Synovial sarcoma is a soft tissue tumor defined by the presence of t(X;18)(p11.2;q11.2), fusing the SYT (SS18) gene on chromosome 18 and one of three SSX genes on chromosome X. T(X;18) results in production of a fusion protein (SYT-SSX) that is thought to underlie synovial sarcoma pathogenesis through aberrant targeting of both activating (trithorax, SWI/SNF) and repressing (Polycomb) transcription factors when expressed in a stem or progenitor-like cellular background. Clinically, synovial sarcomas present considerable diagnostic and therapeutic challenges. Whereas the classical biphasic histology is distinctive, the more common monophasic histology can be difficult to differentiate from other spindle cell tumors. In these situations, detection of t(X;18) is the gold standard for diagnosis, but it is a specialized and time-consuming process. Immunohistochemistry can be helpful, but no marker that is both highly sensitive and specific is available. Here I describe a fluorescence in situ hybridization based method employing an SYT break-apart probe set that can expedite detection of t(X;18). I also report that TLE1, which was identified in gene expression studies as a good discriminator of synovial sarcoma from other mesenchymal tumors, is a highly sensitive and specific immunohistochemical marker for synovial sarcoma. Both of these novel diagnostic techniques are applicable to small tissue samples such as core needle biopsies and are now being used clinically. The diagnosis of synovial sarcoma carries a poor prognosis and the 10-year overall survival rate is approximately 50%, most of whom are young adults. The addition of chemotherapy to surgical resection (the mainstay of treatment) does not appear to improve overall survival. Thus, there is a strong need for development of a clinically effective systemic therapy to improve patient outcome. I describe preclinical studies that demonstrate the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) inhibits proliferation of synovial sarcoma by inducing apoptosis and that this is associated with degradation of multiple receptor tyrosine kinases and disruption of the SYT-SSX-β-catenin interaction. I also identify a subset of synovial sarcoma cells, typified by expression of CD133, which exhibit stem-like properties and are relatively
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Data presented in chapters 2, 3, 4 and 5 are collaborative studies. I am primarily responsible for data analysis and manuscript preparation in chapters 2, 3, 4 and 5 and for identification and design of the research programs and performing the research in chapters 2, 4 and 5. My role in the latter two areas in chapter 3 is minor.
1 INTRODUCTION

1.1 Natural history of synovial sarcoma

Synovial sarcomas account for approximately 5-10% of soft tissues tumors, with an annual incidence of approximately one in 375,000 (1). Ninety percent of cases occur before the 50th year (mean age 26) and show slight predilection for males (1). No predisposing factors have as of yet been identified, although synovial sarcomas have been reported arising in areas previously irradiated for non-related conditions (2, 3). Patients typically present because of the tumor mass or related mass effects such as pain, limitation of motion or parasthesias; despite a propensity for recurrence and metastatic spread, these tumors are typically slow growing and the delay between onset of symptoms and presentation is typically 2-4 years (1). Most (> 90%) synovial sarcomas arise in the limbs, typically in periarticular regions and usually around the knee; the head and neck region is the second most common site (1). These tumors may arise in almost any organ or anatomical location and primary molecularly-confirmed synovial sarcomas have been found in brain, thyroid, heart, lung, liver, kidney, prostate, nerve, bone, blood vessels, pleura, mediastinum, GI tract, scrotum, fallopian tube and vulva (4-22). Simultaneous development of de novo tumors has also been reported (23).

On gross examination, synovial sarcomas are typically rubbery to friable in texture, tan to grey-white, 3 to 10 cm diameter circumscribed tumors with irregular areas of infiltration into surrounding tissues (1). Satellite nodules and multiple cystic areas, sometimes filled with blood or gelatinous material, occasionally occur; hemorrhage and necrosis are less common and usually associated with poorly differentiated histology (1, 24). Microscopically, synovial sarcomas can be subdivided into three major histological subtypes: monophasic, biphasic and poorly differentiated. The monophasic synovial sarcoma subtype is encountered most commonly, followed by biphasic synovial sarcoma and then poorly differentiated synovial sarcoma (1, 25, 26). Monophasic synovial sarcomas are composed entirely of spindle cells, sparse stroma and frequently exhibit a hemangiopericytomatous vascular pattern; epithelioid areas can occasionally be found. Biphasic synovial sarcomas are composed of spindle cells similar to those in the monophasic subtype interspersed with epithelial cells arranged in glandular, papillary or solid cord patterns. Typically, the spindle and
epithelial components of biphasic synovial sarcomas are present in equal proportions but predominantly epithelial or spindle cell variants occur and can lead to biphasic synovial sarcomas being mistaken for adenocarcinomas or monophasic synovial sarcomas, respectively (1). The poorly differentiated subtype is regarded as a dedifferentiated synovial sarcoma and is characterized by sheets of round, dark staining cells similar to other small round cell tumors such as Ewing sarcoma/primitive neuroectodermal tumor (ES/PNET). Despite significant histological differences in morphology, some ultrastructural details of poorly differentiated synovial sarcoma are similar to those of monophasic and biphasic synovial sarcoma (27). Other histological features of synovial sarcoma include fibrous, hyaline or myxoid matrix, calcification, and formation of osteoid or bone; areas of poor differentiation or squamous metaplasia can also be found (1, 28, 29).

Synovial sarcomas were originally named because they were thought to be associated with or derived from synovium based on the original descriptions of periarticular tumors with glandular histology (1). It has been subsequently shown that synovial sarcomas lack many features typical of synovium and synovial differentiation and thus likely bear no significant relationship to synovium; the tissue of origin remains obscure (30-33). The histological overlap between monophasic synovial sarcoma and biphasic synovial sarcoma subtypes, which has been corroborated by ultrastructural studies (28), suggests that monophasic synovial sarcoma and biphasic synovial sarcoma are not discrete entities but rather ends of a histological continuum. This has led to suggestions that synovial sarcoma is actually a carcinoma or carcinosarcoma (30, 34-36), but theories of an epithelial origin have largely been dropped in favor of a neuroectodermal or myoblastic derivation, based on histological, immunohistochemical, ultrastructural, gene expression and transgenic mouse model studies (37-49).

1.2 Pathobiology

Although investigations into the pathobiology of synovial sarcoma began with the initial descriptions and histologic examinations of this tumor over a century go (1), the discovery of a translocation between the X and 18 chromosomes in synovial sarcoma over two decades ago has been perhaps the most important event in the understanding of this tumor thus far (50, 51). Since then, numerous studies have shown that t(X; 18) can be identified in the vast majority (>95%) of synovial sarcomas
and is the only demonstrable karyotypic aberration in up to a third of cases (52, 53). t(X; 18) is present in all tumor cells comprising a synovial sarcoma, including both glandular and spindle cell components of biphasic tumors, indicating that synovial sarcomas are clonal in origin (54-57). Moreover, recurrences of primary tumors and successive xenograft passages derived from primary tumors maintain t(X; 18), demonstrating the importance of this translocation to synovial sarcoma pathology (58-60). The recent revelation that expression of SYT-SSX in myoblastic cells leads to the development of tumors that are histologically identical to synovial sarcoma validates t(X; 18) as the seminal event in synovial sarcoma pathogenesis (49).

1.2.1 T(X; 18)(p11.2; q11.2) and the SYT-SSX fusion gene

T(X; 18)(p11.2; q11.2) results in fusion of two genes, SYT (SYnovial sarcoma Translocated; also known as SS18 and SS7T) and SSX (Synovial Sarcoma translocation, X chromosome), which were initially identified as part of a novel fusion variant (SYT-SSX) detected by screening a synovial sarcoma cDNA library (61). Fusion of SYT to SSX1, SSX2, and rarely SSX4, was subsequently recognized (62-68). Synovial sarcoma cells bearing either the SYT-SSX1 or the SYT-SSX2 fusion variant have been reported to coexist in the same tumor by fluorescence in situ hybridization (FISH) in 10% of cases (69). Interestingly, SYT-SSX2 bearing cells appear to predominate in most of these situations, although the implication of this finding is unclear. Despite the high homology between SSX1, 2 and 3, SSX3 fusion variants have not been described (70), and neither have fusions containing SSX5-9 nor any of several SSX pseudogenes. The SYT-SSX fusion gene also produces two splicing variants that correspond to the SYT splice variants, normal (N, herein referred to simply as ‘SYT) and long (l) (71, 72). Interestingly, appreciable amounts of l-SYT mRNA are found in embryonic tissues only, suggesting that the protein product of l-SYT plays a role in development (73). SYT promoters are similar to those of housekeeping genes, explaining the near-ubiquitous expression of SYT and offering insight to expression of SYT-SSX (74). Downregulation of SYT expression has been observed in a SYT-SSX4 fusion variant tumor; expression of SYT-SSX4 in cell lines also downregulated SYT, suggesting a regulatory role for SYT-SSX in SYT expression and potential alteration of normal SYT function (67). In support, decreased levels of SYT mRNA compared to SYT-SSX have been described in primary synovial sarcomas (61, 64). Interestingly, increased SYT-SSX expression has also been
associated with epithelial/epithelioid areas and cytokeratin expression, suggesting SYT-SSX may promote epithelial differentiation in a dose-dependent manner (48).

Typically, the translocation breakpoints in SYT are clustered at the 5’ end of intron 10 which is rich in repetitive sequences whereas breakpoints in SSX1 and SSX2 occur in intron 4 and are also associated with repetitive sequences, but are not as tightly clustered (75). Otsuka et al. have described an SYT-SSX2 variant in which 15 additional bases from SYT intron 10 and small portions of introns 4 and 5 of the SSX2 gene were incorporated into the breakpoint, resulting from a cryptic splicing acceptor site (76). A variant of the SYT-SSX1 fusion, containing extra base pairs on the 5’ portion of SSX1 has been isolated from an epithelioid monophasic synovial sarcoma (77). Another variant of SYT-SSX1, containing an extra 51 bp at the 5’ end of SSX1 and missing the 3’ terminal 135 bp from SYT has been described in a monophasic fibrous synovial sarcoma synovial sarcoma (78); similar SYT-SSX1 variants missing the 132 and 161 bp from the 3’ terminal of SYT but gaining 144 and 50 bp at the 5’ end of SSX1 (respectively) have also been isolated from monophasic synovial sarcomas (60, 64). Variations of the common SYT-SSX2 transcript have also been described in association with the monophasic synovial sarcoma subtype and involving insertions into the breakpoint (52, 64, 79). Sonobe et al. describe a monophasic fibrous synovial sarcoma that bears a variation of the SYT-SSX1 fusion in which 240 bp of the SSX1 3’ end has been truncated (80). SSX4 fusions exhibit more breakpoint heterogeneity compared to SSX1 and 2; in one case, the KRAB-like domain (which mediates transcriptional repression (81)) is retained without apparent perturbation of the synovial sarcoma phenotype (68). Tornkvist et al. describe a fusion variant involving exon 6 of SSX4 from a typical case of synovial sarcoma, suggesting that the intervening sequence is not crucial to SYT-SSX function (82). Importantly, none of these variations have an appreciable effect on the synovial sarcoma phenotype implying that coding regions distal to the breakpoint are critical to SYT-SSX function.

Attempts to find correlations between fusion variants and tumor biology have uncovered a significant association between the SSX1 fusion variant and biphasic synovial sarcoma histology, whereas the SSX2 and SSX4 variants occur more commonly in the monophasic synovial sarcoma subtype (62, 65-68, 83-85) (although some studies have contradicted this finding (79, 86, 87)). Differences in the gene
expression profile between SYT-SSX1 and SYT-SSX2 expressing synovial sarcomas have been reported (88). Among genes previously identified as upregulated in synovial sarcoma, discriminators between SYT-SSX1 and SYT-SSX2 include the oncogenic receptor tyrosine kinase AXL, transcription factor ZIC2 and neural cell adhesion molecule NCAM1. The authors conclude that SYT-SSX1 and SYT-SSX2 produce different downstream effects and that this may account for some of the differences observed between SSX1 and SSX2 fusion variants. The SSX2 fusion subtype has been reported to occur more commonly in females (the SSX1 subtype appears to exhibit no sexual bias), while a significant geographical variation in the frequency of SYT-SSX1 and SYT-SSX2 fusions between a group of Dutch and Slovenian patients has been reported, implying that controlling for sex and ethnicity is important when attempting to correlate fusion variant to tumor behavior (89, 90). Significant correlations have also been established between fusion variants and underlying tumor biology and patient outcome and will be discussed in more detail where relevant in following sections.

The role of other genetic changes in synovial sarcoma development is less clear. Observations that the karyotypes of established synovial sarcoma cell lines do not differ from those of the parent tumor over years of serial passages suggest that the synovial sarcoma genome is relatively stable over time (91-93). Complex karyotypes have been described in approximately two thirds of synovial sarcomas, particularly in metastatic lesions; in addition to various unbalanced inversions, deletions and additions, monosomies of every chromosome except 7 and trisomies of every chromosome except 1, 3, 10 and X have been described (9, 38, 51, 52, 86, 94-103). Few of these karyotypic abnormalities have been credited with affecting the phenotype of synovial sarcoma, the most remarkable of which is trisomy 8 or gains of portions thereof being significantly associated with tumors over 5 cm, which itself is a negative prognostic indicator (99, 100). Curiously, analysis of the sequence of accumulation of karyotypic aberrations secondary to t(X; 18) in synovial sarcoma suggests that it occurs in a loosely ordered manner that more closely resembles patterns typical of epithelial malignancies rather than other translocationally-defined cancers, particularly hematological malignancies, although the biological relevance of this observation presently remains unclear (104). The use of comparative genomic hybridization (CGH) to examine the synovial sarcoma genome is revealing a greater
level of complexity than previously appreciated (105). CGH has shown that, in
general, copy number changes can be demonstrated in 55% of synovial sarcomas,
gains occur more commonly than losses and that these changes are more frequent in
monophasic synovial sarcoma than biphasic synovial sarcoma (99, 100, 106, 107).
The consequences of many of these changes is unclear, although some involve
genes implicated in other malignancies including gains in MDM2, CDK2, ERBB3 and
CDK4 and losses in HRAS, RASSF1 and CCND1 (107). Thus it appears that synovial
sarcomas harbor more subtle genetic alterations than previously appreciated and
these may play significant roles in progression of this tumor towards a more
aggressive phenotype.

1.2.2 The SYT-SSX fusion protein

T(X:18) is thought to produce synovial sarcoma through expression of SYT-
SSX and its protein product, SYT-SSX. Expression of SYT-SSX1 in rat 3Y1
fibroblasts increases proliferation, anchorage independent growth and formation of
xenograft tumors, while overexpression of either SYT or SSX had a negligible effect
(108); anchorage independent colony formation was found to be dependent on the N-
terminus 181 amino acids of the SYT-SSX fusion protein. The N-terminal domain of
SYT-SSX1 was also found to be required for interaction with the chromatin
remodeling SWI/SNF factor hBRM in both the 3Y1 model and the synovial sarcoma
cell line HS-SY-II. Binding of hBRM to SYT-SSX1 required amino acids 156-205 of
hBRM and expression of this domain inhibits anchorage independent growth.
Together, these results suggest that interaction between SYT-SSX1 and hBRM
contribute to synovial sarcoma pathogenesis. The N and I isoforms of SYT-SSX both
increase proliferation when transformed into the murine embryonic fibroblast cell line
NIH3T3; however, the I isoform (which is primarily expressed in embryonic tissue)
appears to promote increased rates of transcription and proliferation compared to the
N isoform, which the authors speculate is due to enhanced transactivation ability and
interaction with hBRM acquired through the extended QPGY domain of the I variant
(73). Expression of SYT-SSX2 in the human embryonic kidney cell line HEK293 also
increased proliferative rates (109).

In contrast, transient SYT-SSX2 expression has also been reported to inhibit
proliferation when transfected into NIH3T3 cells, as well as the U2-OS
(osteosarcoma), C57MG (murine mammary) and MDCK (canine kidney) cell lines
In this study, the authors associate this inhibition of NIH3T3 cell proliferation with SYT-SSX2-dependent cytoskeletal changes and enhanced ephrin signaling that disrupts cell adhesion and promotes formation of neurite-like extensions of the cell membrane (how SYT-SSX2 mediates this effect is unclear). SYT-SSX1 expression in the human adrenal carcinoma cell line SW13, which lacks detectable BRG1 (another SWI/SNF chromatin remodeling factor similar to hBRM) expression, also retards proliferation, indicating that SYT-SSX induced oncogenesis requires an appropriate cellular context that includes sufficient BRG1.

The recent development of conditional SYT-SSX expressing transgenic mice has demonstrated that synovial sarcomas will only develop when SYT-SSX is expressed in myoblasts at a specific point in mouse development. This seminal study shows that SYT-SSX requires a permissive cellular background to generate the synovial sarcoma phenotype; moreover, this background is progenitor or stem-like in nature and identifies that a primitive myogenic origin for synovial sarcoma is possible. Further characterization of these transgenic mouse models promises significant progress in understanding the underlying biology of SYT-SSX function.

Insight into SYT-SSX function has also been gleaned from studies into the function of SYT and SSX and their interactions with nuclear binding partners. SYT is expressed almost ubiquitously throughout adult tissues. SYT, SSX and SYT-SSX are nuclear localized proteins; SSX is distributed uniformly whereas SYT and SYT-SSX2 exhibit a speckled pattern suggesting the SYT moiety directs localization of SYT-SSX to specific structures within the nucleus. The N-terminal domain of SYT contains the widely conserved SNH (SYT N-terminal Homology) domain and a nuclear localization signal, while the C-terminus is rich glutamine, proline, glycine and tyrosine (the QPGY domain) and contains transcriptional transactivating sequences. Deletion of the SNH domain of SYT increases transcriptional activation, suggesting a negative regulatory function that appears to involve interaction with SIN3A, a component of the histone deacetylase (HDAC) complex. The QPGY domain, which facilitates SYT oligomerization, is preserved in SYT-SSX and enhances SYT-SSX interaction with the SWI/SNF chromatin remodeling factor hBRM compared to SYT alone. The general nuclear co-activator and splicing modulator RBM14 (SIP, CoAA) also binds to the QPGY region of both SYT and SYT-SSX2 and together with the SWI/SNF transcriptional regulators hBRM and/or BRG1,
SYT and RBM14 can activate transcription (109, 119). The histone acyltransferase p300, but not the closely related CBP (CREB binding protein), has been shown co-immunoprecipitate with SYT in lysates from confluent but not subconfluent cell cultures (120). Formation of the SYT-p300 complex is promoted by adhesion to fibronectin and involves activation of β1 integrin and tyrosine phosphorylation of SYT, suggesting a signaling role for SYT in cell adhesion. Interestingly, the SYT paralog SS18L1, also known as CREST (Calcium-Responsive Transactivator), is expressed in the developing brain where it interacts with both CBP and p300 and influences neuron morphology and growth in a calcium dependent manner (121). Any commonalities between the function of CREST and SYT remain unclear, although SS18L1 has been identified in a fusion with SSX7 in what otherwise appears a typical case of synovial sarcoma and both SS18L1 and another SYT paralog, SS18L2, can functionally replace SYT in protein-protein interaction assays (74, 122). Both of these paralogs were identified through similarities with the SNH domain and are thought to have arisen from gene duplication events (123). SS18L2 may also modulate the interaction between the N-terminus of SYT and the C-terminus of AF10, a DNA binding transcription factor involved in translocational fusions with MLL in leukemias (124). Although the exact composition remains unclear, the SYT portion of SYT-SSX appears to recruit a complex of transcriptionally active partners, particularly members of the SWI/SNF complex.

In the adult, SSX1 and SSX2 are expressed at high levels in the testis and to a lesser extent in thyroid tissue; expression is virtually undetectable elsewhere. SSX has also been shown to be expressed in undifferentiated mesenchymal stem cells (MSC), suggesting a part in maintenance of the stem cell phenotype (125). The C-terminus of SSX contains a nuclear localization signal and is responsible for nuclear localization of SYT-SSX and colocalization with SSX2 (116). Removal of the C-terminal portion of SYT-SSX enhances colocalization with SYT, demonstrating that the SSX moiety also directs localization of the fusion protein and likely results in abnormal targeting of SYT related SWI/SNF complexes to normal SSX targets. The C-terminal portion of SSX1 binds histones, suggesting that the SYT-SSX fusion may function by inhibiting the chromatin remodeling function of the SWI/SNF complex, targeting the SWI/SNF complex to inappropriate promoters or sequestering SWI/SNF proteins from their intended promoters, thereby inhibiting appropriate gene...
expression (126). In addition, both SSX and SYT-SSX have been demonstrated to co-localize with the Polycomb transcriptional complex components RING1 and BMI-1, which are typically involved in transcriptional repression, in association with chromatin (114). The C-terminus of SSX has also been assigned transcriptional repressor function (127). Although the nature of this C-terminal repressor domain is not fully understood, a cell line derived from a typical monophasic fibrous synovial sarcoma that bears a variation of the SYT-SSX1 fusion in which 240 bp of the SSX1 3’ end (including the C-terminal repressor domain) has been truncated exhibited relatively slow proliferation (compared to another synovial sarcoma cell line) and was unable to form xenograft tumors (80). More recently, the C-terminal repression domain of SSX has been shown to interact with the DNA-binding transcription factor LHX4, which appears to have some importance to synovial sarcoma pathobiology as SYT-SSX, but not SSX, enhances LHX4-mediated transcription (128). The proteins RAB3IP and SSX2IP interact with the N-terminal portion of SSX2, which is lost in the SYT-SSX fusion; however, the functions of these two proteins and their significance are unclear (129). Thus, the SSX moiety promotes proliferation and likely contributes to pathogenesis through recruitment of Polycomb group proteins and aberrant localization of SYT associated SWI/SNF proteins.

