Molecular Cytogenetic Studies of Soft Tissue Sarcoma:

with focus on Prognosis and Acquired Events

Wen-Hui Weng

Stockholm 2005

Faculty Opponent:

Göran Stenman, Professor

Department of Pathology

Göteborg University, Göteborg, Sweden

Thesis Committee:

Stefan Imreh, Associate Professor

Microbiology and Tumor Biology Center

Karolinska Institutet, Stockholm, Sweden

Jörgen Carlsson, Professor

Department of Oncology, Radiology, and Clinical Immunology

Division of Biomedical Radiation Sciences,

Uppsala University, Uppsala, Sweden

Li-Fu Hu, Associate Professor

Microbiology and Tumor Biology Center

Karolinska Institutet, Stockholm, Sweden

Supervisors:

Catharina Larsson, Professor

Department of Molecular Medicine

Karolinska Institutet, Stockholm, Sweden

Weng-Onn Lui, PhD

Department of Molecular Medicine

Karolinska Institutet, Stockholm, Sweden

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Published and printed by Karolinska University Press
Box 200, SE-171-77 Stockholm, Sweden,
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ISBN 91-7140-241-1

Abstract

Soft tissue sarcomas (STSs) constitute a heterogenous group of highly aggressive tumors of mesenchymal origin that account for approximately 1% of all human malignancies. With the aim of eventually improving the diagnostics and clinical handling of these patients, considerable research efforts have been invested to understand the natural history of these tumors. In this thesis, we applied both molecular and cytogenetic techniques to identify specific genetic events associated with clinical outcome, drug resistance and tumor progression in soft tissue sarcomas. In **paper I**, a panel of 39 primary malignant fibrous histiocytomas (MFH) of high malignancy grade were characterized by comparative genomic hybridization (CGH) analyses. The genetic alterations were then evaluated in relation to the survival and the tumor recurrent during follow-up. Our results suggest that the clinical outcome of MFH is associated with the genetic profiles of the primary tumors. Importantly, a subgroup of MFHs characterized by a low risk of developing metastasis and local recurrence is recognized based on their frequent gains of 17q, and hence a better survival. In **paper II**, we evaluated the prognostic role of ezrin in a series of 50 patients with primary highly malignant STSs using immunohistochemistry and correlated its expression to clinical outcome. Among the STSs analyzed, half of the cases showed positive ezrin immunoreactivity in the membrane and cytoplasm of the tumor cells. Positive expression was significantly associated with death from or with disease as well as development of metastasis. Our findings suggest that ezrin immunoreactivity could be valuable as an additional prognostic marker for this tumor type. In **paper III**, we aimed to establish Picropodophyllin (PPP) resistance in human malignant cells and to investigate the involved mechanisms. Four established human cancer cell lines with documented IGF-1R function and responsiveness to PPP treatment were exposed to increasing concentrations of PPP for up to 80 weeks. Only two cell lines survived the selection process, and in both of them, the resistance development was remarkably slow and limited. During the first 40 weeks, these lines successively developed moderate increase of IGF-1R expression, whereafter the expression returned to normal levels. The increased IGF-1R expression was overlapped by some genetic changes, and the common alteration for both cell lines was gains in chromosome 11p12-q13. None of the resistant cell lines exhibited increase in the expression of multidrug-resistance related proteins MDR1 or MRP1. In **paper IV**, we characterized the chromosomal composition of an MFH case using a combination of SKY, G-banding, CGH, and cDNA array-CGH.This MFH was shown to carry large chromosome markers with a high-level amplification at the regions of 6q21-23, 8p21-pter, 8q24-qter and 12q13-15, suggesting that these regions might harbor oncogenes that could play important role in the tumorigenesis. In **paper V**, we performed CGH, mutation screening, SKY and Southern analyses in a series of 32 STSs from 26 patients. CGH analyses revealed frequent chromosomal imbalances involving several different chromosomes. The most common finding was deletions involving chromosome 13 that was seen in nearly half of the cases. Southern analyses excluded the involvement of candidate suppressors such as RB gene, which frequently deleted region in CLL, and pointed towards the involvement of a more telomeric target. In **paper VI**, we reported a case of two synovial sarcomas occurring synchronously with biphasic feature in a 10-year old girl. Molecular and cytogenetic analyses were performed on these tumor samples, as well as the peripheral leukocytes. A *SS18-SSX2* fusion was detected by both RT-PCR and FISH assays in the tumor samples, but not in the blood sample. An apparently normal karyotype was found in the leukocytes, suggesting that the *SS18-SSX2* fusion detected in the tumor samples is an acquired event.

ISBN 91-7140-241-1

List of articles

Papers included in the study

This thesis is based on the following articles, which will be referred to by their Roman numerals throughout the text.

I **Wen-Hui Weng**[*], Jan Åhlén, Weng-Onn Lui, Otte Brosjö, See-Tong Pang, Anette von Rosen, Gert Auer, Olle Larsson and Catharina Larsson
Gain of 17q in malignant fibrous histiocytoma is associated with a longer disease-free survival and a low risk of developing distant metastasis.
Br J Cancer 2003 Aug 18;89(4):720-6.

II **Wen-Hui Weng**[*], Jan Åhlén, Kristina Åström, Weng-Onn Lui and Catharina Larsson
Prognostic impact of immunohistochemical expression of ezrin in highly malignant soft tissue sarcomas.
Manuscript

III Daiana Vasilcanu, **Wen-Hui Weng**, Ada Girnita, Weng-Onn Lui, Magnus Axelson, Leonard Girnita, Catharina Larsson and Olle Larsson[*]
Malignant cells exhibit limited resistance to the insulin-like growth factor receptor 1 inhibitor PPP.
Manuscript

IV **Wen-Hui Weng**[*], Johan Wejde, Jan Åhlén, See-Tong Pang, Weng-Onn Lui and Catharina Larsson
Characterization of large chromosome markers in a malignant fibrous histiocytoma by SKY, CGH and array-CGH.
Cancer Genet Cytogenet. 2004 Apr 1;150(1):27-32.

