictors of adverse outcome including break-points in 1p1, 1q4, 14q1 and 17q2 and gain of regions 6p1 and 6p2 in a series of 467 mixed STS. These alterations were evaluated in an extended series of 278 STS, in which two of the

initial alterations, a breakpoint in 1p1 and gain of 6p1, predicted risk of metastasis independent of other prognostic factors [167].

A Student's T-test performed identified 2,107 clones that distinguished metastasizing from non-metastasizing tumors. Hereby, 101 regions of continuous and near-continuous clones were identified, which were reduced to 84 significant regions after a SAM analysis (delta value 0.5 and 0% FDR). Hierarchical clustering of the 60 samples based on the 84 regions showed some degree of clustering related to metastatic potential (*Study IV*, Figure 4). In later univariate and multivariate analyses, losses in 6q14 and 7q36 were positively and negatively correlated with development of metastasis. Tumor depth, vascular invasion, size and necrosis were the clinical variables tested in the current data set, of which size and necrosis correlated significantly with development of metastasis in the univariate analysis whereas only necrosis remained significant in the following multivariate analysis. These finding are the first to demonstrate aCGH-based identification of prognostic markers in STS and thereby suggesting the possibility of using copy-number alterations as prognostic factors. Such combined classifiers are likely to improve prognostication and thereby allow selection of high-risk patients in high-grade pleomorphic STS.

Conclusions and future questions

The general aim of this thesis was to characterize STS using microarray-based gene expression profiling and aCGH, and the results can be briefly summarized as below:

- In *Study I*, we demonstrate that intratumor heterogeneity in expression profiles exists in STS, but is not a major source of bias in gene expression analysis, making total RNA extraction from single pieces an appropriate strategy to obtain representative profiles for diagnostic and prognostic use. Intratumor heterogeneity may have a larger impact on the results in small tumor series in which it may limit the validity of the findings. It is therefore important to run large sample sets of tumor pieces carefully selected avoiding necrotic tissue and normal cell infiltration in order to minimize the effects of heterogeneity, normal cell contamination and experimental variability and maximize chances of obtaining reliable and reproducible expression patterns in STS.

- In *Study II*, we report for the first time an expression profile related to gene fusion type in SS confirming that *SS18-SSX1* and *SS18-SSX2* have different downstream effects leading to differential expression of various developmental genes, transcription factors, histones and metallothioneins, which may provide important information regarding the histopathologic and prognostic differences seen in the SS subtypes. Moreover, a metastasis signature was identified in the SS including many cell cycle regulators and proliferation markers.

- In *Study III*, we identified discriminatory genes for the different subtypes and showed that several morphologically and genetically distinct subtypes are characterized by upregulation of several genes within developmental pathways, whereas the pleomorphic and genetically complex STS subtypes are more challenging also when it comes to expression profiling. The latter, however, showed upregulation of genes involved in cell-cycle function, proliferation, adhesion, motility, protein degradation, homeostasis and immune-response. The novel genes and pathways identified herein provide important information about tumor origin and constitute potential therapeutic targets. Furthermore, a prognostic signature, partly characterized by hypoxia-induced genes, was identified in the pleomorphic high-grade STS, which is highly promising and provides information independent of the currently used prognosticators.

- In *Study IV*, high-resolution copy-number profiling of the pleomorphic subtypes MFH and LMS identified multiple recurrent alterations, including high-level amplifications and homozygous deletions in regions harboring novel candidate genes, but also verified previously identified genetic alterations, e.g. cell-cycle checkpoint deregulation. We also identified several copy-number imbalances that correlate with the development of metastasis among which losses in two regions, 6q14 and 7q36, provided prognostic information independent of the currently used prognostic variables.

Genes and pathways identified in these studies, particularly the prognostic profiles, need to be validated in independent tumor materials using different platforms and/or techniques better suited for high-throughput analysis and clinical application. One way would be through the use of TMAs that can provide protein expression data for a single gene across hundreds of tumor sections. The genes identified to correlate with SS prognosis in *Study II*, e.g. *TOP2A*, along with *HIFIA* and its targets identified to be of prognostic value in *Study III,* need

to be confirmed at the protein level using FISH or immunohistochemical staining. The same goes for the many developmental pathways seen to be activated in some of the STS subtypes. This would be of great clinical value not only in identifying diagnostic markers but may also constitute specific pathways that can be targeted using novel inhibitors. We also identified copy-number imbalances harboring candidate genes and those of prognostic importance which need to be validated using techniques such as FISH and TMA. Other techniques such as real-time quantitative PCR and proteomic profiling may also be used to complement gene expression and copy-number profiling studies.

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