1.2.3 Gene expression patterns in synovial sarcoma

Based on studies of SYT, SSX and SYT-SSX function, it has become generally accepted that SYT-SSX-mediated pathogenesis involves dysregulation of gene expression through aberrant localization of SWI/SNF and Polycomb transcriptional complexes, resulting in expression or repression of genes that together produces the synovial sarcoma phenotype (130). Synovial sarcomas should thus exhibit a characteristic and reproducible pattern of gene expression, and this has been confirmed by cDNA microarray analysis. Nielsen et al. demonstrated that synovial sarcoma exhibits a distinct gene expression pattern that separates it from other soft tissue tumors, including leiomyosarcoma, malignant fibrous histiocytoma and schwannoma (40). The synovial sarcoma gene expression pattern is typified by a cluster of 104 genes including upregulated genes involved in development (TLE1, SOX9, FGFR3, ENC1, BMP7), retinoid metabolism (CRABP1, RARG), growth signaling (EGFR, IGFBP2, DACH) and transcription (SSX3, SSX4). Lee et al. found synovial sarcoma could be discriminated from leiomyosarcomas and malignant
fibrous histiocytomas in a similar manner by higher expression of a set of 48 genes involved in development (FGF9, BMP4), signaling (IGF2, EFNBI), cell adhesion (ICAM1), and control of transcription (SSX4) (131). Allander et al. also obtained a similar gene expression pattern, again typified by upregulation of genes related to development (FGFR3, SOX9), retinoid metabolism (CRABP1) and growth signaling (HER2, IGFBP2 and IGF2). In this study, protein level overexpression of HER2 and IGFBP2 was confirmed in immunohistochemical analyses of synovial sarcoma tissue microarrays. A number of genes related to neural development, including TLE2, OLFM1 and CNTNAP1, were also found to be significantly overexpressed, suggesting a possible neuroectodermal phenotype for synovial sarcoma. Comparison of gene expression between fusion variants (i.e. SYT-SSX1 and SYT-SSX2) revealed no significant differences; however, comparison of biphasic synovial sarcoma and monophasic synovial sarcoma tumors revealed differential overexpression of ELF3 in the biphasic synovial sarcoma subtype. ELF3 is a transcription factor involved in epithelial differentiation that is upregulated by HER2 and MET signaling, suggesting a mechanism for epithelial differentiation in synovial sarcoma (42). Nagayama et al. also found upregulation of genes implicated Wnt signaling (FZD10) and the differentiation and migration of neural crest cells, suggesting a potential neuroectodermal origin of synovial sarcoma (41). Baird et al. reported that synovial sarcoma was typified by upregulation of developmental (PBX3, EN2, MEOX2), growth signaling (EGFR, PDGFRA) and Wnt signaling (TLE1, TLE4, FZD1, Wnt5A) related genes (44). Francis et al. found that synovial sarcomas exhibited a characteristic gene expression signature highlighting upregulated expression of multiple development related genes including those involved in the EGF, FGF, TGF-β, Wnt, retinoic acid and hedgehog receptor signaling pathways (47). Upregulation of genes involved in chromatin remodelling was also particularly prominent, as were many neural differentiation genes and the transcription factors SSX1 and 3. When taken together, these studies demonstrate that synovial sarcoma is typified by dysregulation of multiple signaling pathways primarily involved in control of growth and differentiation. What is particularly striking about these gene expression studies is the relatively high degree of concordance between data from different laboratories using separate tissue sources and array platforms, which suggest that these data very likely
represent the SYT-SSX mediated molecular derangements underlying synovial sarcoma pathogenesis.

The finding that exogenous expression of SYT-SSX in a non-synovial sarcoma background dysregulates gene expression in a manner similar to that reported in actual synovial sarcomas further strengthens the theory that SYT-SSX mediates dysregulation of gene expression. SYT-SSX2 expression in the HEK293 cell line resulted in groups of genes that were upregulated throughout SYT-SSX2 expression (SPARC, PRAME), became upregulated over time (SSX3, SYT, IGF2, FGFR3, CCND1), were downregulated throughout SYT-SSX2 expression (LDLR) or became downregulated over time (CD44, VEGF) (109). Uncoupling of signaling pathways, such as Wnt and EGR1, from their target effectors was also observed, providing further evidence for SYT-SSX mediated transcriptional dysregulation. SYT-SSX2 was also able to influence histone modification and promoter methylation of SYT-SSX2-responsive promoters, possibly in conjunction with BRG1, suggesting an epigenetic role for SYT-SSX in synovial sarcoma pathogenesis. Interestingly, a number of genes involved in cholesterol synthesis were upregulated, which has previously been correlated with both carcinogenesis and more specifically with overexpression of IGF2 (132, 133).

1.2.4 Oncogenic processes in synovial sarcoma

Dysregulation of gene expression in a reproducible manner should result in typical changes in protein expression that are the ultimate effectors involved in synovial sarcoma pathogenesis. Many of these effectors are not unique to synovial sarcoma and involve typical modalities that facilitate cancer development, including repression of apoptosis, self-sufficient proliferation and elaboration of an invasive phenotype.

1.2.4.1 Resistance to induction of apoptosis

A cornerstone of cancer development is the ability to resist induction of apoptosis. The tumor suppressor gene p53 plays a central role in cell cycle arrest and induction of apoptosis and is the most commonly mutated gene found in malignancies. Positive immunostaining for p53 has been observed in only 30% of synovial sarcomas, whereas mutations that inactivate p53 have been found in 6-18% of synovial sarcomas (134, 135). Amplification of the MDM2 gene, the product of which binds and inhibits p53 function, has been reported in 40% of synovial sarcomas.
MDM2 expression also correlates with nuclear localization of p53, implying that MDM2-mediated sequestration of p53 is the primary mode of p53 inhibition in these cases. The apoptosis inhibitors BCL2 and BCLX have been reported to be ubiquitously expressed in synovial sarcoma, with higher BLC2 levels in the spindle cell component correlating with lower levels of apoptosis compared to glandular component in biphasic tumors (66, 83, 92, 137-140). BCL2 gene amplification or rearrangement as a cause for increased expression was not found. Interestingly BAX, which promotes apoptosis, is also ubiquitously expressed, however no significant correlation between levels of apoptosis and the expression of BAX has been found (138). The type of SYT-SSX fusion variant involved does not appear to affect expression of BCL2, BAX, p53, p27 or the apoptotic index (83, 141). Doxorubicin, a DNA-damage inducing agent and topoisomerase inhibitor typically employed in synovial sarcoma chemotherapy, appears to induce senescence rather than apoptosis in the synovial sarcoma cell line FU-SY-1 (142). Generally, evasion of apoptosis appears to play a significant role in synovial sarcoma, especially in cells with spindled morphology.

1.2.4.2 Loss of cell cycle control

Increased proliferation is characteristic of many cancers; however, most cancer cells exhibit slower progression through the cell cycle than their normal tissue counterparts. Loss of cell cycle control and a greater fraction of actively cycling cancer cells explain this apparent contradiction. Immunohistochemical studies of the fraction of actively cycling synovial sarcoma cells shows there are significantly more in the glandular component, suggesting a proliferative phenotype compared to the anti-apoptotic phenotype expressed by the spindle cell component (140). Positive (i.e. 10% or more of cells) immunohistochemical staining for cyclin D1, which controls cell replication by facilitating progression through the early G1 phase of the cell cycle, in both monophasic and biphasic synovial sarcoma was found in 59% of cases (143). SYT-SSX1 leads to increased levels of cyclin A and D1, which appears to occur through inhibition of degradation, suggesting an intimate connection between SYT-SSX and deregulated cell cycle progression (144, 145). SYT-SSX1 promotes an increased proliferative rate when compared to SYT-SSX2 tumors, although the reason for this difference and any relationship to cyclin D1 expression is unclear (83,
Mutations in the tumor suppressor PTEN, which inhibits cell cycle progression through expression of the cyclin dependent kinase inhibitor p27, have been reported only in monophasic synovial sarcoma at a frequency of 14%; accordingly, low p27 expression levels are associated with poorer overall survival (147-149). Heterozygous deletion of \textit{CDKN2A}, which encodes the cell cycle progression inhibitor p16, has been found in approximately 80% of synovial sarcomas examined, and 80-90% of these exhibited abnormally low or absent p16 expression with concurrently increased expression of cyclin D1, though no significant correlation between deletion of \textit{CDKN2A} and proliferation rate, tumor grade or fusion subtype has been found (150, 151). Expression of the p53 regulated cyclin kinase inhibitor \textit{CDKN1A} (p21) occurs in the majority of synovial sarcomas and appears to be induced by \textit{SYT-SSX1} expression (111, 143); however, the inhibitory effect of this protein on cell cycle progression does not appear to occur in synovial sarcoma.

Another positive regulator of proliferation, nuclear-localized β-catenin, is a prominent feature of many synovial sarcomas (10, 45, 48, 152-158), where it is associated with increased proliferative rates and poorer prognosis, implying an important pathogenic role. Nuclear-localized β-catenin modulates transcription of many cell cycle related genes including the proliferation promoters \textit{CCND1} (cyclin D1) and \textit{MYC}. Correlation between nuclear localized β-catenin and cyclin D1 expression has been reported in synovial sarcoma, particularly in the spindle cell component in synovial sarcoma (158), although cyclin D1 expression in the absence of prominent nuclear β-catenin can also occur in the glandular component (159). Nuclear localization MYC can be found in 28% of synovial sarcomas, although high proliferative index (found in 26% of cases) did not correlate with MYC suggesting it does not significantly influence proliferative rate (160). Typically, nuclear β-catenin accumulates in malignancies as a result of stabilizing mutations in \textit{CTNNB1} (β-catenin) or \textit{APC}; however, the frequency of these mutations in synovial sarcoma is relatively low (~8-14%) (152, 161). In the absence of β-catenin stabilizing mutations, other factors facilitate nuclear β-catenin localization in synovial sarcoma. FZD10, one of the FZD family of membrane receptors that transmit Wnt signals across the cellular membrane, is overexpressed at the protein level in synovial sarcoma while absent in normal tissues. Inhibition of FZD10 expression suppresses proliferation, demonstrating that canonical Wnt signaling plays an active role in synovial sarcoma.
proliferation (162). The indirect interaction between SYT-SSX and β-catenin may play a more direct part in synovial sarcoma development. Exogenous expression of SYT-SSX2 in NIH3T3 results in recruitment of β-catenin to the nucleus in a non-Wnt dependent manner where it localizes to transcriptional complexes that include SYT-SSX2, suggesting that SYT-SSX2 acts as an anchor to retain β-catenin in the nucleus; this interaction appears to involve one or more unidentified intermediaries (163). In this study, SYT-SSX2-dependent recruitment of β-catenin to the nucleus was not found to promote transcription of known target genes including \textit{CCND1} (cyclin D1) and \textit{MYC}, although it did promote transcription from a β-catenin-responsive reporter plasmid. Taken as a whole, it appears that β-catenin is intricately involved in synovial sarcoma pathogenesis through both typical Wnt signaling and atypical interaction with SYT-SSX.

\textbf{1.2.4.3 Growth factors}

Gene expression studies of synovial sarcoma have identified overexpression of a number proliferation related signaling pathway components, particularly receptor tyrosine kinases such as EGFR. Overexpression of EGFR in synovial sarcoma, which was initially suspected over two decades ago, has been confirmed in subsequent studies and is not attributable to gene amplification (139, 164-167). Overexpression of EGFR is associated with phosphorylation of downstream signaling molecules (167), implying active EGFR signaling in these situations and a part in tumor progression. Activating mutations in \textit{EGFR}, such as those found in gefitinib-sensitive lung cancer, appear to be rare in synovial sarcoma (166-168).

Among other receptor tyrosine kinases that are frequently reported as overexpressed, KIT and PDGFRβ can be detected in approximately half of synovial sarcomas with one or the other being expressed in over 70% of cases; mutually exclusive expression appears to occur more frequently in the monophasic synovial sarcoma subtype (169). When expressed, these receptor tyrosine kinases are phosphorylated and thus activated, implying a role in synovial sarcoma proliferation. Expression of both \textit{KIT} and \textit{SCF} (KIT ligand) mRNA has been found concurrently in ~87% (20 of 23 cases) of synovial sarcomas, suggesting an autocrine feedback loop (170). PDGFRα expression has also been reported to occur in 67% of synovial sarcomas (171). Both hepatocyte growth factor receptor (MET, HGFR) and its ligand (HGF) are also overexpressed in synovial sarcoma. Inappropriate HGF/MET signaling
has been implicated in the development of other malignancies, however the role of HGF/MET signaling in synovial sarcoma development is not well defined. Immunohistochemical expression of HGF, found in approximately 32% of synovial sarcomas, is correlated with higher proliferative rate, suggesting that an autocrine/paracrine stimulatory loop may play a role in proliferation (172). The adaptor protein CRK is required for sustained phosphorylation of MET in response to HGF in synovial sarcoma cell lines, which facilitates increased motility, while downregulation of CRK expression limits tumor formation and invasion in vivo, suggesting that HGF/MET signaling contributes to synovial sarcoma aggressiveness and metastatic potential (173). Concurrent expression of both HGF and MET in synovial sarcoma is restricted to the glandular component; MET expression without HGF has been observed in epithelioid nests of monophasic fibrous synovial sarcoma synovial sarcomas while neither has been found in the spindle cell component (174). This pattern of expression hints at a role for HGF/MET signaling in epithelial differentiation in SS. Fibroblast growth factor (FGF) receptors are also overexpressed in synovial sarcoma. Activation of FGF signaling leads to phosphorylation of extracellularly regulated kinase (ERK) and inhibition of FGF signaling decreases phosphorylation of ERK and slowed proliferation of synovial sarcoma cell lines in vitro and as xenografts, demonstrating that FGF signaling plays an important role in synovial sarcoma proliferation (175).

Growth factor related molecules less commonly associated with synovial sarcoma include PDZK4, which encodes a membrane associated adapter protein potentially involved in modulation of transmembrane signaling, was identified in a gene expression study as being overexpressed in synovial sarcoma; expression is normally restricted to fetal brain. Knock-down of PDZK4 expression in synovial sarcoma inhibited proliferation and exogenous expression of PDZK4 in a non-synovial sarcoma background led to increased proliferation, suggesting that PDZK4 is an oncogene that plays a role in synovial sarcoma proliferation (176). Overexpression of HER2 (c-erb2, neu, EGFR2) has also been documented, although less frequently than EGFR (48, 139, 177, 178). Increased HER2 expression has been correlated with increased levels of SYT-SSX transcript, suggesting a direct role for SYT-SSX in HER2 expression. No significant correlation between HER2 expression and proliferative index has been found and HER2 gene amplification (15% of cases) is
associated with a significantly decreased risk of metastasis (48, 139, 177, 179), implying HER2 overexpression does not play a provocative role in synovial sarcoma pathogenesis. IGF2 is another prominent signaling molecule in the gene expression profile and is a target of epigenetic deregulation by SYT-SSX (109). Expression of IGF-1R, a receptor for IGF2, has been associated with increased proliferative rate and metastatic potential in synovial sarcoma; however, IGF-1R is undetectable in approximately 50% of cases, implying it is not crucial to synovial sarcoma pathogenesis (180). SYT-SSX2 appears to be required to maintain IGF2 expression in a synovial sarcoma cell line and exogenous expression of SYT-SSX1 induces expression of IGF2 in rat 3Y1 fibroblasts, which in turn inhibits apoptosis and promotes tumor formation (181).

Less is known about abnormalities in the growth factor signaling pathways downstream of receptor tyrosine kinases in synovial sarcoma, although one study found oncogenic activating mutations in HRAS in 6% of cases (135).

1.2.4.4 Mesenchymal-epithelial transition

Transgenic mouse models expressing SYT-SSX have substantiated theories of a primitive, progenitor-like phenotype for synovial sarcoma and suggested the origin may be myogenic. There is little evidence for muscular differentiation in synovial sarcoma, though, implying that if the origin is truly myogenic, there is an early obstruction of differentiation. Side populations (a subpopulation with the capacity to efflux the dye Hoechst 33342 typically enriched for stem-like cells and found in some normal tissues and malignancies) with enhanced tumorigenic potential have been reported in synovial sarcoma (182), adding further evidence that synovial sarcomas arise from a progenitor-like origin. Although the identity of this progenitor remains unproven, it is likely mesenchymally or neuroectodermally derived, indicting that the mesenchymal-epithelial spectrum of histologies displayed by synovial sarcomas likely represents transition from a mesenchymal to epithelial phenotype. The glandular portions of biphasic synovial sarcomas exhibit tight junction protein expression identical to normal glandular tight junctions; however, these proteins are abnormally expressed and localized in monophasic synovial sarcoma, poorly differentiated synovial sarcoma and the spindle cell component of biphasic synovial sarcoma tumors, suggesting aberrant mesenchymal to epithelial differentiation (183). Expression of claudins 4, 7 and 10, which are proteins critical to tight junction
formation, occurs in the glandular component of biphasic synovial sarcomas (184). Expression of Integrin α2 has also been detected in the epithelial components of synovial sarcomas (185). Membranous E-cadherin is required for maintenance of cell-cell interactions and epithelial phenotype, and is seen in 31-100% of synovial sarcomas and strongly correlates with biphasic histology and the SYT-SSX1 fusion variant (186-189). Analysis of E-cadherin gene (CDH1) expression found that missense mutations and transcriptional repression by SNAIL accounted for reduced CDH1 expression and loss of epithelial differentiation. Interestingly, SYT-SSX1 preferentially interacts with the transcriptional repressor SNAIL whereas the SYT-SSX2 fusion protein preferentially interacts with the SNAIL-related transcriptional factor SLUG (190). These interactions sequester either SNAIL or SLUG and prevent their binding to the promoter of CDH1 (the E-cadherin gene), resulting in relief of transcriptional repression, E-cadherin expression and expression of epithelial phenotype. Relief of SNAIL repression appears to result in greater E-cadherin expression compared to SLUG, suggesting a mechanism whereby SYT-SSX1 fusion variants are preferentially associated with epithelial differentiation. Interestingly, this study found that neither SYT nor SSX interacted physically with SNAIL or SLUG, demonstrating a gain-of-function for the SYT-SSX fusion protein. Thus, SYT-SSX appears to promote inappropriate expression of E-cadherin and block an apparent attempt at epithelial to mesenchymal transition. SSX downregulation also leads to increased expression of E-cadherin, although this appears to be independent of SYT-SSX (190).

1.2.4.5 Angiogenesis, telomere maintenance and chemotherapeutic resistance

Other cancer-related factors are involved in synovial sarcoma development. In vitro experiments have shown that wild type p53 inhibits expression of VEGF in synovial sarcoma and angiogenesis in Matrigel-supplemented xenografts, suggesting a role for p53 dysfunction in angiogenesis and tumor growth (191). However, VEGF expression does not appear to correlate with vessel density in primary synovial sarcomas, suggesting angiogenesis may be driven by additional factors (192). Inappropriate maintenance or expansion of telomere length is another hallmark of malignancy; however, analysis of telomere length in synovial sarcomas found that telomere lengths do not differ from those of patient-matched non-neoplastic control tissue (193, 194). Chemotherapeutic drug resistance does not lead to development of
cancer per se, but it does significantly impact treatment success and patient outcome. In primary synovial sarcomas, one study found 29.7% and 40.5% express the drug resistance proteins P-glycoprotein (ABCB1) and glutathione S transferase-pi (GST-pi, GSTP1) respectively; however, no significant association between expression of ABCB1 and/or GSTP1 and response to chemotherapy was found (195). BCL2 in synovial sarcoma is not phosphorylated (inactivated) by treatment with chemotherapeutic agents, demonstrating that synovial sarcoma is unable to activate apoptosis and suggesting a mechanism for the relative resistance to chemotherapy (66); downregulation of BCL2 expression should abrogate this effect and is thus an attractive potential adjunct to doxorubicin-based chemotherapy. Joyner et al. have examined this possibility by treating the synovial sarcoma cell line FU-SY-1 with antisense oligonucleotides specific for BCL2 mRNA, which decreased BCL2 levels and increased sensitivity to doxorubicin (196).

1.3 Diagnosis

In addition to the typical gross and histologic appearances already described, the pathological genomic and proteomic changes typical of synovial sarcoma can be exploited as effective diagnostic criteria that are especially useful in difficult diagnostic situations like small biopsies. Data from studies to define optimal diagnostic markers have also generated considerable insight into the pathobiology of this tumor.

1.3.1 T(X:18)(p11.2; q11.2)

Synovial sarcoma is defined by t(X; 18)(p11.2; q11.2), a translocation not found in other neoplasms, and as such t(X:18) is the gold standard molecular diagnostic for this tumor. One study investigating the value of adjunctive molecular testing (RT-PCR and FISH) for t(X; 18) in the diagnosis of synovial sarcoma found that t(X; 18) could be confirmed in approximately 84% of cases where histology identified synovial sarcoma, 74% of cases where synovial sarcoma was favored within a differential diagnosis and in 24% of cases where a diagnosis of synovial sarcoma was considered but not favored, demonstrating that molecular testing for t(X; 18) is a useful diagnostic tool (197). Initially, detection of t(X; 18) was accomplished by cytogenetic analysis (94, 96); however, it is slow, expensive, requires special tissue handling and the resolution is limited. Despite improvement for detecting cryptic t(X; 18) with multi-colored or spectral karyotyping (198, 199), FISH and
reverse transcriptase polymerase chain reaction (RT-PCR) based methods have become the preferred methods (200).

FISH based methods for detection of t(X; 18) are, in general, both highly sensitive and specific for diagnosis of synovial sarcoma and can detect t(X; 18) in situations where PCR product cannot be obtained (86, 87, 201). Initial FISH based methods for detecting t(X; 18) relied on detection of disrupted chromosome 18 or X and have since evolved to incorporate various combinations of centromeric, chromosomal and breakpoint probes (86, 202-207). FISH methods capable of distinguishing between SYT-SSX1 and SSX2 fusion variants have also been recently developed (103). Detection of t(X; 18) by FISH remains popular because of the applicability to formalin-fixed, paraffin embedded (FFPE) tissues (particularly small samples like core needle biopsies) and robust sensitivity and specificity; however, the requirement for specialized training and apparatus has limited the use of FISH to larger, properly equipped centres.

RT-PCR is appreciated as a valuable diagnostic tool in detection of translocations in many tumors, including synovial sarcoma, because of the rapid turnaround time, relatively low cost and applicability to both fresh and formalin-fixed tissues including archival cytological specimens (25, 79, 85, 208-211). RT-PCR-based detection of SYT-SSX is specific but sensitivity is inconsistent and depends on successful PCR amplification, which can fail in up to 10% of cases (87, 212). RT-PCR-based approaches also suffer from the lack of direct histologic correlation with test results, relatively large tissue requirements (compared to FISH), the intrinsic instability of mRNA and errors introduced by novel variant transcripts, which occasionally occur in synovial sarcomas (60, 65, 79, 80, 82). Many of the shortcomings of RT-PCR as a diagnostic tool for synovial sarcoma can be addressed using more expensive and labor-intensive techniques, such as laser capture microdissection of tumor cells to exclude non-tumor tissue, RT-multiplex PCR with capillary electrophoresis when only a small amount of sample is available for analysis and RT-PCR in situ hybridization to allow histologic correlation (57, 213, 214). Differentiation between SSX1 and SSX2 fusions can be accomplished with RT-PCR, however the high degree of sequence similarity between SSX1 and SSX2 requires highly specific detection methods. Gaffney et al. describe a method employing ligation of fluorescently labeled primers complementary to a common portion of SYT-SSX to
either SSX1 or SSX2–specific primers that is faster and potentially more cost-effective than a second round of fusion variant-specific PCR (215). Real time RT-PCR, which differs from standard RT-PCR by allowing monitoring of DNA amplification during, rather than after, the PCR process, has been successfully applied to detection and differentiation of SYT-SSX fusions in fresh frozen and archival (FFPE) synovial sarcoma specimens (216-218). Multiplex real-time RT-PCR capable of detecting various fusion transcripts, including SYT-SSX, in multiple tumor samples under identical PCR conditions has been described (219), which could be valuable in designing an automated test to screen these tumors for their respective fusion transcripts and improve the rapidity of testing.

RT-PCR amplification of SYT-SSX may also be valuable in detecting spread of synovial sarcoma beyond the main tumor, such as microinvasion of surrounding tissue. One study of tissue adjacent to the tumor margin by RT-PCR identified transcripts in 2 of 4 tumors, one of which was assessed histologically as having negative margins (220). In another study, SYT-SSX1 transcripts could be detected in tissue samples 3 cm from the gross tumor margin while histological examination detected microinvasion only up to 1 cm from the gross margin, providing evidence that microinvasion is likely underdetected (221). SYT-SSX transcripts have also been detected by RT-PCR in the blood of synovial sarcoma patients (52, 222). Although promising, these approaches to detection of microinvasion and potentially inadequate resection margins will require more extensive study to determine if they are of any practical clinical use.