V **Wen-Hui Weng**[*]**,** Mikael Lerner, Dan Grandér, Jan Åhlén, Andrea Villablanca, See-Tong Pang, Johan Wejde, Weng-Onn Lui and Catharina Larsson[*]
Loss of chromosome 13q is a frequently acquired event in genetic progression of soft tissue sarcomas in the abdominal cavity.

Int J Oncol. 2005 Jan;26(1):5-16.

VI **Wen-Hui Weng**, Alexander Claviez, Matthias Krams, Olle Larsson, Catharina Larsson[*] and Meinolf Suttorp
A unique case of two synovial sarcomas occurring synchronously in a 10-year old girl.
Manuscript

Other papers on related topics

Jan Åhlén[*], **Wen-Hui Weng**, Otte Brosjö, Anette von Rosen, Olle Larsson and Catharina Larsson
Evaluation of immunohistochemical parameters as prognostic markers in malignant fibrous histiocytoma.
Oncol Rep. 2003 Sep-Oct; 10(5): 1641-5.

Theodoros Foukakis[*], Srinivasan R. Thoppe, Svetlana Lagercrantz, Trisha Dwight, **Wen-Hui Weng**, Ann Svensson, Anders Höög, Jan Zedenius, Göran Wallin, Weng-Onn Lui and Catharina Larsson
Molecular cytogenetic characterization of primary cultures and established cell lines from non-medullary thyroid tumors.
Int J Oncol. 2005 Jan; 26(1): 141-9.

Jan Åhlén[*], Johan Wejde, Otte Brosjö, Anette von Rosen, **Wen-Hui Weng,** Leonard Girnita, Olle Larsson and Catharina Larsson
Insulin-like growth factor type 1 receptor expression correlates to good prognosis in highly malignant soft tissue sarcoma.
Clin Cancer Res. 2005 Jan 1; 11(1): 206-16.

See-Tong Pang[#,*], **Wen-Hui Weng**[#], Amilcar Flores-Morales, Peter Nilsson, Birgitta Byström, Åke Pousette, Catharina Larsson and Gunnar Norstedt
Cytogenetic and expression profiles associated with transformation to androgen-resistant prostate cancer.
Submitted for publication

Cherry Tzu-Ru Chang[#], **Wen-Hui Weng**[#], Andy Shau-Bin Chou, Cheng-Keng Chuang, Anja Porwit-McDonald, See-Tong Pang, Catherina Larsson and Shuen-Kuei Liao[*]
Immunophenotypic and molecular cytogenetic features of the cell-line UP-LN1 established from a lymph node metastasis of a poorly differentiated carcinoma**.**
Anticancer Res., in press.

[*]Corresponding author(s)
[#]Authors contributed equally

Abbreviations

AGS	Angiosarcoma
ASTS	Alveolar soft tissue sarcoma
CDK4	Cyclin-dependent kinase 4 gene
cDNA	Complementary deoxyribonucleic acid
CGH	Comparative genomic hybridization
CHOP	C/EBP homologous protein gene
C-KIT	c-kit receptor tyrosine kinase gene
DAPI	4´-6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
e.g.	For example
FBS	Fibrosarcoma
FISH	Fluorescence *in situ* hybridization
G-banding	Giemsa banding
GIST	Gastrointestinal stromal tumor
GLI	Glioma-associated oncogene homologue
HSR	Homogenously staining region
IGF-1R	Insulin-like growth factor 1 receptor gene
KLF5	Krüppel-like factor 5 gene
LIG4	DNA ligase 4 gene
LMS	Leiomyosarcoma
LPS	Liposarcoma
MASL1	Malignant fibrous histiocytoma amplified sequence 1 gene
Mb	Megabase
MDM2	Murine double minutes gene
MFH	Malignant fibrous histiocytoma
MNS	Mesenchymoma
MPNST	Malignant peripheral nerve sheath tumor
NOS	High grade neurofibrosarcoma
NPM1	Nucleophosmin gene
p	The short arm of a chromosome
PCR	Polymerase chain reaction
PDGFRA	Platelet-derived growth factor receptor alpha gene
PPP	Picropodophyllin
pter	The end of the short arm of a chromosome
q	The long arm of a chromosome
qter	The end of the long arm of a chromosome
RB1	Retinoblastoma gene
SAS	Sarcoma amplified sequence gene
SKY	Spectral karyotyping
SS	Synovial sarcoma
STS	Soft tissue sarcoma

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Papers I-VI

1. Introduction

1.1 The concept of cancer

One of the hallmarks of cancer is that the malignant phenotype is constantly inherited from the mother cell to the daughter cells. This and other observations have led to the present view of cancer as a basically genetic disease that result from a cascade-like series of genetic changes. This multistage theory of cancer evolution includes increasing degrees of genetic abnormalities with sequential loss of tumor suppressor genes and activation of proto-oncogenes, with or without a concomitant defect in the DNA repair machinery (Figure 1) [1]. The model has been applied to all types of tumors including hematological malignancies and solid tumors of carcinoma and sarcoma types.