Studies attempting to improve detection methods of t(X; 18) for diagnostic purposes have also revealed idiosyncrasies with potential biological significance. Cells harboring either SYT-SSX1 or SYT-SSX2 have been found to coexist in the same tumor in 10% of cases, although in all cases the protein products of these fusions were identical (69). An alternate translocation involving the SS18L1 gene (20q13.3), which is closely related to SYT (74, 115), has been identified in a fusion with SSX1 resulting from a translocation between chromosomes X and 20 in a case with typical biphasic synovial sarcoma histology (122). SS18L1 is involved in regulating neural dendrite growth that requires dimerization to function (121, 223). Synovial sarcoma-related translocations involving SS18L2 (3p21), which is also homologous to SYT (74, 115), have not been described. These observations
demonstrate that it is the function of the fusion protein itself, and not necessarily t(X; 18), that is critical for synovial sarcoma development.

1.3.2 Immunohistochemical markers

In situations where histological appearance is not sufficient to make the diagnosis of synovial sarcoma, mainly in monophasic and poorly differentiated tumors, a combination of immunohistochemical markers are usually employed. High levels of BCL2 expression can be detected in many synovial sarcomas and is a marker with good negative predictive value (45, 137-139). Synovial sarcomas can exhibit focal positivity for cytokeratin markers such as Cam5.2, AE1/AE3 and CK1 (10, 14, 28, 29, 224). Cytokeratin reactivity is generally not useful when differentiating monophasic tumors from other spindle cell neoplasms, where other immunohistochemical tests are more valuable, although monophasic synovial sarcoma have been reported to stain with cytokeratin (CK) 7 and/or 19 while few MPNSTs do so (45, 225). Biphasic synovial sarcomas can occasionally present diagnostic dilemmas, particularly with metastatic adenocarcinomas and mesothelioma. Differentiation of biphasic synovial sarcomas from metastatic adenocarcinomas can usually be accomplished by comparison to the primary adenocarcinoma. The epithelial markers BerEP4 (EpCAM) and CK7 can help separate biphasic synovial sarcoma from mesothelioma (226). Discrimination of monophasic fibrous synovial sarcoma and poorly differentiated synovial sarcoma from other spindle and small, blue cell tumors, respectively, can be especially difficult and immunohistochemical markers such as EMA, cytokeratins and BCL2 can be helpful (227). Like the monophasic and biphasic subtypes, monophasic fibrous synovial sarcoma and poorly differentiated synovial sarcoma are rarely, if at all, positive for CD34, smooth muscle antigen (SMA), h-caldesmon and desmin. Diffuse CD99 staining and focal immunoreactivity for CK7 and EMA has been reported in poorly differentiated synovial sarcoma; ES/PNET is frequently CD99 positive and may stain with AE1/AE3 but is typically CK7 negative, making CK7 useful in distinguishing these entities (43, 228, 229). S-100, a protein of unknown function but structurally similar to calmodulin, has been reported to be frequently positive in monophasic synovial sarcomas but less so in biphasic synovial sarcomas tumors; S100 positivity has been reported in 38% of monophasic fibrous synovial sarcoma and 23% of poorly differentiated synovial sarcoma as well, however it is also expressed by other
sarcomas in the differential diagnosis, particularly MPNST (29, 227). Considerable overlap in the immunohistochemical phenotype occurs between synovial sarcoma and certain spindle and small blue cell tumors, such as MPNST and ES/PNET, making proper selection of immunohistochemical markers essential in these situations and highlighting the need for a marker that is both highly sensitive and specific (29, 39, 230, 231).

A number of markers that are not typically used in the clinical setting may nevertheless be useful in select circumstances; moreover, the presence and subcellular localization of many of these proteins carries potentially significant implications for synovial sarcoma pathobiology. Nuclear staining for β-catenin is not common amongst most soft tissue tumors but has been repeatedly observed in a significant proportion of synovial sarcomas, suggesting it may be useful as a diagnostic tool (157). Examination of cadherin and catenin expression in synovial sarcoma demonstrated immunoreactivity for E-cadherin, pan-cadherin, α-catenin, β-catenin and δ-catenin (p120) in all samples; γ-catenin was found in 67% of samples (156). In this study, membrane-localized β-catenin was observed in all cases while nuclear β-catenin was seen in 73% and pan-cadherin expression was greater than E-cadherin, indicating other cadherins are also involved in synovial sarcoma cell-cell adhesion. In primary synovial sarcoma of the lung, nuclear β-catenin was found in 67% whereas only 10% expressed E-cadherin (10). E-cadherin expression occurs in 31-100% of cases in association with glandular and epithelioid components, but has also been detected in the spindle cell component of biphasic synovial sarcoma tumors (59, 186-189). Expression of dysadherin, a membrane glycoprotein that inhibits E-cadherin expression and promotes metastasis, can be demonstrated in 44% of monophasic synovial sarcoma tumors and is inversely correlated with E-cadherin expression (232).

Histologic and ultrastructural features of neural differentiation have been described with expression of neural markers including neuron specific enolase, protein gene product 9.5, B3GAT1 (CD57), CD99, GFAP, NF, S-100 and NCAM1 (CD56); expression of the latter appears to be widespread and may be useful diagnostically (29, 37, 38, 43, 45, 48). Nestin, an intermediate filament typically expressed in neural stem cells and occasionally expressed in immature skeletal
muscle, has been found in 83% of synovial sarcomas in one study (46), although others have reported that nestin is not detectable in synovial sarcoma (45).

Calponin, a smooth muscle protein involved in regulating contraction, has been identified as a sensitive (although not specific) marker of synovial that can be detected in most, if not all, monophasic synovial sarcoma and poorly differentiated synovial sarcoma subtypes, although expression is detectable in less than half of biphasic synovial sarcoma cases (233). Focal expression of CD34, a hematopoietic progenitor and endothelial cell antigen, has been described in a small amount of cases (26, 234). Progestagen-associated endometrial protein (PAEP, Glycodelin), a glycoprotein normally produced by epithelium of the endometrium and seminal vesicles, was detected in 11 of 11 biphasic synovial sarcomas in one study; expression was also detected in an epithelioid area of one monophasic synovial sarcoma (235). PAEP expression frequently occurs in gynecological malignancies and appears to promote neovascularization (236), though its significance in synovial sarcoma is unclear.

An immunohistochemical marker that is both highly sensitive and specific for synovial sarcoma is clearly lacking. Ideally, such a marker would be unique to synovial sarcoma, and in this respect SYT-SSX would be a perfect choice. Although specific immune responses have been generated to SYT-SSX breakpoint peptides (237), an antibody that specifically recognizes SYT-SSX is not currently available. Microarray studies have identified a number of overexpressed genes that discriminate synovial sarcoma from other tumors in the differential diagnosis, and these are likely candidates for effective immunohistochemical markers as well. Nielsen et al. found expression of EGFR and SALL2 to be both sensitive and specific markers for synovial sarcoma when compared to other sarcomas (238). Concordance between reports of EGFR expression are poor and vary from 25-70% of synovial sarcomas; estimates of the frequency of HER2 expression appears to be much less compared to EGFR and restricted to the glandular components of biphasic tumors (42, 139, 165, 166, 177, 178). Interestingly, when primary synovial sarcomas are passaged as xenografts in mice, they lose some immunophenotypic markers, including EGFR, but retain others, such as SALL2 (59), suggesting EGFR may be less critical to synovial sarcoma survival. Expression of KIT can be detected in up to 25% of synovial sarcomas; however it is expressed in other malignancies with similar histology and focal
expression of KIT in synovial sarcoma makes it difficult to interpret negative results, particularly on small specimens (171, 227, 239-241). SYT has been proposed as an immunohistochemical marker for synovial sarcoma, although it appears to have relatively poor sensitivity and specificity (242). The products of other highly overexpressed genes and related proteins, such as TLE, CRABP1 and PRAME, have yet to be explored.

1.4 Prognosis

Prior to the 1970s, synovial sarcoma patients fared poorly, with only 25-50% alive 5 years after diagnosis and a dismal 0-15% surviving to 10 years (1). Advancements in diagnosis and treatment have improved overall survival, which presently is 57-68% at 5 years, 41-50% at 10 years and 45% at 15 years (143, 155, 243-245). Assessing a number of clinical, histological and immunohistochemical predictors of outcome can further refine the prognosis for individual patients.

Some patient characteristics have significant associations with outcome. Presentation at a younger age, typically < 20 years, predicts a better outcome, despite other negative prognostic factors being similar between younger and older patients (243-246). In children and adolescents (< 21 years of age) estimated 5 year overall survival is approximately 80% and event-free survival is 56-72% (25, 247). The incidence of poorly differentiated synovial sarcoma, which predicts a worse outcome, as a proportion of cases increases with age of presentation (12). Duration of symptoms of less than 12 months also implies a poorer outcome (248), presumably because this is an indicator of more aggressive disease.

Gross tumor features, such as size and location, also significantly influence survival. Tumors ≥ 5 cm in diameter are strongly associated with poorer outcomes (143, 243, 246, 249-251). Occurrence in the extremities portends a better overall prognosis, particularly those arising in the arms (29, 245, 246); a prospective study of primary synovial sarcomas of the limb treated surgically revealed the risk of local and distant recurrence within 5 years of diagnosis and treatment was 12% and 39%, respectively; overall 5 year survival was 75% (250). Overall survival is poorer when synovial sarcoma arises in the head and neck region, with one small retrospective study reporting a 5 year overall survival rate of approximately 40% (252). Intrathoracic primary synovial sarcomas are more likely to be larger, occur in older patients and have a poorly differentiated subtype and thus a poorer outcome; the 5 year disease
free and overall survival in patients with intrathoracic synovial sarcoma is 21% and 23%, respectively (10, 26, 244).

Changes at the genetic level have been correlated with prognosis. Tumors harboring the SYT-SSX1 fusion have been shown to predict a poorer outcome. In a retrospective, multi-institutional study of 243 patients, Ladanyi et al. found that those with the SSX2 fusion variant have a significantly improved 5-year survival compared to those with the SSX1 variant (73% vs. 53%) as well as significantly improved overall survival (90). In patients presenting with localized disease, 5 year survival was 61% for SSX1 variants compared to 77% for SSX2 variant. SYT-SSX1 has also been associated with decreased metastasis-free survival, with patients harboring the SSX1 variant up to 7.4 times more likely to develop metastases and up to 8.5 more likely to die than those harboring SYT-SSX2 (52, 84, 90, 100, 141, 146, 253); however, one multicentre study of 165 patients found no significant correlation between fusion type (SYT-SSX1 vs. SYT-SSX2) and patient outcome (254). Other genetic aberrations in synovial sarcoma are uncommon; however, comparative genomic hybridization analyses of synovial sarcomas found that gains in chromosome 8 are significantly associated with tumors over 5 cm and those tumors harboring 3 or more aberrations predicted a poorer clinical outcome (99, 107).

Histology is also significant predictor of patient outcome. Biphasic histology is thought to portend a poorer prognosis (248), although some studies claim biphasic synovial sarcoma patients do the same or better than those with monophasic synovial sarcoma (84, 245, 254). The presence of poorly differentiated synovial sarcoma, however, is an unequivocally strong predictor of local recurrence, metastasis and tumor-related death (243, 245, 246, 248, 255). Histologic features less than 10 mast cells per HPF, high mitotic rate (≥ 10/10 HPF), high histologic grade, high proliferative index (PCNA ≥ 20% or MIB-1 ≥ 10%), prominent apoptosis, rhabdoid morphology, aneuploidy, local invasion of bone, nerves or vessels and histological grade (TMN) ≥ III significantly predict a shorter time to relapse and overall 5 year survival (83, 100, 138, 143, 246, 248, 250, 254, 256-258). Tumor recurrence, whether local or at distant sites, is responsible for the majority of morbidity and mortality in synovial sarcoma patients. In concordance with being a strong predictor of poor outcome, tumor size ≥ 5 cm is the most commonly cited predictor of recurrence and metastasis. Metastases are the first post-treatment recurrence in almost one quarter of patients within a
median of 1.6 years while local recurrence of disease was the first event in 16% of patients with a median time of 1.3 years; approximately half of these patients had simultaneous metastases as well (247). Of those patients who develop local recurrence of disease, approximately 42% will go on to develop metastases (median 1.5 years). Although most recurrent synovial sarcomas arise within 2 years of initial diagnosis, remissive periods as long as 15 years have been reported (245).

A number of immunohistochemical markers detectable in synovial sarcoma are prognostic indicators of patient outcome; the association of these markers with poorer or improved outcomes can provide clues to unraveling the underlying pathobiology of synovial sarcoma. Many of these markers have been discussed with respect to other facets of synovial sarcoma in the preceding sections; additional information is presented here. Significant correlation between poorer overall survival and decreased expression of E-cadherin and α-catenin and increased expression of β-catenin has been consistently reported in multiple studies (10, 152-158). Dysadherin expression in synovial sarcoma predicts significantly poorer overall survival as well, particularly when in combination with reduced E-cadherin expression (232). The relationship of p53 function to prognosis is unclear, as p53 gene mutation, > 10% nuclear localization and increased expression have been associated with poorer disease specific and overall survival in some studies (134, 143) but not in others (100, 135). These apparently discrepant observations are likely due to the paradoxically negative influence functional p53 can have on survival in the setting of adjuvant chemotherapy (259). Kawauchi et al. found that the combination of p27 expression in more than 70% of synovial sarcoma tumor cells with 0.9% or less of the tumor exhibiting apoptosis (by TUNEL assay) predicted a significantly improved overall survival (147).

Expression of proteases capable of degrading extracellular matrix, such as metalloproteinases, can promote local invasion and metastasis. Correlation of matrix metalloproteinase 2 (MMP2), MMP9 and TIMP2 (a metalloproteinase inhibitor) expression with outcomes in synovial sarcoma revealed that expression of MMP2 and lack of TIMP2 expression portends a shorter disease-free survival, but MMP2 expression does not correlate with overall survival (188, 260). Expression of the drug resistance proteins P-glycoprotein and GST-pi in synovial sarcoma has not been significantly related to response to chemotherapy, disease free survival and overall survival (195). Expression of the laminin receptor 1 (LMAR1), which is frequently
upregulated in carcinomas, in greater than 35% of cells is a negative predictor of
disease-free survival (185). Immunohistochemical detection of nuclear-localized
transcriptional factor YBX1 (Yb-1) in synovial sarcoma is significantly associated with
poorer survival (261). Expression of CD44 does not correlate with survival or
recurrence (262). The evidence for most of these potential prognostic indicators
comes from single retrospective studies and requires prospective or independent
external validation before they could be considered for clinical application.

1.5 Therapy

The survivability of synovial sarcoma has been improved in part by
advancements in therapy, the mainstay of which is radical or wide surgical excision,
though the lack of an effective systemic therapy to prevent recurrence remains a
significant impediment to long term treatment success (1). The morbidity frequently
associated with these surgeries has prompted assessment of less aggressive surgery
in select situations and marginal excisions of synovial sarcomas in the distal
extremities followed by local re-excision with or without postoperative radiation has
been reported to produce favorable results, with no recurrence in one report of 21
patients with 2 to 32.2 years (median 14.7) of follow-up (29).

Because synovial sarcoma is relatively uncommon, studies examining the
effectiveness of chemotherapy are usually forced to combine tumors exposed to
dissimilar treatment regimens that are inconsistently applied to achieve statistical
significance; for the same reason synovial sarcomas continue to be tested in
aggregate with other soft tissue tumors with disparate pathophysiologies. As such,
these studies are frequently incongruent and difficult to interpret and advances in
treatment are unlikely to occur in these settings (263). Nevertheless, research has
shown that attempts to control synovial sarcoma with regimens based on doxorubicin
and/or ifosfamide have met with varying, although typically limited, success (250,
264-266). In a study of 271 patients, 41% of whom received chemotherapy
(cyclophosphamide or ifosfamide + doxorubicin or epirubicin), the five year event-free
survival rate was 37%, although this varied with age: 66% for those less than 17
years old, 40% between 17-30 and 31% for those over 30; chemotherapy appeared
to improve metastasis-free survival only in patients with tumors greater than 5 cm
(267). Another report of synovial sarcomas occurring mostly in the limbs found that
46% of patients receiving chemotherapy had a partial response (244). In those
receiving neoadjuvant chemotherapy there was a partial response in 5 of 14 patients and one complete response; 3 other partial responders went on to complete response with melphalan-based therapy and autologous stem-cell rescue. A recent retrospective study of patients receiving radiation either before or after conservation surgery found 5, 10 and 15 year survival rates of 76%, 57% and 51% respectively (there was no comparison to patients receiving surgery only) (251). Some retrospective analyses of outcomes in children treated with surgery and adjuvant chemotherapy and/or postoperative radiotherapy reported some benefit (247, 268, 269), although others disagree (266, 270). Ultimately, prospective randomized trials will be needed to accurately determine the efficacy of chemotherapy in synovial sarcoma.

Minimal response to conventional chemotherapeutic agents is common amongst sarcomas and has prompted the development of more effective molecularly-targeted therapeutics to which synovial sarcoma might be particularly amenable (271). The uniqueness of the SYT-SSX fusion protein makes it a desirable target, however there are presently no drugs that block SYT, SSX or SYT-SSX function. The evidence for overexpression of certain receptor tyrosine kinases in synovial sarcoma and the development of effective inhibitors has led to considerable interest in RTK signaling inhibition. Compounds that inhibit EGFR signaling, such as gefitinib, do so by interfering with the tyrosine kinase domain; however responsiveness of non-small cell lung cancer to gefitinib has been shown to rely on the presence of mutations that enhance tyrosine kinase activity or increased copy number of $EGFR$ (168, 272, 273). A number of studies have determined that the prevalence of activating mutations are rare in synovial sarcoma (166-168), suggesting that EGFR tyrosine kinase inhibitors are likely to be of benefit only in those cases. Synovial sarcomas have been shown to express PDGFRα, and to a lesser extent KIT, suggesting that an inhibitor of these tyrosine kinases such as imatinib mesylate may be of therapeutic value in select cases as well (171). Ishibe et al. have demonstrated that a number of fibroblast growth factor genes (i.e. receptors and ligands) typically expressed in neural tissue are also expressed in synovial sarcoma cell lines (175). Inhibitors of FGF signaling decreased phosphorylation of ERK and both FGF signaling and ERK kinase inhibitors caused growth inhibition of synovial sarcoma cell lines in vitro and as xenografts, demonstrating that FGF signaling is a valid therapeutic target. The HDAC complex
component SIN3A interacts with SYT, suggesting that histone deacetylation may play a role in SYT-mediated transcriptional activation (117). Based on the association of SIN3A and SYT, the HDAC inhibitor depsipeptide FK228 was found to be capable of preventing proliferation of synovial sarcoma in vitro and in mouse xenografts (274). This study implicates deacetylation as an important factor in synovial sarcoma proliferation and identifies FK288, which is presently in phase II clinical trials, as a potentially effective therapy.

Another area of interest in experimental synovial sarcoma treatment is immunotherapy. Cytotoxic T lymphocytes capable of recognizing SYT-SSX-specific peptide can be identified in patients with metastatic synovial sarcoma, and peptides corresponding to the SYT-SSX fusion breakpoint have been shown to bind to HLA I molecules, suggesting that these may serve as specific cell surface antigens (237, 275). Immunotherapy has been attempted employing an immunogenic protein corresponding to the SYT-SSX fusion junction (276). Immunized patients experienced no serious adverse effects and the majority developed a cytotoxic T-lymphocyte response to the peptide; however, no significant therapeutic benefit was observed, though an autologous transplantation of dendritic cells exposed to synthetic SYT-SSX junction peptides produced temporary growth suppression of metastatic synovial sarcoma (277). Just under half of synovial sarcomas express at least one of SSX1-5, suggesting these proteins as a potential targets for vaccine-based immunotherapy as well (278). Cytotoxic T lymphocytes sensitized to SSX2 are able to recognize and lyse synovial sarcoma cells expressing the same antigen, implying the potential for effectiveness of this approach in clinical use (278). Downregulation of HLA I expression commonly occurs in cancer, however, suggesting that targeting intracellular proteins for immunotherapy is likely to have limited long term success. FZD10 is a membrane spanning Wnt signaling molecule that is overexpressed in synovial sarcoma while absent in normal tissues. SiRNA inhibition of FZD10 expression suppresses synovial sarcoma proliferation in vitro, suggesting FZD10 as a valid therapeutic target (162). Antibody raised against the extracellular domain FZD10 has been successful in directing antibody dependent cell-mediated cytotoxicity against synovial sarcoma in vitro and inhibiting growth when injected directly into xenografts in vivo. Thus, immunotherapy or a humanized antibody directed against FZD10 might have potential for clinical use in synovial sarcoma treatment. Other
potential antigens for immunotherapy include MAGE CT and NY-ESO-1CT (279, 280).

Many treatments are aimed at improving outcome in patients where full tumor resection is not possible or is otherwise undesirable, such as those entrapping major nerves and vessels. A study involving photodynamic therapy with acridine orange after tumor reduction surgery in synovial sarcoma of the extremities inhibited residual tumor growth for 19-51 months after treatment (281). Percutaneous radiofrequency thermal ablation has also been used to achieve local control of a pleural synovial sarcoma that was medically inoperable (282).

An important consideration in the development of an effective systemic therapy for synovial sarcoma is the presence of treatment resistant cells. There is preliminary evidence that synovial sarcoma contains a subpopulation of tumor initiating cells (TIC) (182). TIC typically exhibit stem-like properties, including relative resistance to conventional chemotherapeutics like doxorubicin. Persistence of such cells after treatment would explain why the presently used chemotherapeutic regimens are ineffective at preventing tumor recurrence. Efforts to further characterize synovial sarcoma TIC will allow assessment of the efficacy of systemic therapies against these cells and identify drugs more likely to improve patient outcome.

1.6 Summary

Synovial sarcoma is a translocationally defined soft tissue tumor of uncertain histiogenesis. Approximately 100 Canadians, mostly adolescents and young adults, will be diagnosed with synovial sarcoma annually and around half are not expected to survive beyond 10 years. There is both a great need and considerable opportunity for improving care for those afflicted with this tumor, particularly through research directed at developing improved diagnostic techniques and identifying novel targets for systemic therapy. Historically, the infrequency of synovial sarcoma compared to other malignancies has kept it out of the research limelight and consequently care for patients with this tumor has been slow to improve. This changed with the seminal description of the association between t(X; 18) and synovial sarcoma that led to a surge of research interest in the role of t(X; 18) and, in turn, enhanced patient care. More recently, gene expression studies conducted on primary synovial sarcomas by multiple groups using different microarray platforms have revealed consistent abnormalities, which implies a direct relationship with synovial sarcoma pathogenesis.
The hypothesis of this thesis is that those genes and pathways strongly highlighted by these studies represent valid diagnostic and/or therapeutic targets that can be clinically exploited to improve patient outcome.
1.7 References


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2 FLUORESCENCE IN SITU HYBRIDIZATION FOR THE DETECTION OF t(X; 18)(p11.2; q11.2) IN A SYNOVIAL SARCOMA TISSUE MICROARRAY USING A BREAKAPART-STYLE PROBE

2.1 Introduction

The histological diagnosis of synovial sarcoma can often be difficult as these tumors may resemble other sarcomas such as malignant peripheral nerve sheath tumor and Ewing sarcoma. Immunohistochemistry is not always sufficient to make the diagnosis, as a subset of synovial sarcoma has been shown to demonstrate focal or absent reactivity for characteristic markers (1). This can be a particular problem with core needle biopsy material. Detection of t(X; 18) can be invaluable in confirming a clinical diagnosis of synovial sarcoma, particularly when the histologic diagnosis is equivocal (1, 2). However, molecular confirmation of a synovial sarcoma diagnosis is frequently required after the fact or on small biopsy specimens when only formalin-fixed, paraffin-embedded (FFPE) tissue is available, limiting the methods available to detect t(X; 18).

A variety of diagnostic methods exist to detect translocations, including cytogenetic analysis, reverse transcriptase-polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH). FISH-based methods are most amenable to FFPE tissue, particularly small biopsies, and various FISH methodologies have been described to allow the visualization of structural chromosomal abnormalities in archival tissue (3-6). Two color breakapart style FISH assays employ probes that flank the translocation breakpoint and are separated by the translocation event. Co-localization assays utilize probes that lie adjacent to loci on separate chromosomes that are juxtaposed by the translocation event. To date, published studies on the detection of t(X; 18) in synovial sarcoma have used co-localization approaches, SSX breakapart probes, or combinations of chromosome paints and centromere probes (7-10). The interpretation of two color co-localization assays can be difficult when multiple partner loci are involved. In contrast, breakapart assays are well-suited to

* A version of this chapter has been published. Terry, J., Barry, T. S., Horsman, D. E., Hsu, F. D., Gown, A. M., Huntsman, D. G., and Nielsen, T. O. Fluorescence in situ hybridization for the detection of t(X; 18)(p11.2; q11.2) in a synovial sarcoma tissue microarray using a breakapart-style probe. Diagn Mol Pathol 2005; 14(2): 77-82.
detect translocations that involve multiple partner loci, such as in synovial sarcoma where the X chromosome breakpoint lies within one of three SSX genes (11).

The application of FISH to identify t(X; 18) in archival tissues could be very useful for the diagnosis of synovial sarcoma in both clinical and research settings, particularly in small tissue samples such as core needle biopsies and tissue microarrays. However, FISH-based methods are laborious, time consuming and are not easily applied to small FFPE tissue samples (12). Various protocol improvements and modifications have been proposed to enhance the utility of FISH in the clinical setting (10, 13) and the use of chromogenic in situ hybridization (CISH) technology has been applied in an attempt to improve the portability of breakapart probe assays. We have a particular interest in developing an improved FISH-based t(X; 18) assay not only for clinical use but that is applicable to screening for t(X; 18) in tissue microarrays prior to conducting studies to identify potential synovial sarcoma immunohistochemical markers, which will be presented in Chapter 3. Here, we present three novel improvements that will greatly facilitate the use of FISH on small FFPE tissue samples: the application of an “in house” two color breakapart interphase FISH assay for t(X; 18) detection, application of FISH to a tissue microarray (TMA) to assess multiple tumor samples simultaneously, and the use of a simplified scoring algorithm for the interpretation of the resulting data. In addition, we compared these results with the findings obtained with a recently released commercial breakapart FISH probe set using an automated scoring system, and with a commercial CISH probe set.

2.2 Materials and methods

2.2.1 Tissue samples

The synovial sarcoma TMA was constructed from archival paraffin-embedded biopsy samples from cases referred to the British Columbia Cancer Agency and has been previously described (14). Briefly, biopsy samples were collected between 1980 and 2001, fixed in 10% neutral buffered formalin (3 cases were fixed in Bouin’s solution) and routinely processed. We selected 27 patient samples with a molecularly confirmed diagnosis of synovial sarcoma (by cytogenetic analysis, RT-PCR and/or expression profiling) to serve as our known synovial sarcoma sample set, and 29 patient samples representing other sarcomas that are included in the differential diagnosis of synovial sarcoma (5 cases each of Ewing sarcoma, solitary fibrous...
tumor, malignant peripheral nerve sheath tumor, leiomyosarcoma, low grade fibromyxoid sarcoma, and 4 cases of hemangiopericytoma) to serve as our non-synovial sarcoma sample set. 18 patient samples in which a diagnosis of synovial sarcoma was made by histology and immunohistochemistry but without t(X; 18) information were also included as test samples. These were later examined for t(X; 18) by RT-PCR, as described previously (15), on formalin-fixed material, with the FISH scorers blinded to the RT-PCR results. Duplicate 0.6 mm cores were used for TMA construction, as well as samples of murine kidney as a non-human negative control. As a positive control, formalin-fixed paraffin-embedded cell blocks (16) were made from cultures of the t(X; 18) positive synovial sarcoma cell line SYO-1 (17). As a negative control, formalin-fixed paraffin-embedded cell blocks were made from cultures of the sarcoma cell line HTB-93 (SW982), obtained from the American Type Culture Collection (Manassas, VA). HTB-93 has been described by the supplier as synovial sarcoma; however, it does not harbor t(X; 18) (18). The presence of t(X; 18) in SYO-1 and its absence in HTB-93 was confirmed in our laboratory by karyotype analysis.

2.2.2 Probe sets

Two FISH probes flanking the SYT gene on chromosome 18 were generated from the RPCI BAC library (19). The BAC clone RP11-90L7 (166 kb) was used to generate the probe centromeric to SYT (Accession number AQ284487.1) while BAC clone RP11-802C10 (191 kb) was used to generate the probe located telomeric to SYT (Accession number AP002752) (Figure 2.1). The probes were labelled with SpectrumOrange or SpectrumGreen, respectively, using the Nick Translation Kit (Vysis, Downers Grove, IL) and purified to remove excess label using Centri-Sep purification columns (Princeton Separations, Adelphia, NJ) according to the
manufacturer’s protocols. 1 μg of each probe was mixed with 10 μg of Cot-1 DNA and 20 μg of human placental DNA to a total volume of 10 μl. The DNA was then precipitated, centrifuged and dried according to standard protocols. Each purified probe was then resuspended in LSI Hybridization buffer (Vysis) to give 10 ng probe/μl. These probes were tested on samples of normal and synovial sarcoma positive control samples confirmed by cytogenetic analysis prior to using them in this study.

2.2.3 Tissue microarray preparation

Two 4 μm sections of the previously constructed TMA were transferred to glass slides and incubated overnight at 60°C. These slides were then deparaffinized by three 5 minute xylene washes and then dehydrated with two one minute washes in 100% ethanol. The slides were then pretreated by immersion in 0.2 N hydrochloric acid (HCl) for 20 minutes, followed by a 10 minute distilled water wash, a three minute 2x saline sodium citrate (SSC) wash, an incubation in 1 M sodium thiocyanate at 80 °C for 30 minutes and a three minute distilled water wash. The slide was then demasked by incubation in a 0.3% pepsin solution (in 0.01 N HCl) at 37 °C for 15 minutes, washed in distilled water for three minutes, dehydrated in 100% ethanol for one minute and then air dried for 30 minutes. Unless otherwise stated, all incubations were at room temperature.

2.2.4 Hybridization and probe detection

Aliquots containing 100 ng of each probe were added to the dried slide and sealed under a coverslip with rubber cement. The slide was then incubated at 73 °C for five minutes to denature the DNA followed by hybridization in a humidified chamber (HYBrite, Vysis) at 37 °C overnight. The rubber cement was removed and the slide was incubated in post-hybridization buffer (2x SSC with 0.3% NP40) to remove the coverslip. The slide was then incubated in fresh post-hybridization buffer at 72 °C for two minutes, followed by a two minute wash in 70% ethanol and dehydration by a one minute wash in 100% ethanol. Finally, the slide was air dried, counterstained with 10 μl of Vysis DAPI I reagent (1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in antifade mounting solution) and coverslipped.

Three composite images of separate areas in each tissue core in the TMA were captured on an Axioplan 2 fluorescence microscope with a 63x objective lens.
and appropriate filters coupled to an AxioCam MRm cooled CCD camera (Zeiss, Thornwood, NY). Each composite image was constructed by stacking nine images taken at focal planes 0.04 μm apart using Metafer image analysis software (MetaSystems, Belmont, MA). Automated background reduction was applied up to twice in images exhibiting high background fluorescence; otherwise, images were not modified after capture. The composite images were recorded for subsequent visual scoring.

### 2.2.5 FISH analysis and scoring

The total numbers of paired and unpaired signals were enumerated for each of the sample cores. The number of unpaired signals was averaged to give the number of mean unpaired signals. Paired signals are defined as an orange and green signal less than three signal diameters apart or a single yellow (overlapping) signal, while unpaired signals were scored as a single green signal and single red signal separated by greater than or equal to three signal diameters. Signals with abnormal morphology were excluded as were images with less than 50 paired and mean unpaired signals. All signals in each image were counted. Data for paired and unpaired signals in each duplicate core were summed and the ratio of paired to unpaired signals, termed the sample score ratio or SSR, was determined. The mean SSR and the standard error of the mean for the non-synovial sarcoma samples were used to determine a value that would optimally divide the synovial sarcoma and non-synovial sarcoma SSRs with respect to test sensitivity and specificity. This cutoff value was then applied to the unknown samples to determine if an SYT disruption was present.

Statistical analysis of the data was performed using SPSS statistical software (SPSS Inc., Chicago, IL) employing Student’s t-test or the Mann-Whitney U test where appropriate.

### 2.2.6 Commercial SYT FISH probe TMA analysis

The Vysis SYT translocation breakapart probe pair was used to probe the synovial sarcoma TMA according to the manufacturer’s instructions. Automated quantitative morphometric analysis of SYT probe FISH was performed using the MetaSystems Metafer scanning system to enumerate and analyze the FISH signals. Each sample core image is divided using a tiling algorithm that permits localization of the nuclei. Signals within tiles are stratified according to proximity, and those tiles containing differentially labelled signals that are separated by 10 or more image pixels
are considered positive. The percentage of positive tiles is calculated. The threshold for positivity was established from a group of non-synovial sarcoma cases that do not contain t(X; 18). A positive case was defined as a case in which the percent of positive tiles detected is greater than 3 standard deviations above the mean of the negative group.

2.2.7 Commercial SYT CISH probe TMA analysis

The SPOT-Light SYT Translocation CISH breakapart probe pair (cat# 84-2500, Zymed, South San Francisco, CA) and CISH translocation detection kit (cat# 84-9288, Zymed) were used to probe the synovial sarcoma TMA and a cell block of the synovial sarcoma cell line SYO-1 according to the manufacturer’s instructions.

2.3 Results

2.3.1 Efficiency of hybridization and controls

Signals corresponding to each BAC FISH probe were seen in every synovial sarcoma sample core whereas there were no signals visible in the mouse kidney negative control sample cores. In general, the signals were easily visible and enumerated in the majority of cores. Four of the 27 synovial sarcoma samples, 7 of the 18 test synovial sarcoma samples and 6 of the 29 non-synovial sarcoma samples could not be interpreted due to high background which obscured the majority of signals. There was no correlation between uninterpretable samples and the sample origin, sample age, fixation method or physical location in the TMA. Hybridization efficiency varied moderately among the cores from different samples on the TMA slide. There was minimal variation between duplicate cores of each sample.

2.3.2 Determination of mean sample score ratios (SSR) and optimal cutoff ratio

There was no significant difference between the number of paired and unpaired signals (U=262, p=0.956) in the 23 molecularly-confirmed synovial sarcoma samples (Figure 2.2). SSRs for synovial sarcoma samples ranged from 0.38 to 5.63 with a mean of 1.37 (standard error of the mean (SEM) +/- 0.24). In contrast, there was a significant difference between the number of paired and unpaired signals (U=26, p<10^-6) in the 22 interpretable non-synovial sarcoma samples (Figure 2.2). SSRs for the non-synovial sarcoma samples ranged from 3.91 to 32.8 with a mean value of 13.96 (SEM +/- 1.75).

The optimal cutoff ratio was determined by subtracting multiples of the SEM from the mean SSR of the non-synovial sarcoma samples until the best compromise
between sensitivity (the percentage of synovial sarcoma samples with SSRs below the cutoff) and specificity (the percentage of non-synovial sarcoma samples with SSRs above the cutoff) was achieved (Table 2.1). The optimal cutoff ratio was determined to be 3.46 and as such tumor samples with a SSR equal to or above 3.46 will be considered to not carry t(X; 18), whereas those with a SSR below 3.46 will be considered to carry t(X; 18). Using this ratio, 22/23 of the synovial sarcoma samples are below the cutoff and are correctly identified as having an SYT disruption (Fig. 2.3), while 22/22 of the non-synovial sarcoma samples are above the ratio and correctly identified as not carrying the SYT translocation. This equates to a sensitivity of 96% and a specificity of 100% among interpretable cases (Table 2.1).

<table>
<thead>
<tr>
<th>SSR Cutoff (-SEM)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.71 (3)</td>
<td>100% (23/23)</td>
<td>64% (14/22)</td>
</tr>
<tr>
<td>6.96 (4)</td>
<td>100% (23/23)</td>
<td>82% (18/22)</td>
</tr>
<tr>
<td>5.21 (5)</td>
<td>96% (22/23)</td>
<td>86% (19/22)</td>
</tr>
<tr>
<td>3.46 (6)</td>
<td>96% (22/23)</td>
<td>100% (22/22)</td>
</tr>
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Table 2.1 The sensitivity and specificity of the simplified scoring algorithm at different cutoff ratios. (SSR = sample score ratio, SEM = standard error of the mean). To determine sensitivity, 23 synovial sarcomas were used. Specificity is based on 22 other soft tissue tumors in the differential diagnosis.
2.3.3 Analysis of unknown samples with optimal cutoff ratio

The optimized cutoff ratio was then applied to the test samples. 10 of 11 (91%) of these were below the cutoff, indicating the presence of t(X; 18) and the diagnosis of synovial sarcoma. In six of these cases, results were confirmed by diagnostic RT-PCR. The remaining four cases gave no amplifiable product by RT-PCR.

![Distribution of synovial sarcoma (black) and non-synovial sarcoma sample (gray) score ratios.](image)

**Figure 2.3** Distribution of synovial sarcoma (black) and non-synovial sarcoma sample (gray) score ratios.

2.3.4 Commercial SYT probe FISH and CISH TMA analysis

Finally, we tested two commercially available t(X; 18) detection probe sets, the Vysis SYT breakapart FISH probe set and the Zymed SPOT-Light SYT breakapart probe set, on sections from the same synovial sarcoma TMA. The Vysis probe set provided comparable results to the BAC probe set. Of the known synovial sarcoma cases, 17 of 21 (81%) were considered positive for an SYT breakage event, as were 14 of 17 (82%) of test synovial sarcoma samples. Of the non-synovial sarcoma samples, using a cutoff value of 2.4 (which is just over 3 standard deviations above
the mean of the negative group), 0 of 28 cases were found to contain SYT disruptions. Six of the 27 known synovial sarcoma samples, 2 of the 19 test synovial sarcoma samples and 1 of the 29 non-synovial sarcoma samples could not be interpreted due to weak or absent signals.

The Zymed CISH probe set produced weak signals and uninterpretable results in the tissue microarray archival cores using the same conditions where CISH signals were readily observed in freshly-prepared synovial sarcoma SYO-1 cell line formalin-fixed paraffin-embedded blocks.

2.4 Discussion

This study describes the use of a simplified scoring method to interpret the results of a dual-color breakapart style interphase FISH assay to detect the presence of a disrupted SYT gene when applied to a sarcoma TMA. The simplified scoring scheme was developed using positive and negative control samples and correctly identified blinded synovial sarcoma test samples. This study demonstrates the novel use of a breakapart probe FISH-based assay to interrogate a TMA for clinically relevant diagnostic translocations. The success of this approach indicates that it is of use in both clinical (core biopsy) and research (TMA) applications.

There are a number of methodologies that can be used to detect t(X; 18). Cytogenetic analysis has been commonly used; however, this method is time-consuming, expensive and requires fresh tissue. RT-PCR is popular in clinical diagnostic and research use, as it is a relatively rapid and simple procedure that is capable of distinguishing between SSX1 and SSX2 fusions (20-23). The success of RT-PCR in archival tissues, unfortunately, is inconsistent due to variability in the quality of RNA retrieved (as occurred in this study) (22, 24-26). The use of FISH to interrogate archival tissue has the advantages of detecting DNA (which is better preserved than RNA), of identification of the translocation in its histological context, and of ready applicability to small core biopsy samples and routinely-processed material referred from other centres. Some have proposed that FISH should be used for molecular verification of synovial sarcoma when RT-PCR fails (27), whereas others contend that FISH is superior to RT-PCR (12). Presently, the equipment, time and technical expertise required for FISH assays limits its routine clinical use. Nevertheless, the application of a simplified scoring scheme, capacity for detection of translocations as well as amplifications, and the applicability to tiny, variably-fixed
specimens (including multiple tumors in TMA format) all contribute to the potential clinical utility of this technique.

Of note, two of three synovial sarcoma cases fixed in Bouin’s solution produced interpretable FISH results, allowing the detection of SYT rearrangements. In general, Bouin’s-fixed material makes a very poor template for PCR-based studies (28) but this FISH strategy at least partially alleviates this problem, even as employed here on older archival specimens in a TMA format.

TMAs are efficient tools for tumor analysis in that they use less patient tissue, fewer laboratory resources and require less time per sample for analysis. Prior to conducting this study, there was only one report of the use of FISH to detect translocations in TMAs (29). More recently, the commercial SYT probe set produced by Vysis has been applied to a synovial sarcoma TMA with results similar to ours (30). A sarcoma TMA and a simplified scoring scheme to enumerate FISH detection of t(X; 18) was used in this study to ascertain if breakapart probe FISH analysis could be successfully applied to TMAs. Twenty-three percent (17/74) of cores in the synovial sarcoma TMA were discarded as un-scorable, which highlights the main shortcoming of performing FISH on TMAs: the inability to optimize tissue preparation and hybridization conditions individually for each tumor sample. Interestingly, the majority of samples discarded from this analysis were also discarded from the analyses with the commercial SYT FISH probe set as well, suggesting an inherent problem with those particular samples. Optimization of protease concentrations and digestion times is often necessary to reduce background fluorescence and to obtain good levels of hybridization in archival tissue (9, 13). There was no overall correlation between sample interpretability and source block age, hospital of origin or fixation protocol. The development of efficient diagnostic protocols for TMA-based assays could become an important step in the practical application of gene-specific screening tests, which will to play an increasingly crucial role in the diagnosis and treatment of cancer.

Various methods of interpreting the results of FISH-based assays to detect t(X; 18) have been proposed (6-8, 10). All are effective, though most are also relatively labor-intensive. By circumventing the need for certain tissue processing steps (i.e. nuclear disaggregation), the simplified scoring algorithm presented above expedites the FISH process in two ways: it permits the use of TMAs, in which certain tissue
processing steps cannot be easily done, and it directly accelerates both the tissue preparation and scoring procedures. This does not come at a cost in accuracy of the results, as the algorithm identified 10/11 of synovial sarcoma test samples and 22/23 of known synovial sarcoma samples as containing t(X; 18) while identifying 22/22 of non-synovial sarcoma samples as not containing t(X; 18). In general, the results obtained by the commercial SYT FISH probe set were congruent with the in house BAC probes, with the exception that the commercial probe set exhibited somewhat less sensitivity. In our hands, this decrease in sensitivity is likely due to the automated quantitative morphometric analysis software, which employs a selection algorithm that identifies areas (image tiles) by nuclear borders in which signals are counted. The software discards those image tiles that do not meet certain criteria from the final analysis for each tumor sample. Those synovial sarcoma samples that were improperly identified as not carrying t(X; 18) by the automated software had particularly dense areas of tumor tissue, causing an inordinate amount of tumor tile images to be discarded and allowing the non-neoplastic tissue signals to skew the results. Presently, the software criteria for nuclear border recognition cannot be fully modified, which would be expected to improve the sensitivity of this assay. Otherwise, there was no apparent difference in hybridization quality between the in-house and commercial SYT probes. The CISH SYT probe set produced weak signals on the TMA that were very difficult to visualize and prohibited the use of the scoring scheme to interpret the results.

The two synovial sarcoma samples not identified by this scoring scheme as being t(X; 18) positive were initially diagnosed based on typical histology and immunohistochemistry features. The synovial sarcoma samples from the known set had a gene expression profile that clustered with other synovial sarcoma samples (but cytogenetic analysis was not performed at diagnosis, and RT-PCR gave no product). Review of the FISH images of these cores revealed that the “known synovial sarcoma” case exhibited predominantly paired signals with occasional solitary signals of either color. This could occur, for example, from a high proportion of non-neoplastic cells within the tumor sample or multiple copies of the unrearranged chromosome 18. The former possibility is the likely explanation here, as this case had a prominent myxoid matrix and a considerably smaller number of tumor cells relative to non-neoplastic vessels than was seen in other cores. The stable loss of one
derivative chromosome could also explain these findings, as has been reported elsewhere (31). The FISH-negative synovial sarcoma from the test set showed only paired signals on review of images and by this method clearly does not exhibit t(X; 18); RT-PCR assay for t(X; 18) in this case also yielded a negative result. Loss of one derivative chromosome or involvement of an SYT homologue represents biological limitations on the sensitivity of a FISH breakapart strategy using this scoring scheme applied to archival material where metaphase chromosomes are not available.

The high sensitivity and specificity demonstrated by this approach suggests that the correlation of signals to nuclear borders is not essential for accurate detection of t(X; 18). Indeed, scoring schemes that disregard nuclear boundaries in the detection of translocations have previously been shown to produce results similar to those that require definition of the nuclear borders (6, 10). Not requiring nuclear boundaries will hasten the FISH process both by simplifying counting algorithms and by removing nuclear disaggregation steps from the tissue preparation protocol. Disregarding nuclear borders also circumvents problems associated with nuclear overlap and aneusomy (6). This scoring method should also be applicable to the detection of translocations in other tumors.

In conclusion, this study demonstrates that a breakapart interphase FISH-based assay is a sensitive and specific technique for the detection of t(X; 18) in small samples of FFPE tissues. The methods described here could be applied in the clinical setting to expedite the molecular confirmation of unclear synovial sarcoma diagnoses as well as in retrospective studies involving multiple tumor samples. The application of these methods could also prove to be useful in the diagnosis of other tumors with characteristic translocations.
2.5 References


10. Lu YJ, Birdsall S, Summersgill B, et al. Dual colour fluorescence in situ hybridization to paraffin-embedded samples to deduce the presence of the der(X)t(X; 18)(p11.2; q11.2) and involvement of either the SSX1 or SSX2 gene: a diagnostic and prognostic aid for synovial sarcoma. J Pathol 1999; 187(4):490-6.