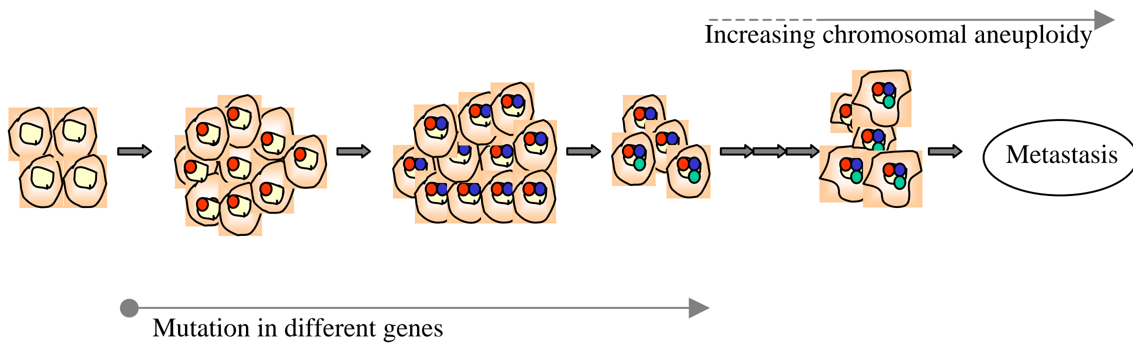

Figure 1. Multistage evolution of cancer.

The genetic progression of cancer leads to increasing degrees of genetic abnormality. This is associated with sequential loss of tumor suppressor genes from several chromosomes, and activation of proto-oncogenes with or without concomitant defects in DNA repair genes.

1.1.1 Models for tumor metastasis

The most fearsome aspect of cancer is the ability to develop metastasis. Once metastases occur, it is usually a sign of poor prognosis giving significantly reduced chances of survival and cure from the disease. Several models have been proposed for how metastasis can occur in tumor progression.

Classical tumor progression theory

The classical hypothesis explaining the form of overt metastasis is that cancer cells must break away from a primary tumor and grow in a new focus (metastasize) in normal tissues elsewhere in the body. These steps include separation from the primary tumour, invasion through surrounding tissues and basement membranes, entry and survival in the circulation, lymphatic or peritoneal space, arresting in a distant target organ, usually, but not always followed by extravasations into the surrounding tissue, survival in the foreign microenvironment, proliferation, and induction of angiogenesis, all the while evading apoptotic death or immunological response [2, 3]. Nonetheless, the inability of a cell to complete and one step of this cascade could results in metastatic failure [4, 5].

"Seed -and-soil" theory

A non-random pattern of metastasis formation was also proposed by Stephen Paget already in 1889, who postulated his " seed -and-soil" theory, which suggests a cross-talk between certain cancer cells (the "seed") and specific organ microenvironments (the "soil"). Later, in 1928, James Ewing proposed that metastastic growth is a result of blood supply and mechanical tumor cell arrest [6, 7].

Genetic background theory

Recently, it has been proposed that metastasis propensity can be predicted from gene expression profiles characteristic of the entire primary tumor cell population, and not only representing a subpopulation of the tumor cells. Hence, the metastatic phenotype is not regarded as an acquired event during progression. Instead, this theory emphasizes that the metastastic ability could be pre-programmed in tumors as a direct effect of the initiating oncogenic mutation [8-10]. However, there are presently no evidence to suggest that the metastasis suppressors, which are associated with genetically defined regions, have any apparent molecular defects nor expression level differences between the high and low metastatic genotypes [10, 11, 12].

1.2 Role of cytogenetics in cancer research

The term cytogenetics refers to the study of chromosomes and the related disease states that are caused by numerical and structural chromosomal abnormalities. These abnormalities may be found in somatic cells where they are frequently associated with a cancer phenotype. Chromosomal abnormalities found in germ-line also give rise to heritable disorders of both neoplastic and non-neoplastic types.

The history of modern genetics can be traced back to Gregor Mendel who first suggested the existence of biological elements called genes in 1865. In the 1880s Flemming and Arnold first observed the human chromosomes [13]. The correct number of human chromosomes was established in 1956 by Tjio and Levan [14], following which the human cytogenetics was founded. In 1890, David von Hansemann described the existence of mitotic aberrations in tumor cells, and was also the first scientist to observe the correlation between chromosomal aberrations and disease [15]. Soon thereafter, Theodor Boveri proposed and provided the evidences for a causative role of chromosomal aberrations in cancer [16]. The power of cytogenetic analyses became evident in the late 1960s when Torbjörn Caspersson and colleagues developed staining protocols for production of highly reproducible patterns of dark and light bands along the length of each chromosome [17]. In the meantime, the "Philadelphia chromosome" was discovered, which result from a translocation between chromosomes 9 and 22 and is a common observation in chronic myeloid leukemia (CML) [18]. Later, in 1973, Janet Rowley identified the product resulting from this specific translocation [19]. Following these initial discoveries, the cytogenetic techniques have been much improved from innovations in molecular biology, chemistry and instrumentation [13]. As a result of these molecular cytogenetic studies, extensive information has been obtained concerning the etiologies of human disease.
However, the causes and mechanisms by which chromosome translocations and rearrangements occur are still partly unknown. Although some of them might happen just by chance, specific mechanisms may also be involved such as inappropriate use of DNA recombination mechanism [20, 21].

1.2.1 Chromosomal alterations in cancer

Human cancers present a wide variety of chromosomal aberrations that may affect the chromosome content or the number or structure of single chromosomes. Alterations in the total chromosome content of a cell (ploidy) largely decrease or increase the chromosome number, most commonly in the form of a number doubling. Numerical alterations of the individual chromosomes are very frequent, as well as structural rearrangements between one or more chromosomes. Chromosomal translocations are believed to be landmarks for tumor development that could be early and necessary events for tumor progression [20, 22, 23]. Other structural aberrations of a single chromosome contribute to altered expression of tumor-suppressor genes and oncogenes following inhibition or activation, respectively. In addition, cancer genes located on the rearranged loci can achieve new functions and oncogenic capacity, which result in disturbed cell regulation, uncontrolled growth and cancer development.