3 TLE1 AS A DIAGNOSTIC IMMUNOHISTOCHEMICAL MARKER FOR SYNOVIAL SARCOMA EMERGING FROM GENE EXPRESSION PROFILING STUDIES *

3.1 Introduction

Correct diagnosis of synovial sarcoma based on histology alone can be challenging especially in small biopsies, as monophasic synovial sarcomas can appear similar to other spindle cell tumors (including malignant peripheral nerve sheath tumor (MPNST), fibrosarcoma and hemangiopericytoma), and poorly differentiated synovial sarcomas can resemble several tumor types including Ewing sarcoma. T(X; 18) is the diagnostic gold standard for synovial sarcoma. Improvements in methodology to facilitate detection t(X; 18) by FISH in FFPE tissue are described in Chapter 2; however, this method is not widely available because most hospital diagnostic laboratories lack the specialized equipment to detect fluorescent probes. Unfortunately, the commercial chromogenic SYT breakapart ISH probes presently available, which would facilitate use of this assay in such centers, work poorly. Identification of an immunohistochemical marker with very high sensitivity and specificity, ideally approaching that of t(X; 18) molecular diagnostic testing, would be an ideal alternative. Immunoreactivity for epithelial markers such as cytokeratin and epithelial membrane antigen (EMA) is frequently used to aid in differentiating synovial sarcoma from other spindle cell neoplasms; however, these markers not only lack specificity (1, 2), but also are limited in sensitivity because these markers are only focally expressed in many synovial sarcomas and are completely negative in a subset of monophasic cases.

Gene expression profiling, using different platforms, comparison groups and informatics approaches, has consistently shown a major association of the Wnt signaling pathway with synovial sarcoma (3-9). One prominent gene related to the Wnt pathway is TLE1, which has been found to be a good discriminator of synovial sarcoma in multiple studies (Table 3.1) (6, 9-11). TLE1 is one of four TLE (Transducin-Like Enhancer of split) genes that encode human transcriptional

Table 3.1 Gene microarray expression profiling studies identify TLE as a good discriminator for synovial sarcoma from other sarcomas.

<table>
<thead>
<tr>
<th>Study</th>
<th>TLE1 Rank*</th>
<th>Array Type</th>
<th>Ranking Parameter</th>
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<th>Comparison Group</th>
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<td>12600 cDNA spotted</td>
<td>Weighted p-value</td>
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<td>1 ASPS; 1 CCS; 1 CSa; 5 DFSP; 19 EWS; 7 FSa; 5 GIST; 6 HPCT; 17 LMS; 33 LPS; 38 MFH; 2 MMMT; 5 OSa; 6 BS; 3 MPNST; 6 RMS; 10 NOS</td>
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<td>Laé Ref #10</td>
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<td>22215 probe set Affymetrix U133A</td>
<td>p-value</td>
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<td>28 EWS; 28 DSRCT; 23 ARMS; 12 ASPS</td>
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<tr>
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<td>42000 cDNA spotted</td>
<td>p-value</td>
<td>13</td>
<td>24 MPNST</td>
</tr>
<tr>
<td>Segal et al. Ref #9</td>
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<td>12626 probe set Affymetrix U95A</td>
<td>p-value</td>
<td>5</td>
<td>6 CCS; 5 DDLS; 5 GIST; 8 FSa; 6 LMS; 11 MFH; 3 PLS; 4 RCLS</td>
</tr>
</tbody>
</table>

ARMS: Alveolar rhabdomyosarcoma; ASPS: Alveolar soft parts sarcoma; BS Benign schwannoma; CCS: Clear cell sarcoma; CSa: Chondrosarcoma; DFSP: Dermatofibrosarcoma protuberans; DDLS: Dedifferentiated liposarcoma; DSRCT Desmoplastic small round cell tumor; EWS: Ewing sarcoma; FSa: Fibrosarcoma; GIST: Gastrointestinal stromal tumor; HPCT: Hemangiopericytoma; LMS: Leiomyosarcoma; LPS: Liposarcoma; MFH: Malignant fibrous histiocytoma; MMMT: Malignant Mixed Mullerian tumor; MPNST: Malignant peripheral nerve sheath tumor; NOS: Unclassified sarcoma; OSa: Osteosarcoma; PLS: Pleiomorphic liposarcoma; RCLS: Round-cell liposarcoma; RMS: Rhabdomyosarcoma; SS: Synovial sarcoma

*Rank of TLE1 within total gene list when sorted by ability to positively discriminate synovial sarcoma

repressors homologous to the Drosophila corepressor groucho (12). Differential overexpression of TLE2, 3 and 4 has also been demonstrated in synovial sarcoma (3, 6, 8). TLE proteins are temporally expressed in embryogenesis where they are involved in developmental processes including neurogenesis, body patterning and hematopoiesis (12-15). The repressive effect of Groucho and TLE1 is dependent on phosphorylation status and involves histone deacetylase (HDAC) activity (16-20). The HDAC inhibitor FK228 has recently been shown to inhibit proliferation of synovial sarcoma, supporting the idea that TLE1 overexpression may play an important role in synovial sarcoma pathobiology and identifying TLE1 as a potential therapeutic target (21, 22).

The specificity of TLE1 gene expression for synovial sarcoma, particularly when compared to other sarcomas, suggests that TLE1 may be clinically exploitable as an immunohistochemical marker. Here, the protein expression of TLE1 in synovial sarcoma and in a broad range of mesenchymal neoplasms is investigated using
tissue microarrays that have been screened for t(X; 18), using the methodology developed in Chapter 2, to assess the value of TLE1 as a diagnostic marker for this sarcoma.

3.2 Materials and methods

3.2.1 Tumor samples and tissue microarrays

Tissue samples were retrieved from the archives of the Vancouver General Hospital (Vancouver, BC), Stanford Medical Center (Stanford, CA), University of Washington (Seattle, WA), Cleveland Clinic (Cleveland, OH), Oregon Health & Science University (Portland, OR), and Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY). Slides corresponding to each archival sample were reviewed by at least two staff pathologists with expertise in bone and soft tissue tumors, and representative areas in each original tissue block identified. The TA-19 synovial sarcoma tissue microarray has been previously described (23) and contains 44 molecularly-confirmed synovial sarcomas and 29 other sarcomas with related histologies. The MSKCC synovial sarcoma tissue microarrays contains 52 molecularly confirmed synovial sarcomas each represented by one 3 mm and one 1 mm core (on different arrays). The TA-138 tissue microarray contains 44 cases of NF-1 related MPNST, 24 sporadic MPNST, 15 synovial sarcoma, 8 localized neurofibroma, 24 plexiform neurofibroma, 11 diffuse neurofibroma, 7 cellular schwannoma, 15 typical schwannoma, 4 perineuroma, 10 melanoma, 5 clear cell carcinoma, and 5 cases of dermatofibrosarcoma protuberans with fibrosarcomatous change. The sarcoma tissue microarrays TA-34 and TA-35 contain 421 benign and malignant soft tissue tumor specimens representing over 50 diagnostic entities and have been previously described (24), as has tissue microarray 03-008, which contains 121 cases of chondroid and osseous tumors (25). For each of these, a tissue microarrayer (Beecher Instruments, Sun Prairie, WI) was used to extract duplicate 0.6 mm or 1.0 mm cores from representative areas of each original formalin-fixed paraffin-embedded tissue block and to transfer to the recipient microarray block, except for one of the MSKCC tissue microarrays for which 3 mm cores were obtained using a manual punch instrument. All tissue samples were collected according to protocols approved by the ethics committees at the contributing institutions.
3.2.2 Molecular confirmation of synovial sarcoma cases

The presence of t(X; 18) in the synovial sarcoma cores and absence of t(X; 18) in the non-synovial sarcoma cores with positive TLE staining in the TA-138, 03-008, TA-34 and TA-39 microarrays were verified using the method described in Chapter 2. Synovial sarcoma cases in which the presence of t(X; 18) could not be verified were excluded from analysis. Cases on the MSKCC tissue microarrays were individually validated by SYT-SSX RT-PCR on frozen or formalin fixed paraffin embedded material, as described previously (26), and included 35 SYT-SSX1 cases and 17 SYT-SSX2 cases.

3.2.3 Immunohistochemistry

Heat-induced epitope retrieval was performed by heating 4 μm sections of each tissue microarray for 30-40 minutes in 10 mM EDTA buffer pH 8. Monoclonal rat anti-human pan-TLE antibody, which recognizes the highly conserved WD-40 domain, has been previously characterized (12) and was graciously provided by S. Stifani (Montreal Neurological Institute, Montréal, QC). Polyclonal rabbit anti-TLE1, which also cross-reacts to a lesser extent with TLE2, 3 and 4, was purchased from Santa Cruz Biotechnology (M101, cat# 9121, Santa Cruz, CA). Each microarray section was hybridized with a 1:2 dilution of monoclonal anti-pan-TLE or a 1:20 dilution of M-101 polyclonal anti-TLE1 using a Ventana automated immunostainer (Tucson, AZ) for 30 minutes followed by washing and hybridization with a 1:1000 dilution of HRP-conjugated anti-rat or anti-rabbit IgG antibody, respectively (Abcam, Cambridge, UK). M-101 staining for comparative purposes was also performed manually using a 1:200 dilution and 4°C overnight incubation. Endogenous peroxidase activity was quenched and antibody visualized by incubation with 3,3'-diaminobenzidine for 10 minutes. TLE immunostaining was graded as “3+” (strong) if greater than 50% of tumor cells per core exhibited intense nuclear staining visible with a 4x low power objective lens, “2+” (moderate) if 10%-50% exhibited intense nuclear staining obvious at low power or greater than 50% nuclear staining well above background when assessed with 10x objective magnification, “1+” (weak) if less than 50% of cells exhibited weak to moderate nuclear staining and “0” (negative) for no visible nuclear staining. Where scores of duplicate cores were discrepant, the higher score was used. Uninterpretable sets of duplicate cores (i.e. no tumor cells, absence of viable cells or folded/lost tissue core) were excluded from analysis.
Tumors with a score of 2+ or 3+ on at least one examined tissue microarray core were considered positive for TLE. Sections of each microarray were also stained with hematoxylin and eosin (H&E) using standard methods for histologic reference.

3.2.4 Digital images

Digital images of immunostained and H&E stained microarrays were acquired using a BLISS imager (Bacus Laboratories, Lombard, IL, USA). Images were not digitally modified after capture. A relational database was constructed that correlates scoring and identification information with images of each core. This information is publicly accessible at https://www.gpecimage.ubc.ca/tma/web/viewer.php.

3.3 Results

3.3.1 TLE1 as an immunohistochemical marker

The consistent identification of strong TLE expression in synovial sarcoma, from several gene expression profiling studies in different laboratories using different DNA microarray platforms (Table 3.1) led us to investigate its value as a diagnostic immunohistochemical marker. Two antibodies against TLE1, a monoclonal antibody recognizing an epitope in the C-terminal WD-40 domain, previously shown to work in immunohistochemical applications (12), and a commercially-available polyclonal antibody raised against TLE1, were tested against 693 cases of adult soft tissue tumors including 94 molecularly-validated synovial sarcomas using a tissue microarray format.

Both antibodies gave intense, easy-to-interpret nuclear staining in positive cases. Examples of the four grades of staining, as described in materials and methods, are presented in Figure 3.1. Original images of tissue cores are available for public review at https://www.gpecimage.ubc.ca/tma/web/viewer.php, and include H&E, pan-TLE monoclonal and M101 TLE1 polyclonal immunostains on the same (TA-138) tissue microarray for comparative purposes.

The two antibodies (monoclonal anti-pan-TLE and M101 polyclonal anti-TLE1) were tested on sequential sections of the TA-138 tissue microarray and found to be almost equivalent (8 discrepancies among 177 cases, Kappa statistic 0.78, \( p < 10^{-25} \)). Overall, the intensity of optimized staining with M101 was slightly less than with the monoclonal anti-pan-TLE; all discrepancies in scoring (two MPNST, 2 solitary fibrous tumor, 1 schwannoma and 3 synovial sarcomas) between the two antibodies were
Figure 3.1 TLE immunostaining in representative 0.6 mm cores using pan-TLE antibody. Examples of 3+ staining in monophasic synovial sarcoma (A), higher power view of 2+ staining in a biphasic synovial sarcoma (B), 1+ staining in MPNST (C) and negative (score = 0) staining in MPNST (D).

cases scored positive by pan-TLE and negative by M101. For rigor, tissue microarrays assessing antibody specificity among various sarcomas were assessed with the more sensitive but less specific monoclonal anti-pan-TLE, and the MSKCC arrays, which only contained synovial sarcoma cases, were assessed with the less-sensitive M101. Staining results for each tumor type are summarized in Table 3.2.

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<th>Tumor Type</th>
<th>n</th>
<th>3+</th>
<th>2+</th>
<th>1+</th>
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<th>Positive</th>
<th>% Positive</th>
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DFSP: Dermatofibrosarcoma Protuberans, DSRCT: Desmoplastic Small Round Cell Tumor, GIST: Gastrointestinal Stromal Tumor, MFH: Malignant Fibrous Histiocytoma, MPNST: Malignant Peripheral Nerve Sheath Tumor, NOS: Not Otherwise Specified

* Eight of the DFSP cases contained fibrosarcomatous change
** Includes 2 cases of epithelioid hemangioendothelioma, 4 capillary hemangioma and 3 intramuscular hemangioma.

Table 3.2 Summary of TLE immunohistochemistry results.

Of the 35 bone and soft tissue tumors types with at least 5 cases included in this study, synovial sarcomas were the only type displaying a high proportion of
positive TLE1 staining, with 91/94 (97%) cases exhibiting intense and/or diffuse nuclear staining (i.e. 2+ or 3+; Table 3.2). All cases of biphasic synovial sarcoma exhibited staining in both the epithelial and spindle cell components (Figure 3.1B), with the epithelial component showing equivalent or stronger intensity. Four cases had the histology of poorly-differentiated synovial sarcoma, and all of these were positive for TLE (three cases scored as 3+, one as 2+). For the synovial sarcoma cases with known SSX subtype, all 25 SYT-SSX1 and all 17 SYT-SSX2 were positive for TLE. TLE staining was weak (i.e. 1+) in only 3/94 synovial sarcomas while none of the synovial sarcomas had absent (score = 0) staining. TLE staining in a core biopsy of a synovial sarcoma is demonstrated in Figure 3.2.

In contrast to synovial sarcoma, TLE staining was low to absent in other spindle cell tumors, including those in the differential diagnosis of synovial sarcoma. Solitary fibrous tumor/hemangiopericytomas (6/20) and schwannomas (6/22) were occasionally positive, whereas fibroxanthoma (1/4), clear cell sarcoma (1/7), carcinosarcoma (1/7), high grade chondrosarcoma (1/8), Ewing sarcoma (1/13 cases), MPNST (4/88), gastrointestinal stromal tumor (1/34) and leiomyosarcoma (1/41) were rarely positive for TLE (Table 3.2). The remaining tumors in this study, including malignant fibrous histiocytoma (synonymously termed pleomorphic undifferentiated sarcoma), fibrosarcoma and dermatofibrosarcoma protuberans with

Figure 3.2 Monoclonal anti-pan-TLE immunostaining of a core needle biopsy, taken from a 6 cm wrist mass in a 49 year-old male. H&E showed a nonpleomorphic spindle cell sarcoma, which was negative for pankeratin, CK7 and EMA by routine diagnostic immunohistochemistry protocols. Subsequent diagnostic FISH assay was positive for a split at the SYT locus, confirming synovial sarcoma 9 days after the TLE immunostaining result. Main image 12.5x, inset 400x. Full biopsy slide available for viewing at https://www.gpecimage.ubc.ca/tma/web/viewer.php.
fibrosarcomatous change, were negative for TLE staining in all examined cases. The positive predictive value (PPV) of TLE immunohistochemistry for synovial sarcoma versus the most problematic entity in the differential diagnosis MPNST, is 95.5% (91/95) and the negative predictive value NPV is 96.6% (84/87) (Table 3.3). When the test is applied to a population of 94 synovial sarcomas and 602 other tumors, the PPV is 79.1% (91/15) and the NPV is 99.5% (578/581) (Table 3.3).

3.4 Discussion

Distinguishing synovial sarcoma from other spindle cell tumors can present a diagnostic challenge, particularly in those cases that do not exhibit biphasic histology. In these situations immunohistochemical markers can be valuable in confirming the diagnosis of synovial sarcoma. Many attempts have been made to identify immunomarkers that have high positive predictive value for synovial sarcoma, but to date there have been no markers identified which are both consistently specific and sensitive for this tumor (1, 2, 27). Keratin and/or EMA immunostains, which are commonly used, can be sensitive markers for synovial sarcoma, however their expression is frequently focal and is not specific, as many tumors, including MPNST, can express epithelial antigens (1, 28, 29).

Gene expression studies in synovial sarcoma have repeatedly shown overexpression of members of the TLE family of genes, particularly TLE1, in synovial sarcoma. Analysis of this gene expression data has identified TLE1 as one of the best discriminators for synovial sarcoma when compared to histologically and/or biologically similar sarcomas such as MPNST or Ewing sarcoma. These data, from multiple groups using different expression profiling platforms, suggest that TLE1 may be a valuable diagnostic marker for synovial sarcoma.

This study demonstrates that immunohistochemical detection of TLE1 is not only very highly sensitive for synovial sarcoma, but also specific in the context of other mesenchymal neoplasms. Results are consistent with either a monoclonal antibody recognizing pan-TLE or a commercially available polyclonal antiserum that recognizes TLE1 (and cross-reacts to a lesser extent with TLE2, 3 and 4). We defined positive staining for TLE1 as moderate to high level staining as described in the materials and methods. Even using stringent criteria, the vast majority of synovial sarcomas (97%) were positive. Other tumors commonly mistaken for synovial
sarcoma exhibited lower levels of positive staining for pan-TLE, including schwannomas (27%), Ewing sarcomas (8%), MPNST (5%) and MFH (0%). Notably, TLE1 expression distinguishes synovial sarcoma from MPNST, a particularly problematic entity in the differential diagnosis, with a high degree of specificity. The majority of the remaining tumor types exhibit low to absent levels of TLE1 immunostaining, suggesting that TLE1 is useful in differentiating synovial sarcoma from these tumors. Since a high proportion of cells within most of the synovial sarcoma tissue microarray cores exhibited TLE1 staining (i.e. 2+ or 3+), high sensitivity on small tissue samples, such as core biopsies, would be seen (Figure 3.2). Using synovial sarcoma tissue microarrays, over 35 established and novel immunohistochemical markers of synovial sarcoma have been assessed (23), none of which perform as well as TLE1.

The prominence of TLE1 and other components of the Wnt/β-catenin signaling pathway in synovial sarcoma gene expression studies suggest that they may well play an important role in the development of this tumor. The observation that high levels of nuclear-localized β-catenin are a feature of synovial sarcoma (22), and recent experimental data showing that SYT-SSX expression leads to accumulation of β-catenin in a nuclear complex that includes SYT-SSX, provides evidence that downstream components of this pathway that mediate its effects on gene transcription are probably important in the pathogenesis of synovial sarcoma (30). The present study demonstrates protein-level correlation of TLE1 mRNA overexpression in synovial sarcoma. TLE1 competes with activated β-catenin for TCF/LEF transcription factors in the nucleus; TLE1 binding displaces β-catenin to form transcriptionally repressive TLE1-TCF/LEF complexes (20, 31). A recent study demonstrated that β-catenin and SYT-SSX2 interact to potentiate β-catenin-mediated transcription (32), suggesting that TLE1 overexpression may represent a compensatory response to excessive β-catenin signaling or serve to limit transcriptional activation of certain genes. TLE1 is also known to associate with other transcription factors, such as HES, where it recruits histone deacetylases to multiprotein complexes involved in Notch signaling-mediated transcriptional repression (13). In this context, TLE may serve to repress genes involved in differentiation and maintain the relatively undifferentiated histopathologic state seen in synovial sarcoma. Of interest, strong growth inhibition has been observed in synovial
sarcoma models that were treated with clinically-applicable histone deacetylase inhibitors (21, 22). Further functional studies are required to delineate the role of TLE proteins in synovial sarcoma pathogenesis.

In summary, TLE1 expression is a consistent and prominent feature of synovial sarcoma whereas it is low to absent in other tumors in the differential diagnosis. Reproducible immunohistochemical staining of TLE1 with negligible background can be obtained with both monoclonal and commercially available polyclonal anti-TLE1 antibodies, and this approach can be applied in most diagnostic hospital laboratories at minimal expense. TLE1 is a sensitive and specific immunohistochemical marker for synovial sarcoma, performing better than other known immunohistochemical markers, and can significantly aid in the pathologic diagnosis of this tumor.
3.5 References


4 THE HEAT SHOCK PROTEIN 90 INHIBITOR 17-ALLYLAMINO-17-DEMETHOXYGELDANAMYCIN PREVENTS SYNOVIAL SARCOMA PROLIFERATION VIA APOPTOSIS IN IN VITRO MODELS *

4.1 Introduction

Approximately 50% of patients will die from recurrent synovial sarcoma within ten years of diagnosis (1). The benefit of conventional chemotherapeutics to overall survival has not been definitively established, although combination doxorubicin/ifosfamide therapy has been reported to offer a modest survival advantage in advanced cases (2, 3), suggesting that drugs directed against specific metabolic and signaling pathways crucial to synovial sarcoma may be more efficacious.

The unique SYT-SSX fusion protein underlying synovial sarcoma development is an ideal target; however, no drugs presently exist that block its function or that of the component transcription cofactors SYT and SSX. Other potential therapeutic targets in synovial sarcoma have been consistently identified by gene expression profiling studies, including known oncogenes (4-8). Among these oncogenes are tyrosine kinases (RTKs) and associated ligands, such as epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 3 (FGFR3) and insulin-like growth factor 2 (IGF2, encoding the ligand of IGF-1R). EGFR is overexpressed at the protein level in synovial sarcoma (9), but is not associated with gene amplification (10). Protein level expression of FGFR3, KIT, and human epidermal growth factor receptor 2 (HER2) has also been reported in synovial sarcoma (9, 11-13), while IGF-1R expression has been linked with a more aggressive tumor phenotype and IGF2 expression appears to be requisite for tumorigenesis (14, 15). Specific inhibitors for a number of these oncoproteins exist, some of which are in clinical use or clinical trials (16-21), and are of interest as potential systemic therapies for synovial sarcoma; gefitinib (Iressa, ZD1839; EORTC protocol 62022) and trastuzumab (Herceptin; NCI protocol CDR0000413703) are already the subject of synovial sarcoma clinical trials. Recent research has also demonstrated that small molecule inhibitors of FGFR and

an antibody directed against IGF2 can inhibit the proliferation of synovial sarcoma in vitro cultures (15, 22).

Another therapeutic strategy is to employ an inhibitor that affects multiple RTKs simultaneously. Heat shock protein 90 (Hsp90) performs numerous cellular functions, including promotion of proper protein folding and stabilization of oncogenic proteins (23, 24). Small molecule inhibitors of Hsp90 like the geldanamycin derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) prevent Hsp90-mediated stabilization (25, 26). Blocking expression of Hsp90 client proteins including RTKs such as EGFR, HER2, IGF-1R and KIT and producing anti-cancer effects (27-31). These observations have led to clinical trials of 17-AAG that have demonstrated its safety and prospective therapeutic efficacy in a number of malignancies (32). The potential for 17-AAG activity specifically against synovial sarcoma, however, remains unexplored.

The involvement of RTK overexpression in the pathogenesis of various malignancies has been well established. Considering this, the overexpression of RTKs observed in synovial sarcoma suggests that one or more of these receptors is involved in the development and growth of this malignancy. As such, inhibiting one or more of these receptors may prevent proliferation and identify a novel therapeutic strategy. To test this hypothesis, the effects of drugs that inhibit RTK activity on the survival of in vitro synovial sarcoma models are examined.

4.2 Material and methods

4.2.1 Reagents

17-AAG was provided by the Developmental Therapeutics Branch of the National Cancer Institute (Bethesda, MD). The EGFR inhibitor gefitinib (Iressa, ZD1839) (17) was provided by AstraZeneca PLC (London, UK). The IGF-1R inhibitor NVP-AEW541 (18) was provided by Novartis-Pharma AG (Basel, Switzerland). The FGFR3 inhibiting antibody PRO-001 (16) was provided by ProChon Biotech Ltd. (Rehovot, Israel). The FGFR1 & 3 inhibitor SU5402 (19) was purchased from Calbiochem UK (Merck Biosciences, Nottingham, UK), the HER2 inhibitor trastuzumab (Herceptin) (20) was purchased from Genentech (South San Francisco, CA) and the KIT/PDGFR inhibitor imatinib mesylate (Gleevec) (21) was purchased from Novartis-Pharma AG. All other chemicals were purchased from Sigma (St.
Louis, MO) unless otherwise specified. Cell culture media and materials were purchased from Invitrogen (Carlsbad, CA).

4.2.2 Monolayer and spheroid cell culture

The monophasic synovial sarcoma cell line Fuji (33) and the biphasic synovial sarcoma cell line SYO-1 (34), both of which carry the SYT-SSX2 fusion, were kindly provided by Dr. Kazuo Nagashima (Hokkaido University School of Medicine, Sapporo, Japan) and Dr. Akira Kawai (National Cancer Centre Hospital, Tokyo, Japan) respectively. The presence of t(X; 18) in the synovial sarcoma cell lines was confirmed by diagnostic cytogenetic karyotyping, RT-PCR and FISH analysis. The MCF-7, SKOV-3 and HeLa cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Monolayer cultures were grown under standard incubation conditions (37°C, 95% humidity, 5% CO2) in RPMI 1640 medium supplemented with 5% (HeLa) or 10% (SYO1, Fuji, SKOV3) fetal bovine serum (FBS), with the exception of MCF-7, which was cultured in DMEM medium supplemented with 10% FBS. Spheroid cultures were produced by trypsinization of confluent monolayer cultures to single cell suspensions and 5x10^3 cells in 500 µL were transferred to each well of an agar-coated 24 well plate. The plate was then incubated for 24 hours with gentle rocking and a further 48 hours without rocking to maximize spheroid size. All spheroid cell cultures were grown in RPMI 1640 supplemented with 5% FBS on an underlay of 1.4% agar in RPMI 1640 supplemented with 5% FBS under standard incubation conditions.

4.2.3 Proliferation assay

Monolayer culture proliferation was assessed by measuring the reduction of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Methylthiazolyldiphenyl-tetrazolium bromide or MTT). MTT was added to a final concentration of 1 mg/mL per well at each time point and incubated under standard conditions for 2 hours, the media removed and an equal volume of DMSO added. Dissolved MTT formazan for each vessel well was transferred to a 96-well plate in triplicate and the absorbance measured at 562 nm in a PowerWaveXplate reader with KinetiCalc KC4 software (Bio-Tek Instruments Inc., Winooski, VT). The average reading for each vessel well was used to determine the overall mean for each treatment time point. The 50% proliferation inhibitory concentration (IC50) values were
determined using non-linear regression dose(inhibitor)-response best fit curve analysis (R² >0.95) on GraphPad Prism 5 software (San Diego, CA).

### 4.2.4 Flow cytometry

To determine the levels of apoptosis, treated synovial sarcoma monolayer cultures were stained with Annexin V (BD Biosciences – Pharmingen, San Jose, CA), according to the manufacturer’s instructions. Briefly, trypsinized cells were washed in ice cold PBS, resuspended in Annexin V binding buffer and stained with Annexin V and propidium iodide. The cells were then analyzed on an EPICS MXL flow cytometer (Beckman-Coulter, Fullerton, CA). Treated spheroid cultures were prepared for flow cytometric analysis as described previously (35), with the following modifications. Treated spheroids were transferred to a single microfuge tube in a minimal amount of media. Trypsin was added and spheroids disaggregated by gentle pipetting. The resulting cell suspension was filtered to remove any residual cell aggregates and stained with Annexin V and propidium iodide as described above.

### 4.2.5 Cell block preparation

Formalin-fixed, paraffin-embedded spheroid fibrin cell blocks for histological analysis were produced as described previously (36) with the following modifications. Eight to ten spheroids were removed from culture for each treatment group in a minimal amount of media and combined in a microfuge tube. Reconstituted normal plasma (HemosIL; Instrumentation Laboratory, Lexington, MA) was then added and the spheroids resuspended by gentle agitation. Thrombin was then added and the mixture gently agitated to maintain the spheroids in suspension until clotting occurred. The spheroid cell blocks were then formalin fixed, processed and stained with hematoxylin and eosin using standard techniques.

### 4.2.6 Sequencing of **EGFR**

Sixteen formalin-fixed paraffin embedded (FFPE) synovial sarcoma tumor samples of both monophasic and biphasic subtypes were obtained from Vancouver General Hospital. Genomic DNA was extracted from the FFPE synovial sarcoma tumor samples by xylene deparaffinization, overnight Proteinase K digestion at 56°C and ethanol precipitation. Genomic DNA was prepared from the Fuji and SYO-1 cell lines using the DNeasy tissue kit (Qiagen, Mississauga, ON) according to manufacturer’s instructions. Previously described primers were used to PCR amplify exons 18, 19 and 21 of the **EGFR** gene (37) and the PCR products were sequenced.
using the Big Dye Terminator kit v3.1 and an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions.

4.2.7 Immunoblot analysis

Primary antibodies were purchased from the following sources: anti-EGFR (cat# CSA-330) from Stressgen/Assay Designs (Ann Arbor, MI); anti-c-KIT (cat# 3392) from Cell Signaling Technology (Beverly, MA); anti-HER2 (cat# ab2428) and anti-FGFR3 (cat# ab52247) from Abcam (Cambridge UK); anti-β-catenin (cat# 06-734) and anti-non-phosphorylated β-catenin (8E4, cat# 05-601) from Upstate Biotechnology (Charlottesville, VA); anti-SYT (H-80, cat# sc-28698), anti-histone H1 (AE-4, cat# sc-8030), anti-TLE1 (M101, cat# sc-9121) and anti-β-actin (C-4, cat# sc-47778) from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary anti-mouse-HRP antibody (cat# SAB-300) was purchased from Stressgen/Assay Designs and anti-rabbit-HRP antibody (cat# sc-2004) from Santa Cruz Biotechnology. Total cellular lysates of SYO-1, Fuji and HeLa monolayer cultures were prepared and immunoblotting performed according to standard protocols. Cultures treated with 1 μM camptothecin for 24 hours were included in the caspase 3 immunoblots as positive apoptosis controls. 5 μg total protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membrane (BioRad, Hercules, CA) using standard methods. Immunoblots were visualized using the SuperSignal West Femto chemiluminescent detection kit (Pierce, Rockford, IL) as per manufacturer’s instructions. Nuclear and cytoplasmic proteins were fractionated with the NE-PER kit (Pierce).

4.2.8 Immunofluorescent analysis

Cells were grown on glass slides, treated and methanol fixed for 10 minutes at -20°C. Immunofluorescence analysis of β-catenin localization was performed with fluorescein isothiocyanate (FITC)-labelled anti-β-catenin antibody purchased from BD Transduction Laboratories (610155, San Jose, CA) at 2 μg/mL final concentration according to manufacturer’s instructions. Nuclei were counterstained using DAPI I reagent (Vysis, Downer’s Grove IL) and images captured on an Axioplan 2 fluorescence microscope (Zeiss, Thornwood, NY) using Isis image analysis software (MetaSystems, Belmont, MA). Automated background reduction was applied up to
twice in images exhibiting high background fluorescence; otherwise, images were not modified after capture.

4.3 Results

4.3.1 The effect of specific receptor tyrosine kinase inhibitors on synovial sarcoma monolayer cultures

The overexpression of RTKs in synovial sarcoma suggests that one or more of these pathways is involved in the pathogenesis of this disease, as has been demonstrated in other cancers. To investigate the possibility that inhibiting specific RTKs will prevent the proliferation of synovial sarcoma, we obtained small molecule inhibitors to RTKs for which the receptor itself or activating components of the pathway are expressed: EGFR (gefitinib), FGFR1 & 3 (SU5402), IGF-1R (NVP-AEW541), and KIT (imatinib mesylate). We also obtained humanized inhibitory antibodies to HER2 (trastuzumab) and FGFR3 (PRO-001). These inhibitors were tested for their ability to prevent the proliferation of SYO-1 and Fuji monolayer cultures.

To assess the effect of EGFR inhibition on synovial sarcoma proliferation, monolayer Fuji and SYO-1 cultures were exposed to varying concentrations of gefitinib and compared to vehicle treated control cultures (Figure 4.1A). The concentration of gefitinib inhibiting 50% of proliferation (IC$_{50}$) was 266 μM for SYO-1 and 265 μM for Fuji. These concentrations are significantly higher than those described to inhibit the proliferation of gefitinib-sensitive cell lines (38). Activating mutations in EGFR affecting the kinase domain have recently been identified to bestow sensitivity in non-small cell lung cancer to gefitinib (37). We sought similar sensitizing mutations in exons 18,19 and 21 of EGFR in each synovial sarcoma cell line and 16 FFPE synovial sarcoma tumor specimens that strongly expressed the EGFR protein (9). No such mutations were found.

Expression levels of IGF-1R are variable in synovial sarcoma and those tumors exhibiting relatively high levels of IGF-1R display a more aggressive phenotype (14). Our previous gene expression profiling data found the IGF-1R ligand IGF2 to be highly expressed in synovial sarcoma (4), and antibody raised to IGF2 has been reported to promote apoptosis (15), suggesting a role for IGF-1R signaling. The
Figure 4.1 The EGFR inhibitor gefitinib (A), IGF-1R inhibitor NVP-AEW541 (B) and KIT/PDGFR inhibitor imatinib (C) reduce proliferation of the synovial sarcoma cell lines SYO-1 and Fuji after 72 hours only at concentrations higher than are clinically applicable. Vehicle control and doxorubicin are included for comparison. Bars represent the 95% confidence interval.

IGF-1R inhibitor NVP-AEW541 produced an IC\textsubscript{50} of 29.4 μM for SYO-1 and 19.8 μM for Fuji (Figure 4.1B). These IC\textsubscript{50} values are 10 to 50-fold higher than that described
to inhibit IGF-1R phosphorylation in cell-free assays and prevent proliferation of NIH 3T3 cells overexpressing human IGF-1R (18).

Expression of KIT and the closely related tyrosine kinase receptors PDGFRα and PDGFRβ has been reported in synovial sarcoma (11, 39), implying that imatinib, which inhibits both KIT and PDGFR (21), may inhibit synovial sarcoma growth. The IC$_{50}$ of imatinib was 6.34 μM for SYO-1 and 43.46 μM for Fuji (Figure 4.1C), which are higher than those reported for imatinib-sensitive cell lines (21, 40, 41).

The FGFR1 & 3 inhibitor SU5402 did not inhibit the proliferation of SYO-1 and Fuji monolayer cultures at concentrations shown to inhibit proliferation and induce apoptosis in cell lines sensitive to this inhibitor (42). The humanized antibodies targeting FGFR3 (PRO-001) and HER2 (trastuzumab) did not inhibit growth inhibition by MTT assay in either cell line.

**4.3.2 17-AAG inhibits the proliferation of synovial sarcoma monolayer cultures**

The Hsp90 inhibitor 17-AAG has been shown to induce degradation of multiple RTKs, many of which are expressed in synovial sarcoma. To assess the effect of 17-AAG on synovial sarcoma, SYO-1 and Fuji were grown in monolayer culture and exposed to varying concentrations of 17-AAG, with doxorubicin treated cultures included for comparison and vehicle treated cultures included as controls. 17-AAG significantly inhibits cell proliferation in all synovial sarcoma cell lines (Figure 4.2).

![Figure 4.2](image-url)

**Figure 4.2** 17-AAG inhibits proliferation in the synovial sarcoma cell lines SYO-1 and Fuji. MTT assays of monolayer cultures after 72 hours of treatment as indicated reveals 17-AAG associated inhibition of proliferation. Bars represent the 95% confidence interval of the mean.
The 17-AAG IC₅₀ values for synovial sarcoma monolayer cultures treated for 72 hours were 23 nM for SYO-1 and 27 nM for Fuji. For comparative purposes we repeated this assay on the breast cancer cell line MCF-7, which has been reported to be relatively sensitive to Hsp90 inhibitors, and the ovarian cancer cell line SKOV-3, which are relatively resistant (43-45). The IC₅₀ values for MCF-7 were approximately 17 times higher and SKOV-3 approximately 232 times higher than the IC₅₀ values for the synovial sarcoma cell lines (Table 4.1), demonstrating that the synovial sarcoma cell lines are particularly sensitive to 17-AAG. Importantly, significant synovial sarcoma growth inhibition occurs below serum concentrations achievable in humans (1600 to 3000 nM) (46, 47). Treatment of each synovial sarcoma cell line with radicicol, another Hsp90 inhibitor that is structurally dissimilar to 17-AAG (48), produced similar results, confirming that Hsp90 inhibition prevents proliferation in these synovial sarcoma cell lines.

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<td>Fuji</td>
<td>27 (16 - 47)</td>
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<tr>
<td>MCF-7</td>
<td>397 (221 - 618)</td>
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<td>SKOV-3</td>
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**Table 4.1** Concentrations (nM) of 17-AAG inhibiting 50% of proliferation (IC₅₀) after 72 hours of treatment. The synovial sarcoma cell lines SYO-1 and Fuji are compared to the Hsp90 inhibitor sensitive cell line MCF-7 and the Hsp90 inhibitor resistant cell line SKOV-3. The mean is derived from at least 3 independent experiments. The 95% confidence interval is included in brackets (CI).

**4.3.3 17-AAG inhibition of proliferation correlates with induction of apoptosis**

To determine if apoptosis underlies 17-AAG-induced inhibition of proliferation, the levels of apoptosis in treated synovial sarcoma monolayer cultures were assessed by immunoblot analysis of caspase 3 activation and by flow cytometric measurement of Annexin V binding. The results of these experiments demonstrate that 17-AAG treatment causes activation of caspase 3 in a dose dependent manner (Figure 4.3A) and increased staining of SYO-1 monolayer cells with Annexin V prior to staining with propidium iodide (Figure 4.3B), each of which are indicators of apoptosis. Similar Annexin V staining was observed when the assay was repeated on
Figure 4.3 17-AAG induces apoptosis in the synovial sarcoma cell lines SYO-1 and Fuji. Immunoblotting of SYO-1 and Fuji cultures treated for 24 hours with vehicle, 17-AAG or camptothecin (CPT) demonstrates 17-AAG associated cleavage and activation of caspase 3 (A). Annexin V flow cytometric analysis of SYO-1 cultures treated for 24 hours shows 17-AAG increases staining with Annexin V (cells in early apoptosis) and propidium iodide (late apoptotic/necrotic cells) (B).

Fuji monolayer cultures. Induction of apoptosis occurs in a time scale consistent with that observed for proliferation inhibition in the monolayer MTT assays, demonstrating that 17-AAG is inhibiting synovial sarcoma monolayer culture growth by inducing apoptosis. Similar results were also obtained with radicicol treated Fuji and SYO-1 monolayer cultures, suggesting that Hsp90 inhibition induces apoptosis in these synovial sarcoma models. Treatment with doxorubicin resulted in apoptosis as well, but to a lesser extent (Figure 4.3B).

4.3.4 17-AAG induces apoptosis in synovial sarcoma spheroid cultures

Spheroid cultures have been shown to more closely resemble in vivo tumor biology and are intrinsically more resistant to chemotherapeutic agents than monolayer cultures because of changes in cell-cell interactions and limited drug diffusion (35, 49-51). Consequently, spheroids represent a more stringent in vitro model for evaluation of cytotoxic drugs. Spheroid cultures were generated from each synovial sarcoma cell line to assess the ability of 17-AAG to induce apoptosis under...
these more rigorous conditions. Vehicle (0.1% DMSO), 17-AAG and doxorubicin treated Fuji and SYO-1 spheroids were formalin fixed and paraffin embedded. Hematoxylin and eosin stained sections of representative Fuji spheroid cell blocks are presented in Figure 4.4. Apoptosis and necrosis is widespread in the 17-AAG-treated spheroids (Figure 4.4 B) compared to the more focal apoptosis observed in doxorubicin-treated spheroids and the lack of apoptosis in the untreated spheroids (Figure 4.4 A+C). 17-AAG induces significant levels of apoptosis in both SYO-1 and Fuji spheroids and to a much greater extent than equimolar concentrations of doxorubicin.

4.3.5 17-AAG induces degradation of receptor tyrosine kinases

17-AAG is well known to promote RTK degradation, which could explain the 17-AAG associated induction of apoptosis in the synovial sarcoma cell lines. The effects of 17-AAG treatment on EGFR, HER2, FGFR3, IGF-1R and KIT expression were examined in SYO-1 and Fuji monolayer cultures (Figure 4.5 A). With the exception of IGF-1R in Fuji cells, all these RTKs were expressed in the cell lines at levels detectable by immunoblot. Treatment with 17-AAG for 24 hours caused a dose-
**Figure 4.5** 17-AAG treatment results in dose dependent degradation of the receptor tyrosine kinases EGFR, HER2, FGFR3, IGF-1R and KIT in the synovial sarcoma cell lines SYO-1 and Fuji (A). 17-AAG treatment had no appreciable effect on total cellular levels of TLE (B). Total protein was isolated after 24 hour treatment with 17-AAG as indicated. β-actin is included as a loading control.

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<th>SYO-1</th>
<th>Fuji</th>
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<td></td>
<td>0.1% DMSO</td>
<td>0.05 μM 17-AAG</td>
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Dependent reduction in the levels of each of these RTKs in both synovial sarcoma cell lines, with HER2 and FGFR3 appearing most sensitive. 17-AAG does not significantly change total cellular levels of TLE protein in both synovial sarcoma cell line models (Figure 4.5 B).

**4.3.6 17-AAG promotes disruption of the SYT-SSX-β-catenin interaction and decreases levels of nuclear β-catenin**

RTK signaling promotes redistribution of membrane associated β-catenin to the nucleus, leading to expression of proliferation-related genes, which under inappropriate circumstances contributes to oncogenesis (52). Nuclear-localized β-catenin is an immunohistological feature of synovial sarcoma and is associated with increased proliferative rates and poorer prognosis (53-60). 17-AAG-mediated loss of RTK expression may lead to relocalization of β-catenin to the membrane and to depletion of nuclear-localized β-catenin, as has been demonstrated in malignant melanoma cell lines (61). Examination of the subcellular distribution of total β-catenin
Figure 4.6 Immunofluorescent analysis of the effect of 17-AAG on membrane localization of β-catenin in monolayer synovial sarcoma cell lines. Treatment with 5 μM 17-AAG for 24 hours does not appreciably change levels of membrane associated β-catenin, although non-specific globular collections of β-catenin were observed (arrows). HeLa is included as a non-synovial sarcoma cell line control and all cultures were exposed to 5 μM doxorubicin for 24 hours as a treatment control.

Within synovial sarcoma monolayer cultures treated with or without 5 μM 17-AAG for 24 hours revealed no appreciable increase in membrane-associated β-catenin (Figure 4.6). The appearance of globular nuclear β-catenin collections (Figure 4.6, arrows) were observed, although these were also present in doxorubicin treated samples and the HeLa cell line signifying they are not specific to 17-AAG treatment.

Interestingly, the level of nuclear β-catenin in the synovial sarcoma cell lines appeared to decrease with 17-AAG treatment over time (Figure 4.7A), suggesting that 17-AAG decreases nuclear levels of β-catenin. 17-AAG mediated loss of nuclear β-catenin was confirmed by biochemical separation of the nuclear fraction and immunoblotting for β-catenin (Figure 4.7B). The lack of detectable β-catenin in the HeLa nuclear fractions is consistent with immunofluorescent observations (Figure 4.6). Treatment with 5 μM 17-AAG did not appreciably change the total cellular levels of β-catenin in the synovial sarcoma cell lines over 48 hours (Figure 4.7C).
Figure 4.7 Treatment of monolayer SYO-1 cultures with 5 μM 17-AAG for 12-48 hours does not appreciably change the level of membrane associated β-catenin (green); however, levels of nuclear β-catenin decrease between 12 hours (arrows) and 48 hours (arrowheads) (A). DAPI staining indicates nuclear boundaries (blue). Exposure of synovial sarcoma cell lines to 5 μM 17-AAG decreases levels of nuclear-localized β-catenin over time. Nuclear β-catenin was undetectable in the HeLa nuclear fraction (5 μg total protein) (B). Exposure of synovial sarcoma cell lines to 5 μM 17-AAG (17) or 5 μM doxorubicin (Doxo) for 48 hours does not change total cellular β-catenin levels compared to vehicle control (DMSO) (C). Histone H1 and β-actin are included as loading controls. IB = Immunoblot.

demonstrating that β-catenin is not dependent on Hsp90 function for expression in these cells lines and that loss of nuclear β-catenin results from primarily from movement of β-catenin out of the nucleus. Interestingly, SYT-SSX2 has been recently reported to anchor β-catenin in the nucleus through indirect interactions with an unknown intermediary, thereby increasing nuclear β-catenin levels (62).

Given that Hsp90 inhibition with 17-AAG causes the loss of fusion proteins such as BCR-ABL and NPM-ALK (63, 64), 17-AAG may promote degradation of SYT-SSX2, thereby allowing β-catenin to diffuse out of the nucleus. The effect of 17-AAG on stability of SYT-SSX2 in the cell lines SYO-1 and Fuji was also assessed. 17-AAG treatment did not reduce total cellular levels of either SYT-SSX2 or SYT in SYO-1 and Fuji, and SYT levels in HeLa were also unaffected by 17-AAG, demonstrating that expression of these proteins is not Hsp90 dependent (Figure 4.8A). Treatment with 5 μM 17-AAG for 24 hours does, however, decrease co-immunoprecipitation of SYT-
Figure 4.8 Effect of 17-AAG on expression of SYT-SSX2 and β-catenin. Treatment of the synovial sarcoma cell lines SYO-1 and Fuji with 17-AAG for 24 hours did not affect levels of SYT-SSX2 (upper band) or SYT (lower band) in the synovial sarcoma cell lines (A, DMSO = vehicle control). Treatment with 5 μM 17-AAG for 24 hours inhibits co-immunoprecipitation of SYT-SSX2 and SYT with β-catenin and β-catenin with SYT-SSX2 and SYT (B). IB = Immunoblot, IP = Immunoprecipitation.