At present, several specific chromosomal alterations have been revealed by cytogenetic and molecular analyses in human cancer, and which have also been linked to distinct histopathological entities. Some well-known examples in the field of soft tissue sarcoma (STS) include t(X;18)(p11;q11) in synovial sarcoma and t(12;16)(q13;p11) in myxoid liposarcoma [24, 25]. Moreover, several genes in specific regions are amplified in human STSs, and therefore characterization of genomic areas comprising DNA copy number changes is another important task. Molecular cytogenetic characterizations of cancer are today performed using a variety of novel FISH based methods such as comparative genomic hybridization (CGH), spectral karyotyping (SKY), and multicolor FISH (mFISH) [26].

The genetic instability known to be present in human cancer is observed at two principal levels. In most tumors, the instability is usually found at the chromosomal level, which result in variations of chromosome number and structure. Besides the chromosome instability (CIN), many tumors also exhibit instability at the nucleotide level. This may result in base substitutions, and other discrete alterations [27]. All these aberrations can be regarded as secondary to the underlying defect giving the genetic

instability. Nevertheless, when the chromosomal and discrete genetic changes have occurred, they can lead to dysregulation or different function of the genes affected.

Variations in chromosome number

The number of chromosome sets is called the ploidy. A normal human individual has two sets of chromosomes in each somatic cell. Each set contains one sex chromosome and 22 different autosomes, giving a total of 46 chromosomes in a normal diploid cell ($2n$). In contrast, cancer cells frequently carry extra sets of chromosomes as compared to the normal (euploid) state and are then called polyploid. Aneuploidy is a more general term referring to all situations with gain or loss of individual chromosomes from the normal set of 46. In addition to ploidy changes, numerical alterations of single chromosomes may be observed resulting in monosomy (1 copy), trisomy (3 copies), tetrasomy (4 copies) and so on. Table 1 shows the chromosomal constitutions in a normal diploid organism with two chromosomes (labelled a and b) in the basic set together with common numerical aberrations.

Table 1. Chromosomal constitutions in a normally diploid organism with two chromosomes in the basic set.

Term	Designation	Constitution	Number of chromosomes
Monoploid	n	ab	2
Diploid	2n	aabb	4
Triploid	3n	aaabbb	6
Tetraploid	4n	aaaabbbb	8
Monosomic	2n-1	abb	3
		aab	3
Trisomic	2n+1	aaabb	5
		aabbb	5

Variations in chromosome structure

Structural chromosomal rearrangements are caused by breakages of the DNA double helices at one or more locations. This is then followed by rejoining of the broken ends and production of a novel chromosome, with subsequent expression of a chimeric gene or disruption or relocation of a normal gene. These events may in turn give either loss of a normal function, activation of a dormant function or creation of a new function. On the chromosomal level, several types of rearrangements are seen, including e.g. deletions, duplications, inversions, and translocations (Figure 2).

1.2.2 Effects of chromosomal mutations

Chromosomal alterations found in tumors have long been grouped into three categories: primary changes of importance for initiating the tumor, secondary changes that contribute to the progression, and noise that are without specific function. It is generally accepted that recurrent chromosomal aberrations affect genes that are important for tumor development. Among the aberrations, recurrent chromosomal translocations are typically detected in hematological malignancies but are also seen in sarcomas, and sometimes in solid tumors [20, 28-30]. Translocations involving proto-oncogenes can lead to tumor formation by two different mechanisms: (1) the transcriptional activation of proto-oncogenes by transposition to an active chromatin domain or a strong promoter (e.g. translocations involving *PLAG1* in pleomorphic adenomas and lipoblastomas) [31, 32], and (2) the creation of fusion genes (Figure 4). In addition to chromosome translocations, gene dosage effects are another type of recurrent aberration that plays an important role in tumor development. In many cases, regions with copy number alterations contain known oncogenes or tumor suppressor genes whose expression levels are altered by the genomic changes. Double minutes (DMs) and homogeneously staining regions (HSRs) are karyotypic abnormalities that occur through redundant replication of genomic DNA (i.e. gene amplification), which are frequently found in human cancers [33]. However, gene amplifications resulting from ring and giant rod chromosomes are more commonly revealed in sarcomas, particularly in well-differentiated liposarcomas [34-36]. Another example is described here in Paper IV. Oncogenes, such as *MDM2*[35] and *C-MYC* [37], are frequently amplified in sarcomas. Identifying the important cancer-related genes in recurrent abnormalities is not always straightforward because the amplicons often contain multiple genes and more than one may be important. For example, the chromosomal region 12q13-15 that is highly amplified in well-differentiated liposarcoma (LPS), contain several known oncogenes (*SAS, CDK4, MDM2*). Altered expression of multiple genes included in this amplicon probably contributes to the tumor phenotype. In contrast to the amplifications, deletions likely leads to the inactivation of tumor suppressor genes. As shown in the study of Paper V and in previously reported studies, loss of chromosomal region 13q14-21 occurs frequently in STSs. Several candidates within this region, such as *RB1, DLEU2, NPM1, KLF5,* and *LIG4* have been suggested [38-40].

In general, most of the aberrations found in solid tumors are regarded as noise caused by the genetic instability of the tumor cells. However, seemingly random aberrations may also be the result of selection during evolution of the tumor [41]. For example, the *C-KIT* or *PDGFRA* mutations are likely to be the initiating events in the development of GIST, while the recurrent chromosomal aberrations detected by karyotyping or CGH in the same tumors are likely to represent secondary events in tumor progression.