4.4 Discussion

This study was undertaken in an effort to identify a novel and effective systemic therapy for synovial sarcoma. With a view to expediting the progression of a potential treatment from the laboratory to clinical use, the initial investigation focused on drugs that are presently in clinical use or being tested in at least phase II trials, so that pharmacology, toxicity, dosing and side effects are known.

Gefitinib does not inhibit synovial sarcoma model proliferation at the maximum concentrations achievable in human serum (756 ng/mL or 1.7 μM (17)) although it does at higher concentrations. Similarly negative results were obtained with the IGF-1R inhibitor AEW541 and the KIT inhibitor imatinib mesylate, which led us to assess the effect of 17-AAG, an inhibitor of multiple RTKs. 17-AAG inhibits proliferation by inducing apoptosis in monolayer synovial sarcoma cell lines at concentrations that are achievable in human serum, and which correlate with inhibitory concentrations observed in other pre-clinical cancer models (46). Of note, the inhibition of synovial sarcoma culture proliferation was greater than that observed for cell lines previously
described as Hsp90 sensitive or resistant, suggesting that synovial sarcoma is particularly susceptible to 17-AAG.

In vitro monolayer assay conditions differ greatly from those found in patients, however, and it is well known that cell lines can significantly differ from the tumors from which they were derived and tend to be more susceptible to chemotherapeutic agents (65). Thus, the results we have observed in monolayer cell culture assays may not accurately predict the anti-tumor effect of 17-AAG and its efficacy relative to doxorubicin in a clinical setting. Three dimensional cancer models, particularly spheroid cell cultures, have been shown to more closely approximate in vivo tumor biology and to be more resistant to chemotherapeutic agents than monolayer cultures. We assessed the effect of 17-AAG on synovial sarcoma spheroid cultures to obtain a more accurate prediction of the potential clinical effectiveness of 17-AAG. 17-AAG was observed to induce high levels of apoptosis in spheroid culture, similar to that observed in monolayer culture; in contrast, the cytotoxic effect of doxorubicin was moderate in spheroid culture. Unlike monolayer cultures, spheroids include a basal level of necrotic cells, the result of a complex interplay between culture conditions, nutrient diffusion and cell-cell signaling (50, 51); however, the levels of apoptosis observed in the 17-AAG treated spheroids are much higher than this inherent baseline.

Gene expression studies indicate that synovial sarcoma is a disease typified by expression of multiple oncogenes, a number of which have been verified as expressed at the protein level. 17-AAG has been shown to cause the degradation of multiple oncoproteins, which made it appealing to us as a potential systemic therapy for synovial sarcoma. We observed that the level of 17-AAG-induced growth inhibition and apoptosis was similar between each synovial sarcoma cell line tested. We found similar sensitivity to radicicol, suggesting that the survival of the synovial sarcoma cell lines is dependent on one or more Hsp90 clients. 17-AAG-mediated induction of apoptosis coincides with degradation of EGFR, HER2, FGFR3, IGF-1R, and KIT, implying that one or more of these events are related to induction of apoptosis. Accordingly, 17-AAG inhibits the growth of other malignancies typified by overexpression of RTKs such as EGFR and HER2, and this correlates with degradation of these receptors (29). FGFR3 is a candidate target to explain the effect of 17AAG on synovial sarcoma. This protein not only has a documented role in
promoting mesenchymal growth, but expression profiling studies from ourselves and others have highlighted expression of FGFR3 in synovial sarcoma (4, 7), and recent studies have demonstrated anti-proliferative activity of FGFR inhibitors in synovial sarcoma (16, 22). Our data shows FGFR3 degradation is induced by 17-AAG, although we were unable to demonstrate growth inhibition by small molecule or antibody inhibitors of FGFR3 in our assays. Similarly, others have shown that media supplementation with stem cell factor increases proliferation in KIT-dependent cell lines (66); however, we did not add exogenous stem cell factor to our assays to promote KIT-dependent growth that would accentuate the growth-inhibitory effect of imatinib. Our results were similar to those for non-sensitive sarcoma cell lines treated with imatinib without the addition of SCF (40). HER2 is another candidate target to explain the effect of 17-AAG in synovial sarcoma, as it is a well-documented client protein of Hsp90 (25). HER2 has been proposed as a therapeutic target in synovial sarcoma (13), although previous tissue microarray studies have found minimal protein expression (9). We found trastuzumab to have a negligible effect; however, our negative findings with trastuzumab (and PRO-001) must be viewed with caution as in vitro assays exclude any potential beneficial interaction with the human immune system and may underestimate the effect of therapeutic antibodies (67). Since many proteins are Hsp90 clients and Hsp90 itself performs other cellular functions apart from promoting proper protein folding (23), additional mechanisms for 17-AAG mediated inhibition of synovial sarcoma proliferation should also be considered.

The strong correlation identified between expression of TLE1 (an inhibitor of β-catenin signaling) and synovial sarcoma identified in Chapter 3 suggests that aberrant β-catenin signaling plays an important role in synovial sarcoma development, and there is considerable histological, clinical and functional evidence to support this premise. Considering that RTK activity promotes nuclear localization of β-catenin, and nuclear localized β-catenin is a feature of most synovial sarcomas, the role 17-AAG mediated RTK degradation on subcellular localization of β-catenin was investigated. Presumably RTK loss would lead to relocalization of β-catenin to the membrane, however this was not observed. The discontinuous pattern of membranous β-catenin distribution between adjacent cells was consistent with previous reports (57). Despite no obvious increase in membranous β-catenin localization, a 17-AAG-mediated decrease in nuclear-localized β-catenin was observed and confirmed by immunoblot
analysis of nuclear subfractions. SYT-SSX2 has been demonstrated to indirectly interact with β-catenin and increase nuclear β-catenin levels (62), suggesting that 17-AAG mediated loss of SYT-SSX2 may underlie the loss of nuclear β-catenin; however, in cell lines harboring the SYT-SSX2 fusion (SYO-1 and Fuji), 17-AAG did not appreciably change the total cellular levels of β-catenin and SYT-SSX2. 17-AAG did inhibit co-immunoprecipitation of SYT-SSX2 with β-catenin and β-catenin with both SYT and SYT-SSX2, implying that 17-AAG affects the undefined bridge linking SYT-SSX2 and β-catenin. Characterization of this interaction is an area for future investigation. Total cellular TLE levels were resistant to 17-AAG treatment, indicating that TLE depletion is likely not a factor in 17-AAG-mediated apoptosis.

Presently, more than half of those diagnosed with synovial sarcoma will ultimately die of this malignancy because there is no consistently effective systemic therapy to treat or prevent recurrence. We have provided in vitro evidence that 17-AAG, a clinically applicable drug with known pharmacology and toxicity, inhibits synovial sarcoma proliferation by inducing apoptosis and may prove to be a novel effective systemic therapy for this deadly disease.
4.5 References


5 IDENTIFICATION AND CHARACTERIZATION OF TUMOR INITIATING CELLS IN SYNOVIAL SARCOMA*

5.1 Introduction

Similarities between malignant cells and the tissues from which they arise suggest that cancers result from transforming events that occur in normal stem-like cells or that confer stem-like properties on previously normal cells (1). Persistence of tumor initiating cells (TIC) could lead to tumor recurrence. Characterization of TIC, which can behave in a stem-like manner (referred to as cancer stem-like cells or CSLC), and their relationship to pathogenesis thus carries significant implications for all aspects of cancer research, particularly in the development of effective therapeutic strategies. Identification and purification of novel TIC can be a complex process, although prospective subpopulations can be identified by assaying for functional stem cell-like qualities and for expression of markers that are associated with stem and stem-like cells (2). In this manner, TIC have been identified in acute myelogenous leukemia, medulloblastoma, glioblastoma, as well as carcinoma of the breast, pancreas and colon (3-10).

There is evidence that TIC exist in synovial sarcoma. Clinically, synovial sarcomas display functional stem cell-like properties, such as the ability to recapitulate the original tumor and relative resistance to conventional chemotherapy, which manifest as high rates of tumor recurrence and associated mortality (11-13). At the microscopic level, synovial sarcomas can contain both epithelial and mesenchymal elements (often within the same tumor) despite being clonal in origin, suggesting aberrant differentiation of malignant sarcoma cells from a common, unidentified precursor (14, 15). Histological, immunohistological and ultrastructural studies of primary synovial sarcomas have identified features consistent with a neuroectodermal derivation and evidence of neural differentiation (16, 17). cDNA microarray studies have further revealed characteristic gene expression patterns that include upregulation of genes (e.g. TLE1, SOX9, CRABP1, COL9A1, EPHB3) implicated in neural crest cell migration and differentiation, leading to speculation that synovial sarcoma arises from a primitive progenitor aberrantly recapitulating neural

* A version of this chapter will be submitted for publication. Terry, J and Nielsen, T. O. Identification and characterization of tumor initiating cells in synovial sarcoma.
crest cell development (18-22). Tumors with identical histology to synovial sarcoma arise when SYT-SSX is expressed in myoblasts, but not myocytes, of transgenic mice (23), indicating that a primitive progenitor background is necessary for the development of this tumor and adding a myogenic precursor to the list of potential origins of synovial sarcoma. Recently, subpopulations of a number of sarcomas, including synovial sarcoma, have been shown to exhibit enhanced tumorigenic potential, suggesting the presence of TIC (24). Thus, there is substantial evidence for the presence of TIC in synovial sarcoma, although they have yet to be isolated and characterized.

The potential relationship between synovial sarcoma TIC and treatment failure highlights the considerable clinical relevance that characterizing synovial sarcoma TIC may have in facilitating development of novel therapies. This has particular relevance to the potential role 17-AAG in synovial sarcoma treatment, as Hsp90 inhibitors have been reported to destroy leukemic cancer stem-like cells in chronic myelogenous leukemia (25) and 17-AAG may have similar activity in synovial sarcoma. The phenotypic similarities between synovial sarcoma and cells derived from the neural crest suggests that expression of markers used to identify neural progenitors may also be useful in identifying and isolating synovial sarcoma TIC.

CD133 is a transmembrane glycoprotein of uncertain function that associates with cholesterol-based lipid rafts and has been identified as a marker for both hematopoietic and non-hematopoietic stem cells and other progenitors, including neural progenitors (26-29). A monoclonal antibody recognizing human CD133 has been used successfully to identify and enrich for both normal tissue stem cells and stem-like cells from solid primary tumors of neural origin, as well as colon and prostate (6, 7, 9, 10). Stem-like CD133-enriched subpopulations have also been isolated from cancer-derived cell lines using CD133 positive selection (30, 31). Thus, CD133 may be a useful marker for the identification and isolation of potential synovial sarcoma TIC from representative cell lines and facilitate characterization of CD133 expressing (CD133+) and CD133 non-expressing (CD133-) subpopulations, including sensitivity to 17-AAG.
5.2 Materials and Methods

5.2.1 Reagents and cell culture

17-AAG was provided by the Developmental Therapeutics Branch of the National Cancer Institute (Bethesda, MD). All other reagents were purchased from Sigma (St. Louis, MI) and cell culture supplies from Invitrogen (Carlsbad, CA) unless otherwise stated. The monophasic synovial sarcoma cell line Fuji (32) and the biphasic synovial sarcoma cell line SYO-1 (33), which both harbor the SYT-SSX2 fusion, were kindly provided by Dr. Kazuo Nagashima (Hokkaido University School of Medicine, Sapporo, Japan) and Dr. Akira Kawai (National Cancer Centre Hospital, Tokyo, Japan). The human teratocarcinoma cell line NT2 was purchased from the ATCC (cat# CRL-1973, Manassas, VA). Monolayer cultures were grown under standard incubation conditions in RPMI 1640 supplemented with 5% (HeLa), 10% (Fuji,) or 10% to 20% (SYO-1) FBS with the exception of NT2, which was grown in DMEM supplemented with 10% FBS. Differentiation of NT2 cells was induced by plating at low density in medium containing 10 μM all-trans retinoic acid (ATRA). Penicillin G (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL), were purchased from Calbiochem (San Diego, CA) and added to media for sorting experiments.

5.2.2 Flow cytometry and cell sorting

CD133 expressing cells were quantitated by staining 1x10^7 unfixed cells with phycoerythrin (PE) conjugated anti-CD133/2 monoclonal antibody (293C3, cat# 130-090-853, Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions, with the exception of a 30 minute incubation in media at 37°C prior to labelling to allow re-expression of CD133. All subsequent steps were performed on ice. CD133 expressing (CD133+) cells were defined as those exhibiting a level of fluorescence greater than that of approximately 99% of cells stained with a PE-labelled mouse IgG2b isotype control (IS6-11E5.11, cat# 130-092-215, Miltenyi Biotec). Non-CD133 expressing (CD133-) cells were defined as those exhibiting a level of fluorescence equal to or less than that of approximately 99% of isotype control-stained cells. Dead cells were excluded based on propidium iodide permeability. Prior incubation with anti-human CD32 (Fc receptor) blocking antibody (cat# 14551, StemCell Technologies, Vancouver, BC, Canada) did not affect CD133 or isotype control staining. Dilutional analysis of the anti-CD133 antibody revealed that a 10-fold
decrease below the manufacturer’s recommended concentration was required before CD133+ staining was negatively affected. Approximately 5% of CD133+ cell staining is lost per hour with standing on ice after labelling, thus all experiments were analyzed within 2 hours of labelling. Measurement of CD133 staining was performed on an EPICS MXL cytometer (Beckman-Coulter, Fullerton, CA) with 1x10^4 to 5x10^5 total cells analyzed per experiment. Cell sorting was performed on either a FACSVantage SE (BD Biosciences, San Jose, CA) or InFlux (Cytopeia, Seattle, WA) cytometer. Cell purity was typically greater than 95% for both CD133+ and CD133- fractions. Unsorted cells remaining after the completion of sorting experiments were reserved as the unsorted fraction.

5.2.3 In vitro analyses of proliferation and self-renewal of CD133+ cells

The proliferative capacity and susceptibility to 17-AAG and doxorubicin hydrochloride of unsorted, CD133+ and CD133- fractions were measured by plating 1000 cells per well in a 96 well plate and proliferation was measured by MTT reduction assay as described in Chapter 4. The capacity for self-renewal of unsorted, CD133+ and CD133- cells was assessed by plating 1000 cells per well in a 6 well plate and analysing the relative fraction of CD133+ and CD133- cells at 7 days by flow cytometry. Experimental results were discarded if the purity of the CD133+ and/or CD133- fractions was less than 95% and/or CD133+ cells were detected in parallel cultures of purified CD133- cells. All plating experiments were performed at least in triplicate and statistical analysis of results performed with Microsoft Excel (Redmond, WA).

5.3 Results

5.3.1 Synovial sarcoma cell lines contain a subpopulation of CD133 expressing cells

CD133 has been used as a marker for the identification, quantification and purification of stem-like and progenitor cells, including neural progenitor cells. Based on the hypothesis that synovial sarcoma arises from TIC with similarities to neural progenitors, CD133 expression was examined in two model synovial sarcoma cell lines. TIC are detectable as a subset of cells in many cancer-derived cell lines and cell lines are particularly useful as models for studies of TIC because of their biologic stability over time and absence of non-tumor tissue (e.g. blood) (30, 31, 35-37). Prior
to investigating CD133 expression in the synovial sarcoma cell lines, CD133 expression was first characterized in positive and negative control cell lines. The teratocarcinoma cell line NT2 has features of neural progenitors and is known to express CD133, which is downregulated upon prolonged exposure to ATRA (27).

Figure 5.1 Positive and negative CD133 staining controls. The NT2 cell line, which expresses CD133, contains a significant CD133+ subpopulation that is diminished by ATRA treatment. HeLa cells, which do not express significant levels of CD133 mRNA, do not contain a significant subpopulation of CD133+ cells. Typical results for each cell line are presented.

Approximately 38% of NT2 cells expressed CD133 (Figure 5.1), which decreased to approximately 13% after treatment of the cells with 10 μM ATRA for 10 days, indicating that the anti-CD133/2 antibody was detecting CD133. The cervical carcinoma cell line HeLa was included as an additional negative control as CD133 mRNA levels have been previously reported as undetectable in these cells (38, 39). Consistent with these reports, we did not find any evidence of CD133 expression in HeLa cells (Figure 5.1).

In multiple (>10) independent experiments examining CD133 expression in the two synovial sarcoma cell lines, an average CD133+ subpopulation of 3.2% of SYO-1 (range: 0.08-11.8%) and 7.7% of Fuji (range: 1.4-27.7%) cells was found. Results of representative experiments for each cell lines are presented in Figure 5.2. Interestingly, the optical size (forward scatter) of the CD133+ subpopulations from each synovial sarcoma cell line was at the lower end of the range for the total population, indicating that the CD133+ synovial sarcoma subpopulation is composed of relatively smaller cells. Unlike the NT2 cell line, treatment of the synovial sarcoma cell lines with 10 μM ATRA for 10 days does not appreciably change the size of the CD133+ subpopulation. Thus, all of the synovial sarcoma cell lines models examined contain a consistent subpopulation of cells expressing increased levels of CD133.
Figure 5.2 CD133 expression in synovial sarcoma cell lines. The synovial sarcoma cell line models SYO-1 and Fuji contain a subpopulation of CD133+ cells (CD133+). Typical results for each cell line are presented.

5.3.2 CD133+ synovial sarcoma cells exhibit an increased proliferative rate and capacity for expansion

In subsequent experiments, the SYO-1 cell line was selected for studies of the CD133+ subset. In the cancer stem-like cell model of tumor development, TIC give rise to progeny that make up the bulk of the tumor but have limited capacity for self renewal and tumor growth is thought to be dependent on the long-term proliferative activity of the TIC (2). Accordingly, CD133+ TIC from other malignancies have been shown to exhibit increased long term proliferative rates in prolonged culture when compared to their CD133- counterparts (6, 30). If the CD133+ subpopulation represents synovial sarcoma TIC, then CD133+ cells would be expected to behave in a manner similar to CD133+ TICs from other malignancies, such as exhibiting increased proliferative potential. In experiments examining the proliferative capacity of CD133+ SYO-1 cells in prolonged culture, increased proliferative activity of CD133+ SYO-1 cells compared to unsorted and CD133- fractions typically became apparent within 4 days of separation; this increased proliferative activity is more pronounced in cells cultured in 20% FBS compared to 10% FBS (Figure 5.3; representative experiment). By ten days, CD133+ cells are clearly proliferating faster than the
unsorted cells, which were in turn expanding more rapidly than the CD133- cells (Figure 5.3). These differences in proliferative rates (CD133+ > CD133-) correlate with the relative amount of CD133+ cells present in each SYO-1 fraction, indicating that increasing the proportion of CD133+

**Figure 5.3** Proliferation of unsorted (diamond), CD133+ (square) and CD133- (circle) SYO-1 cells. CD133+ cells expand more quickly than unsorted and CD133- cells. Error bars represent the 95% confidence interval of the mean. A = absorbance. Results of a typical experiment are presented.

cells enhances proliferative capacity. Of note, the CD133+ fraction initially appears to proliferate more slowly in the first few days after sorting, particularly in cultures supplemented with 10% FBS.

To more directly assess the capacity of CD133+ synovial sarcoma cells for self-renewal, 1000 unsorted, CD133+ and CD133- SYO-1 cells were grown in monolayer culture and the proportion of CD133+ cells analyzed 7 days after sorting. The proportion of CD133+ cells in cultures expanded from purified CD133+ cells decreased from greater than 95% to approximately 23% within 7 days, indicating that CD133+ cells are capable of generating CD133- cells (Table 5.1). During this period,
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<th>SYO-1 culture fraction</th>
<th>Proportion of CD133+ cells</th>
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<td>CD133+</td>
<td>23.1% (+/- 3.6%)</td>
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<tr>
<td>CD133-</td>
<td>0.5% (+/- 0.2%)</td>
</tr>
<tr>
<td>Unsorted</td>
<td>3.7% (+/- 1.1%)</td>
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Table 5.1 The proportion of CD133+ and CD133- cells arising from unsorted, purified CD133+ and purified CD133- SYO-1 cell line fractions 7 days after sorting. CD133+ cells give rise to both CD133+ and CD133- cells, whereas CD133- cells do not. Brackets indicate the 95% confidence interval of the mean.

the absolute number of CD133+ cells also rose, demonstrating that CD133+ cells also self-renew. Purified CD133- cells give rise to additional CD133- cells but did not appear to generate an increase in CD133+ cells (Table 5.1). This was confirmed when no significant increase in CD133+ cells (0.8% +/- 0.7%) was observed during extended culturing of CD133- subpopulations for an additional 14 days (21 days total), indicating that generation of CD133- cells by CD133+ cells is a unidirectional event. The proportion of CD133+ cells in extended cultures of purified CD133+ fractions returned to level similar to those of unsorted cells cultured in parallel. On prolonged incubation (1-2 months), the CD133+ derived cultures survive whereas cultures derived from purified CD133- cells typically stop expanding and die off. Together, these results establish that CD133+ synovial sarcoma cells self-renew and can regenerate the cell population from which they were derived, whereas CD133- synovial sarcoma cells cannot.

5.3.3 The Hsp90 inhibitor 17-AAG prevents proliferation of both CD133+ and CD133- synovial sarcoma cells

The demonstration of potential TIC in synovial sarcoma suggests that resistance of these TIC to current therapies could be an important cause of tumor recurrence and therapies targeting these cells could significantly improve patient survival. To examine this, the sensitivity of CD133+ and CD133- SYO-1 subpopulations to doxorubicin hydrochloride and the Hsp90 inhibitor 17-AAG was assessed by MTT assay. In the presence of 1 μM doxorubicin (peak human serum concentrations with a single 60 mg/m² dose of 1 to 2 μM (40)), CD133- cells were prevented from proliferating and the cultures usually died within 10 days; however,
CD133+ cells continued to proliferate, albeit at a greatly reduced rate when compared to vehicle treated control (representative experiments, Figure 5.4). In contrast, exposure to 1 μM 17-AAG (achievable human serum concentrations of 1.6 to 3.0 μM (41, 42)) blocked proliferation of both CD133+ and CD133- fractions and eventually killed all cells in cultures derived from both subfractions. Unsorted SYO-1 cells exhibited a pattern of sensitivity to doxorubicin and 17-AAG that was intermediate between the CD133+ and CD133- fractions (Figure 5.4). These results demonstrate that CD133+ synovial sarcoma cells are less sensitive to doxorubicin, but not 17-AAG, when compared to CD133- cells.

**Figure 5.4** Comparison of the antiproliferative effect of 1 μM doxorubicin (triangle) and 1 μM 17-AAG (square) on purified CD133+ and purified CD133- SYO-1 cells. 17-AAG blocks proliferation of both CD133+ and CD133- cells whereas CD133+ cells continue to proliferate in the presence of doxorubicin. Vehicle control (0.1% DMSO, diamond) and unsorted cells are included for comparison. Error bars indicate 95% confidence interval of the mean. Note that the Y-axis is log10 scale. Results of typical experiments are presented.