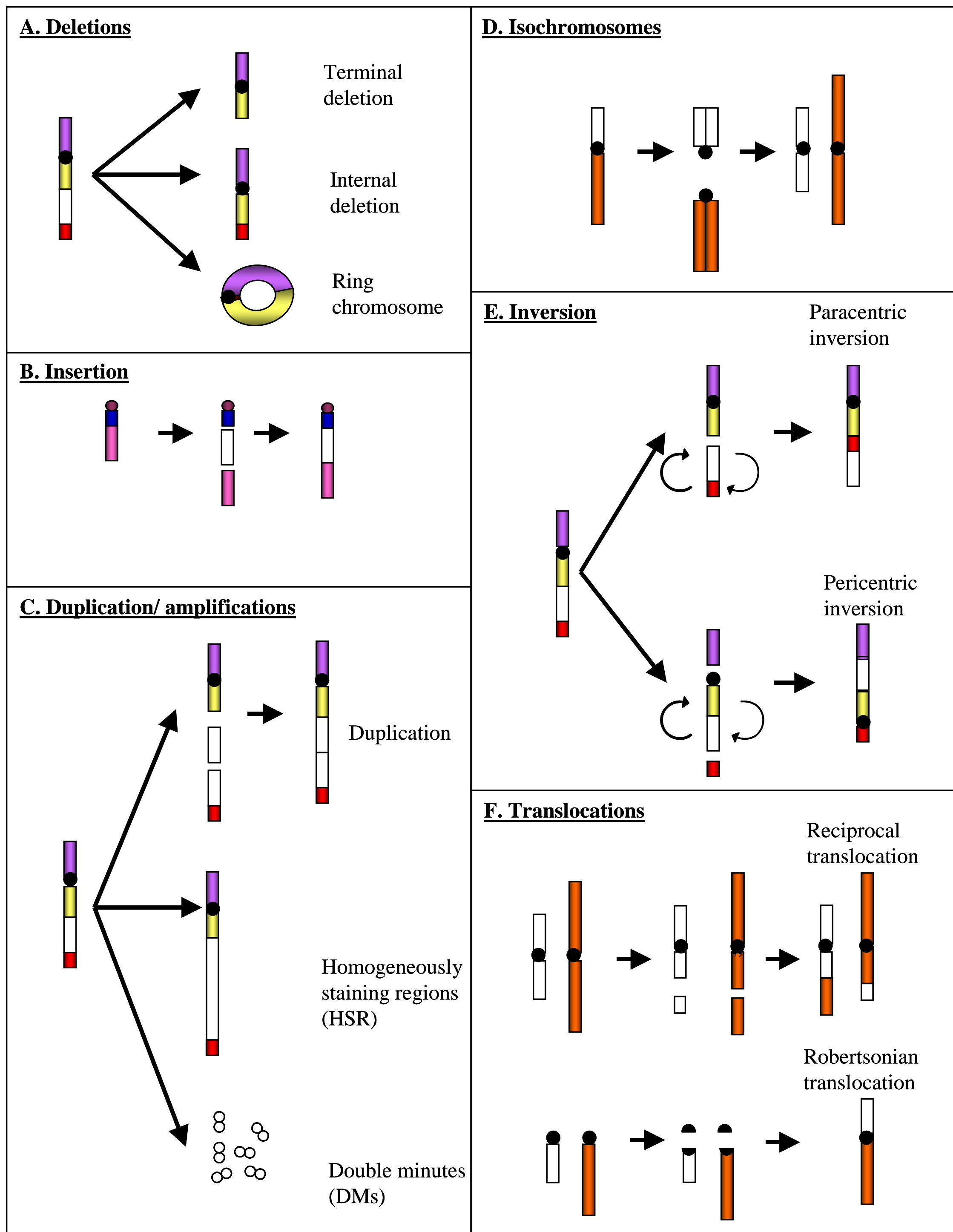

Figure 2. Different types of chromosomal aberrations seen in human cancers.

2. Genetic aspects of soft tissue sarcomas (STSs)

2.1 Natural course and genetic features of STSs

The term sarcoma originates from the Greek language and means "fleshy growth". Sarcomas are malignant tumors of mesenchymal origin that can arise almost anywhere in the body. The most common location is in the extremities (the arms, legs, hands, or feet) where about 50% of the tumors are found. Another 40% occur in the trunk (chest, back, hips, shoulders, and abdomen), and 10% occur in the head and neck. Sarcomas account for approximately 1% of all adult malignancies and 20% of pediatric malignancies [42, 43]. One of the clinical features of sarcoma is that in the early stages they do not usually cause clinical symptoms. This is related to the fact that soft tissues are relatively elastic, and the tumors can grow rather large, pushing aside normal tissue, before they are felt or cause any problems. Sarcomas generally are capable of invasive or destructive growth and the patients frequently develop recurrence and distant metastasis forming secondary tumors. Radical surgery is usually required to ensure total removal of these tumors. The natural course of sarcomas is highly variable. For example, some sarcomas such as dermatofibrosarcoma protuberans rarely metastasise, while other types such as malignant fibrous histiocytoma (MFH) do so with alacrity. Because of the general aggressiveness of STSs and the frequent use of extensive surgery, development of prognostic and diagnostic markers is advocated. Until today, parameters such as tumor size, location, necrosis, intra-vascular invasion, histopathological malignancy grade, and the treatment of the tumor have been shown to have prognostic value [44-46].

The histopathological classification is a challenging task. More than 50 subtypes of proliferative soft tissue lesions are presently defined [43, 47, 48]. Until today, the most common types of STS diagnosed have been malignant fibrous histiocytoma (MFH) and liposarcoma (LPS) that together account for 35% to 45% of all sarcomas [49, 50]. The classification of STSs has not stayed constant over the years, but is regularly re-evaluated and re-formulated. For example, the MFH entity that was introduced forty-two years ago by Ozzello *et al* [51] has recently been challenged to its mere existence

by Fletcher and co-workers [52]. Similarly, the gastrointestinal stromal tumor (GIST) entity was introduced by Stout [53] and Martin et al. in early 1960s [54] and today several abdominal STSs are classified as GIST that would have been diagnosed as e.g. leiomyosarcoma a few years earlier [55]. Nevertheless, many of the tumors still lack clear-cut diagnostic foundation, especially when the tumors exhibit an undifferentiated morphology. Therefore, new specific molecular genetic markers are expected to become increasingly useful in the clinical evaluations of STSs.