### 5.4 Discussion

Identification of a CD133+ subset of cells in two synovial sarcoma cell line models implies that a CD133 subpopulation is a common feature of synovial sarcomas. Although concordance between CD133+ expression in cell lines and primary tumors has been reported in hepatocellular carcinoma (31), the prevalence of the CD133+ subpopulation in primary synovial sarcomas has yet to be determined.
The proportion of cells showing positive CD133 staining in the synovial sarcoma cell lines was less than that reported for the established hepatoma cell line Huh-7 (~25-50%) (30, 43), but similar to that observed for primary colon adenocarcinoma (~2-25%) and the hepatocellular carcinoma cell line SMMC-7721 (~0.1-2%) (9, 10, 31). Methodological factors influenced CD133+ staining in the control and synovial sarcoma cell lines. Increased CD133 antibody incubation times (up to 30 minutes) and higher temperature (e.g. room temperature) led to increases in the proportion and intensity of CD133 staining relative to unstained and isotype stained controls in each cell line. This increase in CD133 staining was associated with smaller differences in proliferative rate between CD133+ and CD133- subpopulations, indicating that increased non-specific staining was leading to contamination of the CD133+ fraction with CD133- cells during sorting. Another factor is the use of trypsin to generate a single cell suspension for cytometry, as trypsin digests CD133 and temporarily decreases CD133 immunoreactivity (44). Allowing at least 20 minutes between trypsinization and CD133 staining for re-expression of CD133 increased the proportion of CD133 positive cells and greatly reduced experiment-to-experiment variability in levels of CD133+ cells within each cell line. Culture density may also play a role in CD133 expression in synovial sarcoma, as confluent and hyperconfluent cultures typically exhibited larger CD133+ subpopulations when compared to subconfluent cultures stained under identical conditions. The relationship between CD133 expression and culture density in these synovial sarcoma cell lines has yet to be defined but likely accounts for the residual inter-experimental variance in the proportion of CD133+ cells for each cell line.

The characteristics of CD133 staining in the two synovial sarcoma cell line models indicated that the SYO-1 cell line would be best for conducting proliferative capacity and self-renewal assays of CD133+ and CD133- cells. A significant (95% confidence interval) increase in proliferation in the CD133+ subpopulation compared to CD133- fractions was observed in longer term cultures (≥ 10 days), which is similar to that reported for stem-like cells isolated from other malignancies (6, 7, 30). CD133+ cultures expanded more quickly than unfractionated and CD133- derived cultures, which is consistent with the proportion of CD133+ cells in each of these subpopulations and indicate that CD133+ cells are responsible for increasing the rate of culture expansion. Purified CD133+ SYO-1 synovial sarcoma cells are also
capable of giving rise to other CD133+ cells (self-renewal) as well as CD133- cells; however, purified CD133- cells did not give rise to CD133+ cells. Together with the results of the proliferation assays, this indicates that CD133+ SYO-1 cells are responsible for culture maintenance and suggest that CD133+ subpopulation contains synovial sarcoma TIC. Interestingly, the proliferative rate of the CD133+ fraction was observed to initially lag behind the CD133- and unsorted fractions, suggesting that CD133+ cells replicate more slowly than CD133- cells. This lag is mitigated in SYO-1 by increasing the concentration of FBS in the culture media from 10% to 20%, suggesting growth factors play a role in determining CD133+ cell renewal rate. The increased proliferative rate of CD133+ derived cultures is explained by the limited replicative potential of CD133- cells and the associated progressive decrease in overall proliferation in longer term cultures.

Subsets of cells within a malignancy, such as TIC, may exhibit increased resistance to chemotherapeutic agents and promote tumor recurrence after initial treatment. Considering that present systemic treatment regimens for synovial sarcoma do not significantly impact the survival of most patients, identifying subsets of chemotherapy resistant synovial sarcoma cells would allow novel drugs to be tested for activity against such resistant subpopulations. The Hsp90 inhibitor 17-AAG, which inhibits proliferation of unsorted synovial sarcoma cell lines, has activity against stem-like cancer cells (25), suggesting it may have a similar effect in synovial sarcoma. To assess this, the susceptibility of CD133+ and CD133- subpopulations of SYO-1 cells to doxorubicin and to 17-AAG was examined. CD133+ cells were able to persist in the presence of 1 μM doxorubicin whereas 1 μM 17-AAG killed CD133+ cells within 10 days of exposure; CD133- cells were destroyed by both doxorubicin and 17-AAG. These results indicate that CD133+ cells are more resistant than CD133- cells to doxorubicin, adding to the evidence that CD133+ subpopulation contains cells that play a stem-like role in synovial sarcoma. Although considerable care must be taken when extrapolating the potential clinical efficacy of drugs based on bench top experiments, these results bolster the findings in Chapter 4 that 17-AAG may be a more effective systemic therapy than doxorubicin.

Synovial sarcoma TIC are poorly defined and further study requires development of a method to reproducibly identify and separate potential TIC from the overall tumor cell population. This study presents evidence that the stem cell marker
CD133 is expressed in a subpopulation in both synovial sarcoma cell line models, and that enrichment for CD133 expressing cells also enriches for basic functional characteristics associated with stem-like cells. These results identify CD133 as a marker for stem-like cells in synovial sarcoma, and selection for CD133 as a valid method for separating this subset of cells from the total population. Furthermore, CD133+ synovial sarcoma cells exhibit relative resistance to doxorubicin. Importantly, 17-AAG was able to prevent proliferation of CD133+ cells, suggesting this clinically available drug may better be able to reduce or prevent progression of this disease.
5.5 References


6 CONCLUSIONS AND FUTURE DIRECTIONS

The impetus for studying gene expression patterns in tumors like synovial sarcoma is the hope that consistent irregularities will be uncovered that may offer insight into the underlying pathobiology. This is certainly true for synovial sarcoma, and many clues to the development of this tumor have been gleaned from such studies; however, the applicability of this wealth of information to directly improving patient care is a work in progress to which the research presented here constitutes a significant contribution.

Demonstration of t(X; 18) is presently the gold standard for diagnosis of synovial sarcoma and the application of novel and evolving methodologies continues to improve its practical diagnostic value. Clinically, the core needle biopsy is an increasingly popular method to obtain tumor tissue for analysis that minimises procedural risk for the patient; however, RT-PCR methods to detect t(X;18) are difficult to apply to the small amounts of tissue obtained and FISH based detection of t(X; 18) in small tissue samples had not been systematically surveyed. In addition, tissue microarrays are excellent platforms for assessing potential immunohistochemical markers and the development of a robust assay for t(X; 18) applicable to the small tumor cores in this format would be a significant enabling step for such assessments. Accordingly, the applicability of FISH based detection of t(X; 18) in small tissue samples was investigated by applying an SYT breakapart assay to a synovial sarcoma tissue microarray using a unique method to simplify interpreting the results. This novel approach demonstrated that FISH-based detection is a robust method for this application. Probe hybridization could be obtained in the majority of samples under common incubation conditions, and a simplified scoring system can be used to determine the presence of t(X; 18) with high sensitivity and specificity, findings which both have significant implications for the practical clinical use of this technique. In local clinical practice, this SYT breakapart FISH method is now being used instead of RT-PCR for detection of t(X; 18) in core biopsies, and has been applied by other groups in recently published research applications (1, 2). This method was successfully applied to automated scoring, which suggests an application in high-throughput detection of t(X; 18), although the rarity of synovial sarcoma will likely make automated detection practical only in research applications. This method could be further refined to incorporate methodologies to differentiate
between fusion variants (3). This simplified scoring scheme could also be applied to other translationally defined malignancies to similarly facilitate the use of FISH-based assays.

*TLE1* is an effective discriminator of synovial sarcoma from other sarcomas in the differential diagnosis at the level of gene expression. This is also true at the protein level when studied in almost 700 samples comprising fifty soft tissue tumor types. As an immunohistochemical marker, TLE1 exhibits high sensitivity and specificity for synovial sarcoma compared to other sarcomas that can be difficult to distinguish histologically, like ES/PNET and MPNST, although some hemangiopericytomas, schwannomas and solitary fibrous tumors do appear to express moderate levels of TLE1 as well. TLE1 immunohistochemistry is now being used locally and at collaborating sites for the clinical diagnosis of synovial sarcoma and the sensitivity and specificity of this marker will continue to be refined. This work has been presented by our colleagues at the International Academy of Pathology Long Course on molecular diagnostics, has been invited for inclusion as a chapter in an upcoming textbook of diagnostic immunohistochemistry, and has been referenced in the most recent version (5th edition) of Enzinger and Weiss' *Soft Tissue Tumors* pathology textbook, and so is likely to become clinically used in a broader variety of diagnostic laboratories. Future prospective investigations into the concordance of positive TLE1 immunostaining and t(X; 18) status (already underway by my supervisor and funded by NCIC) will further define the role of TLE1 in synovial sarcoma diagnosis, perhaps supplanting the need for direct demonstration of t(X; 18).

The demonstration that TLE1 is highly expressed in the vast majority of synovial sarcomas also has significant biological implications, particularly considering the vigorous expression of TLE1 also observed in neural progenitor cells (4). Further examination of TLE1 function in synovial sarcoma will undoubtedly help unravel the nuclear signaling pathways underlying development of this tumor.

Aberrant gene expression patterns have highlighted certain signaling and metabolic pathways that may serve as potential therapeutic targets in synovial sarcoma. Principal among these are overexpression of genes encoding RTKs and/or associated ligands. The effect of clinically available drugs designed to specifically inhibit EGFR, HER2, IGF-1R, FGFR and KIT/PDGFR signaling was examined on *in vitro* synovial sarcoma models and we found that none of these had an appreciable
impact on proliferation at clinically achievable concentrations. 17-AAG, which is known to promote degradation of multiple tyrosine kinases simultaneously, inhibited proliferation and induced apoptosis in association with degradation of multiple RTKs. 17-AAG produced similar results in more stringent spheroid cultures, indicating that Hsp90 inhibition, with 17-AAG or other clinically applicable Hsp90 inhibitors, could be an effective therapeutic strategy for synovial sarcoma. Preliminary investigations into the effect of 17-AAG on synovial sarcoma tumor xenograft growth have been less promising. In a preliminary study of established subcutaneous xenografts in nude mice, 17-AAG marginally slowed tumor growth, an effect that became apparent after approximately 2 weeks of treatment under conditions where doxorubicin (which is usually used clinically in a palliative setting) had no inhibitory effect (Appendix B, Figure B.1). These results suggest that 17-AAG may be less effective in vivo than the in vitro studies show; however, given that mortality from synovial sarcoma arises primarily from metastatic recurrence, a metastatic model may be more appropriate. In this regard I have developed a modified SYO-1 cell line that stably expresses firefly luciferase (SYO-1 luc+), appropriate for use in conjunction with Ivis live mouse imaging apparatus to monitor metastatic tumor development and response to treatment in vivo (Appendix A). Luminescent tumors have been successfully obtained in a preliminary test of this system, and one mouse was found to have developed clinically imperceptible peritoneal deposits (Appendix B, Figure B.2), demonstrating the value of this system for monitoring SYO-1 luc+ metastatic models. A full-scale study employing this model comprises a significant area for future research. The recently described SYT-SSX-expressing transgenic mice would also be an excellent model for testing Hsp90 inhibitor action, although monitoring tumor growth would be more difficult (5). A number of other clinically applicable Hsp90 inhibitors have been developed, some of which are orally available and may avoid some of the side effects of 17-AAG identified in phase I trials (6-8). These will need to be tested in a similar manner; many are presently in clinical trials for other cancer indications (9).

Hsp90 inhibition has been shown to sensitize some malignancies to common chemotherapeutics like paclitaxel, doxorubicin and etoposide; additive and synergistic affects have been observed with rapamycin, bortezomib, Iressa, Gleevec and even dimeric 17-AAG (10-17). More detailed analyses of these drug combinations will be required to confirm additive, synergistic or antagonistic relationships with 17-AAG in
synovial sarcoma. Monoclonal antibody-based therapeutics, such as Herceptin, act in part by selectively directing the immune system towards malignant cells overexpressing their target (18). Our assays cannot account for this effect and would require assessment in a living system; in this respect, the SYT-SSX transgenic mouse model would be a perfect candidate (5). Another area for further investigation is the mechanisms of action behind the 17-AAG mediated inhibition of proliferation in synovial sarcoma, as Hsp90 inhibition is known to affect multiple pathways simultaneously (19). 17-AAG-mediated RTK degradation did not promote substantial membrane localization of β-catenin, nor did 17-AAG promote degradation of SYT-SSX; however, 17-AAG did promote disruption of the SYT-SSX-β-catenin complex and decreased levels of nuclear β-catenin, suggesting that 17-AAG also affects the nuclear activity of β-catenin. Presumably 17-AAG disrupts β-catenin-SYT-SSX interaction through degradation or modification of a critical shared binding partner; immunoprecipitation studies with mass spectrometric analysis of co-immunoprecipitants lost with 17-AAG treatment would be a good path for further investigation. Both SYT and SYT-SSX2 co-immunoprecipitate with β-catenin, which is likely due to multimers formed between SYT-and SYT-SSX2 through the homo-oligomerization domain of SYT (20); however, neither SYT nor β-catenin co-immunoprecipitate with SLUG, which selectively binds SYT-SSX2 in approximately the same physical location as the homo-oligomerization domain (Appendix B, Figure B.3). This preliminary result implies that SYT and SLUG may compete for SYT-SSX2 binding and that SYT is required for β-catenin co-immunoprecipitation, which suggests SYT is part of the SYT-SSX2-β-catenin bridge. Furthermore, 17-AAG does not appear to affect SLUG-SYT-SSX2 interaction (Appendix B, Figure B.3).

Identification of a labile bridging protein, as part of characterization of the SYT-SSX-β-catenin transcriptional complex, is an attractive area for further study. 17-AAG also inhibits β-catenin signaling in SYO-1 and Fuji (Appendix B, Figure B.4), suggesting that the SYT-SSX2-β-catenin interaction is required to maintain β-catenin signaling in synovial sarcoma.

The clinical effectiveness of any systemic therapy for synovial sarcoma depends on all cells comprising a tumor being sufficiently sensitive to that therapy, stressing the importance of identifying any potentially resistant subgroups within that cell population. A subpopulation of cells expressing increased levels of CD133, a
neural stem cell marker, exists in synovial sarcoma cell lines and that these cells exhibit functional stem-like characteristics. Assessment of expression of other neural stem cell-related markers, such as nestin or NCAM (CD56), will figure prominently in the further characterization of the CD133+ subpopulation. Importantly, the CD133+ subpopulation is sensitive to Hsp90 inhibition, but less so to doxorubicin, suggesting that Hsp90 inhibitors such as 17-AAG will be more effective than doxorubicin in controlling or preventing clinical tumor recurrence. The argument for tumor initiating cells (TIC) in synovial sarcoma will be strengthened by further characterization of the CD133+ subpopulation, particularly by identifying additional biomarkers that select for stem-like properties to enhance identification and purification. In this respect, the synovial sarcoma cell lines SYO-1 and Fuji both express a side population (Appendix B, Figure B.5), which is typically enriched for TIC (21, 22). More recently, side populations from two of three primary synovial sarcomas have been reported as enriched for TIC (23). Correlating in vitro functional studies with CD133+ cells isolated from primary tumors will lend considerable weight for the use of Hsp90 inhibitors clinically. Ideally, these studies will facilitate isolation of a single t(X; 18) bearing cell from a primary tumor capable of regenerating that tumor, which will constitute ideal evidence cancer stem-like cells in synovial sarcoma.

The presence of CD133+ stem-like cells in synovial sarcoma bolsters the theory of a neural crest origin. In the developing embryo, neural crest cells (NCCs) are induced from progenitors by signals from the adjacent mesoderm, resulting in transition from a mesenchymal to epithelial phenotype and delamination and migration into the periphery where NCCs ultimately differentiate into various tissues including bone, neurons and connective tissue (24, 25). Specification of murine trunk NCCs involves the coordinated expression of the transcription factors Slug, FoxD3 and Sox9, each of which appears to influence distinct segments of NCC development (25). In this respect, Sox9 expression is not required for induction of NCCs; however, NCCs lacking Sox9 undergo apoptosis prior to or during commencement of delamination and migration into the periphery, demonstrating that Sox9 is necessary for NCC survival during EMT (26). Interestingly, one of the NCC-related genes identified as overexpressed in synovial sarcoma is SOX9, the human homologue of murine Sox9 (27, 28). Overexpression of SOX9 may play an important role in synovial sarcoma biology, similar to its anti-apoptotic role in NCC delamination, by preventing
apoptosis while in this suspended state of aberrant differentiation. If true, then synovial sarcoma should be dependent on SOX9 expression and interruption should lead to apoptosis. This hypothesis suggests an interesting and likely rewarding future direction, as does the examination of the role of other stem and progenitor related nuclear signaling molecules, particularly TLE and β-catenin.

The objectives of this body of research were to test the hypothesis that genes and pathways highlighted prominently in these gene expression studies represent valid areas to focus appraisal of potential diagnostic markers and therapeutic targets with the intention of clinical use. The research presented in this thesis tangibly demonstrates that this hypothesis is true and in doing so has led to significant advances in the diagnosis, treatment and understanding of synovial sarcoma.
6.1 References


APPENDIX A

A.1 Materials and methods
A.1.1 Tumor xenograft studies

Four to six week old *nu/nu* female mice were obtained from Harlan (Indianapolis, IN) and housed in sterile, ventilated cages following institutional guidelines for animal care and protocols approved by the University of British Columbia animal care committee. Mice were injected subcutaneously over the flank region with 5x10^6 SYO-1 cells resuspended in RPMI. Tumor dimensions were measured by caliper and tumor volume was calculated using the formula: \( \pi/6 \times \text{larger dimension} \times \text{smaller dimension} \times \text{height above surrounding skin} \). Mice with established tumors of 50 mm³ were selected for treatment (n = 5-6 mice per group). Stock solutions of 17-AAG and doxorubicin were prepared and diluted in PBS containing 0.05% Tween 80 (Sigma, St. Louis, MO) to give the appropriate weight-based dosage for each mouse just prior to each injection. 17-AAG was administered by intraperitoneal injection at a dose of 50 mg/kg on the first, 3rd and 5th day of each week for three weeks. Control mice were dosed in a likewise fashion with vehicle only. Doxorubicin was administered by intraperitoneal injection at a dose of 6 mg/kg on the first day of each week for three weeks. Mice were euthanized by CO₂ and tumors harvested. Luciferase expressing SYO-1 (SYO-1 luc+) was generated as previously described (1). Stable expression of luciferase in the absence of selective pressure was confirmed to occur for at least 12 weeks. SYO-1 luc+ exhibited growth characteristics (both *in vitro* and as a subcutaneous xenograft) and sensitivity to 17-AAG (*in vitro*) identical to the parent SYO-1 culture. SYO-1 luc+ cells were inoculated subcutaneously as for SYO-1 cells and visualized by intraperitoneal injection of luciferin (Sigma) as previously described (1). Images were captured in an Ivis 100 imaging system (Xenogen, Hopinkton, MA).

A.1.2 SLUG immunoprecipitation and immunoblotting

SLUG immunoprecipitation and immunoblotting was performed as described in Chapter 4 using anti-SLUG antibody (H-140, cat# 15391, Santa Cruz Biotechnology, Santa Cruz, CA).
A.1.3 β-catenin signaling luciferase assays

The Super8xTOPflash/FOPflash firefly luciferase-based β-catenin reporter system (2) was kindly provided by Dr. Randall Moon (University of Washington). Cell cultures were co-transfected with either Super8xTOPflash or Super8xFOPflash and Renilla control plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, with the exception of the Fuji transfections in which the amount of TOP/FOPflash was doubled. Transfected cultures were treated as indicated for 24 hours. Treated cultures were then prepared and assayed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) according to manufacturer’s instructions on a MicroLumat Plus luminometer (EG&G Berthold, Bad Wildbad, Germany). Data presented is representative of at least 3 independent experiments.

A.1.3 Side population analysis

Side population analysis was performed as previously described (3), with minor modifications. Confluent monolayer synovial sarcoma cell cultures were trypsinized and resuspended in RPMI + 10% FBS containing 5 μg/mL Hoechst 33342 and incubated at 37°C with intermittent shaking for 90 minutes. Side populations were verified by co-treatment of parallel samples with either 100 μM verapamil (Sigma) or 100 μM reserpine (Sigma). The cells were then pelleted and resuspended in 500 μL ice cold PBS, filtered to remove cell clumps and propidium iodide (Sigma) was added to a final concentration of 2 μg/mL to identify dead cells. Cytometric analysis was performed on a FACSVantage (Beckman-Coulter, Fullerton, CA). Hoechst 33342 emissions were measured at 525 nm (blue) and 670 nm (red).
A.2 References


B.1 Supplementary data

Figure B.1 The effect of 17-AAG and doxorubicin on the growth of synovial sarcoma cell line SYO-1 subcutaneous tumor xenografts. 17-AAG slows, but does not prohibit, the growth of established SYO-1 xenografts. Error bars represent standard error of the mean.
Figure B.2 Example of a subcutaneous SYO-1 luc+ xenograft tumor. The initial subcutaneous injection site is the right rear flank; metastatic deposits that likely occurred from inadvertent introduction of cells into the peritoneal cavity are visualized on in the region of the left shoulder. The scale indicates absolute luminosity and allows relative comparison of tumor density.
**Figure B.3** SLUG co-immunoprecipitates SYT-SSX2 but not β-catenin.

Immunoprecipitation of SLUG, a transcription factor that binds SYT-SSX2 but not SYT, from SYO-1 lysate co-precipitates SYT-SSX2 but neither SYT nor β-catenin. Treatment with 0.5 μM (0.5) or 5 μM (5) 17-AAG does not affect association of SLUG with SYT-SSX2. IP = Immunoprecipitation, IB = Immunoblot.
Figure B.4 The effect of 17-AAG on β-catenin signaling. Exposure to 17-AAG decreases β-catenin mediated signaling from the artificial reporter plasmid 8xTOPflash within 24 hours of treatment in the SYO-1 and Fuji cell lines. HeLa does not exhibit a significant response to 17-AAG. Error bars represent standard deviation.
Figure B.5 The synovial sarcoma cell line SYO-1 contains a side population. Plot of Hoechst 33342 dye staining of hyperconfluent SYO-1 monolayer culture. The side population (green) accounts for approximately 0.4% of the total population. Similar results were obtained with hyperconfluent Fuji cultures.
APPENDIX C

C.1 Animal care committee certificate

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-1449
Investigator or Course Director: Torsten O. Nielsen
Department: Pathology & Laboratory Medicine
Animals: Mice nude 86

Start Date: September 28, 2006 Approval Date: November 10, 2006
Funding Sources:
Funding Agency: Canadian Institutes of Health Research
Funding Title: Synovial Sarcoma – Translating Gene Expression into Clinical Treatment
Funding Agency: National Cancer Institute of Canada
Funding Title: Synovial sarcoma: Translating gene expression into patient care

Unfunded title: n/a

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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