STS tumors constitute a highly heterogenous group of tumors, for which genetic characterization is still limited. Therefore, the aims of this thesis has been to extend the knowledge of genetic and molecular alterations involved in the progression and metastasis formation of this tumor group.

2.2 Chromosomal events in STSs

The value of cytogenetic and molecular analyses is already well established in basic and clinical investigation of hematological malignancies, and specific genetic alterations are used in clinical practice for diagnostic, prognostic and therapeutic purposes. A similar situation is presently evolving for sarcomas, where several specific chromosomal alterations, mostly reciprocal translocations, have been associated with distinct histopathological entities. In some situations, different oncogenic fusion genes are associated with a single type of cancer, while in other situations one gene can fuse to different partner genes, resulting in distinct neoplastic phenotypes.

2.2.1 Specific translocations result in fusion genes in STSs

On the genomic level fusion genes commonly result from breakage within introns of the two partner genes whereby exons with the same 5´- 3´ orientation are joined in frame, enabling the translation of a functional new protein from the resulting fusion transcript. A series of specific translocations and fusion genes have already been reported and associated with certain STS subtypes (Figure 4). For example, a translocation t(12;16)(q13;p11) is found in more than 90% of myxoid LPS. Through this rearrangement the fusion gene *FUS-CHOP* is created and expressed specifically in the tumor cells. Although the same genes are involved in each case, variations on the base-pair level are frequently seen which are mainly attributed to varying

breakpoints in the FUS (TLS) gene (Figure 3) [56-59]. Furthermore, myxoid LPS without a typical t(12;16)(q13;p11) may instead carry a *EWS-CHOP* fusion gene resulting from a t(12;22)(q13;q12). However, both these fusions lead to the same sarcoma subtype displaying indistinguishable histopathological features [60]. Altogether at least 25 fusion oncogenes have been described in STSs (Figure 4). Most recently, a novel fusion gene *FUS-BBF2H7* resulting from t(7;16)(q33;p11) was found in low grade fibromyxoid sarcoma [61]. Notably, it is common that the same partnergene is involved in different fusions, which can each be associated with different tumor phenotypes. For example, the *EWS* gene has been found fused to one of nine partner genes, giving rise to five different types of STS (Figure 4) [62-70]. Some of the *EWS*-fusions are associated with very aggressive clinical tumor progression, e.g. desmoplastic small round cell tumor [69]. The *FUS* gene is reported to be involved in three different fusion genes [61, 71, 72], that are each associated with a different type of STS.

Obviously, these specific fusion genes and their phenotypes are tightly linked with each other, but the exact mechanism behind the specificity is still unclear. The expected pathogenic importance of the fusion genes is supported by the observations of chromosome translocations as the sole cytogenetic anomaly in a significant proportion of STSs (Figure 4). However, in several instances it still needs to be established whether STS fusion genes represent the first tumor initiating events or are proceded by other events not detectable on the chromosome level.

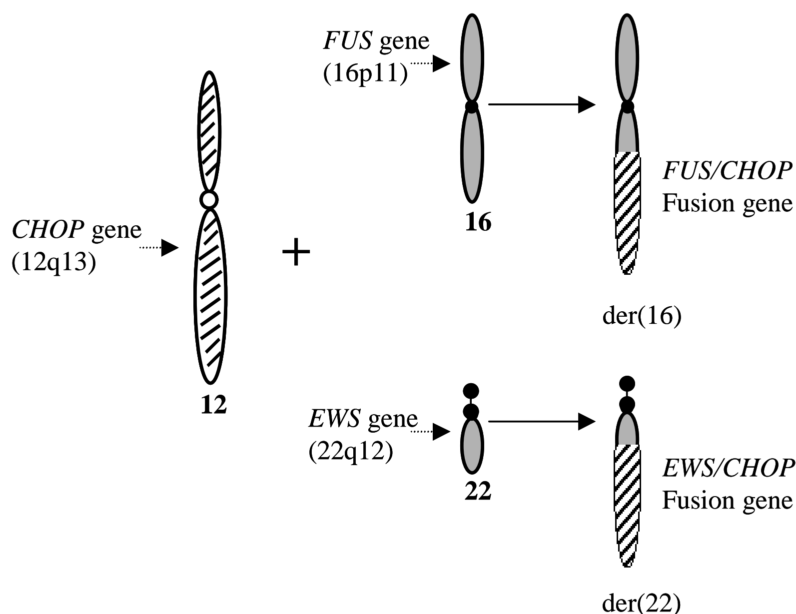

Figure 3. Schematic illustration of the translocations t(12;16) and t(12;22) in myxoid LPS.

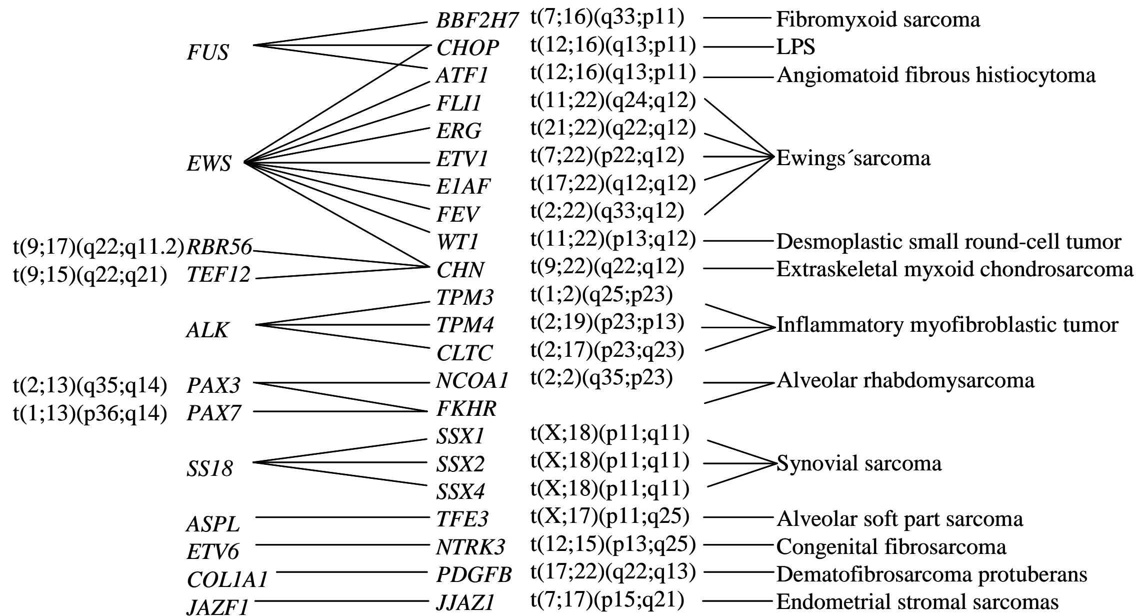

Figure 4. Chromosomal translocations frequently observed in STSs.

2.2.2 Amplicons are common findings in STSs

In addition to chromosome translocations, other recurrent aberrations are also found in STSs such as double minutes, ring chromosomes and giant rod chromosomes. These alterations are non-randomly distributed and commonly involve amplifications and over-expression of genes in the target regions. Ring or giant rod marker chromosomes with amplification of 12q13-15, play a key role in lipomatous tumor development [73, 74]. Well-differentiated LPS frequently involve several genes known to be amplified in human STSs, e.g. *MDM2*, *SAS*, *GLI*, *CHOP* and *CDK4* in the chromosomal region 12q13-15 [35, 75]. Recently, Tallini et al. showed that the *HMGA2* gene is commonly over-expressed in well-differentiated LPS with ring or rod chromosomes and amplification of 12q13-15 [76].

The region 1q21-23 is also commonly involved in amplifications, and includes the *COAS2* gene as reported by Nilsson et al. [77]. By FISH, the most common localization of extra *COAS2* signals in lipomatous tumors was demonstrated to be in supernumerary ring and giant marker chromosomes [73, 77].

In malignant fibrous histiocytoma (MFH), the *MASL1* gene has been suggested to be the oncogenic event driving the amplifications of the chromosome region 8p23.1 [78].

2.3 Mutational events in STSs

2.3.1 Mutation of the C-KIT and PDGFRA genes in GIST

In addition to specific alterations that can be revealed at the cytogenetic level, STSs also demonstrate recurrent genetic alterations of more discrete types. A good example is provided by the gastrointestinal stromal tumors (GISTs) that show mutations and/or over-expression of the *C-KIT* and *PDGFRA* genes. GISTs are the most common mesenchymal tumors of the gastrointestinal tract, representing approximately 20-30% of all STS in this location. The majority of GISTs exhibit mutations in *C-KIT* that cluster in four hot spot exons (9, 11, 13 and 17) and are especially frequent in exon 11. In GIST, the *C-KIT* mutations regularly alter or delete one or more amino acids, but are always in frame. This then leads to changes in the juxtamembrane domain of the c-kit protein and tyrosine kinase activation without binding of the stem cell factor (SCF) ligand. The resulting constitutive expression of c-kit in turn results in altered cell proliferation and tumorigenesis (Figure 5) [79]. GISTs with *C-KIT* mutation are more likely to be of high malignancy grade, and are characterized by more frequent recurrencies and a higher mortality rate than tumors with wild-type *C-KIT* only [80]. GIST tumors with a *C-KIT* mutation are also responsive to treatment with Imatinib, a drug that inhibits the c-kit tyrosine kinase, and which is applied to patients with inoperable or metastatic disease. However, acquired resistance to Imatinib may develop after a period of treatment. Additional mutation of *C-KIT* is one possible explanation for the observed resistance. Most recently, Heinrich and co-workers found that approximately 35% of GISTs lacking C-*KIT* mutations carried activating mutations in the related receptor tyrosine kinase gene, platelet-derived growth factor receptor alpha (*PDGFRA*) [79]. Tumors expressing *C-KIT* or *PDGFRA* oncoproteins were found to be indistinguishable with respect to activation of downstream signaling intermediates and cytogenetic changes associated with tumor progression. Therefore *C-KIT* and *PDGFRA* mutations appear to be alternative and mutually exclusive oncogenic mechanisms in GISTs [79]. There are two hot spot exons for *PDGFRA* mutations, i.e. exons 12 and 18. Taken together both *C-KIT* and *PDGFRA* mutations contribute to more than 80% of GISTs [79].

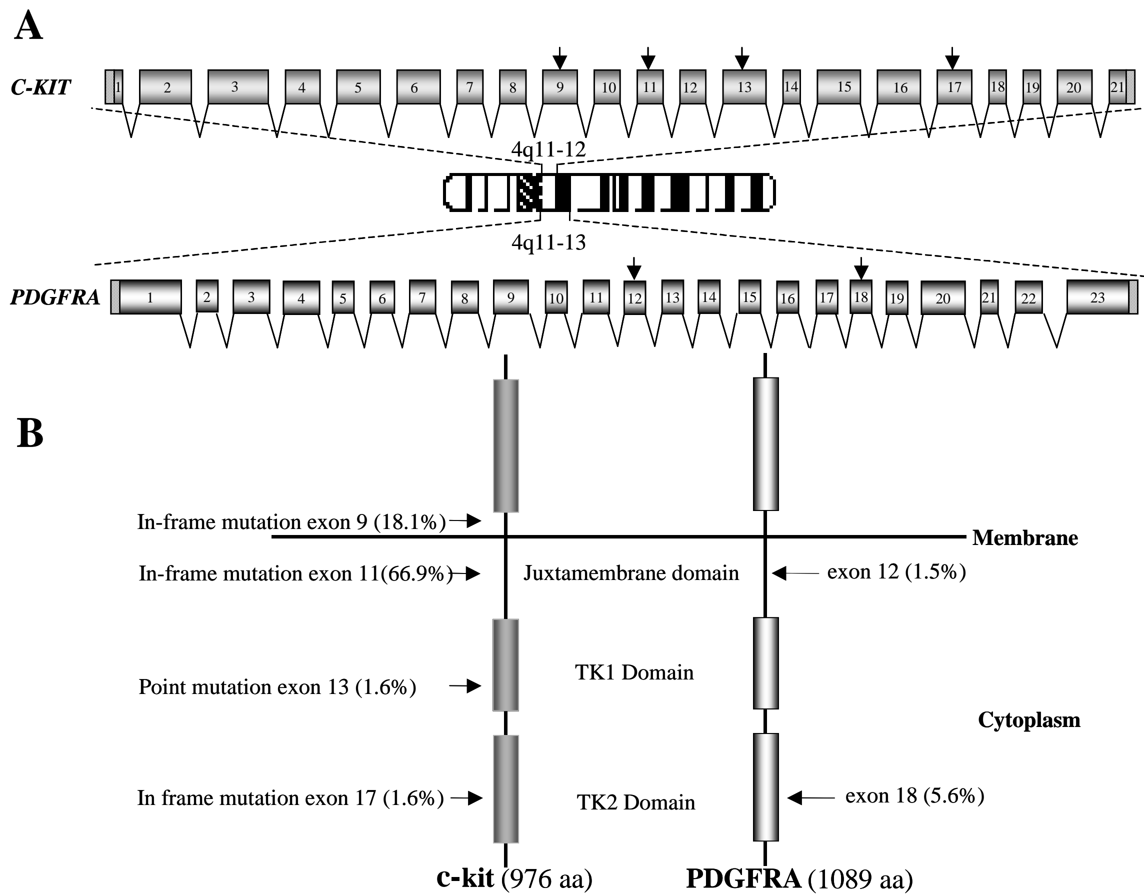

Figure 5. A) Schematic illustration of the *C-KIT* and *PDGFRA* genes located in chromosome 4q, and their gene structure. Arrows show the mutated exons reported so far in the litterature. B) The c-kit and PDGFRA proteins and the locations of frequent mutations.

3. Prognostic aspects of soft tissue sarcomas

3.1 Histopathological markers

Clinical and histopathological markers of documented prognostic value include e.g. malignancy grade (high grade III or IV), tumor size (>8 cm or >11 cm), tumor depth (deep location), tumor localization and surgical margin. Presence of necrosis and high mitotic count are similarly established markers. [44, 83-87]

As shown by us and others one of the best parameters is tumor size, [44, 88, 89], which in turn is also related to the location of the tumor. For example, STSs located in the distal extremities are often small and superficial when diagnosed. On the other hand, the prognosis is usually better compared to the tumors located intra-abdominally [89], where the tumors are usually rather large already at the initial diagnosis.

The value of malignancy grade as prognostic variable for STSs has been reported by many groups [86]. The features that define the grade are strongly linked with the degree of cellularity, differentiation, necrosis as well as the number of mitosis, that may also on their own be of prognostic value.

3.2 Immunohistochemical markers

Vascular invasion, metastasis and local recurrence are features of an aggressive tumor phenotype. Many immunohiostochemical markers studied are therefore chosen to reflect the three cornerstones of tumor growth, i.e. cell proliferation, apoptosis, and angiogenesis. For example, factor VIII measures vessel density, Ki-67 is a marker of proliferation, and the p53, p27 and Bcl2 proteins are all related to the regulation of the cell cycle and hence linked to apoptosis. Increased expression of IGF1-R was seen in some malignancies in cases with metastatic disease [90-93], while in high grade STS the IGF-1R expression was associated with favourable outcome [87, 94]. Furthermore, expression of CD44 and ezrin are associated with cell adhesion, and related to the cell migration and metastasis.

3.2.1 Over-expression of ezrin is related to poor outcome

The tumor phenotypes that will usually lead to metastastic behaviour include the capacity of tumor cells to migrate within tissues, transmigrate through vessels and to adhere to the metastatic organs [4, 5]. Since metastasis are the main cause of death in cancer the identification of genes that regulate tumor cells migration may therefore lead to improved therapeutic strategies.

Recently, ezrin was identified as a key component in the metastasis of tumors as reported by several authors [95-97]. In general, the role of ezrin in tumor metastasis was based on two of the major reasons: A) ezrin is best known to connect membrane proteins to the actin cytoskeleton [98, 99], through the adhesion molecules that are known to depend on the ezrin-mediated linkage to actin, such as CD44, and are directly related to the invasion and metastasis of tumors [96, 98, 100, 101]; B) The ability of ezrin to confer metastatic capabilities to tumors, which has been proved in experimental models, for example, mouse model of osteosarcoma and osteosarcoma in dogs [96].

The over-expression of ezrin in malignant tumors and its relation to poor outcome have been reported in carcinomas, such as, prostate cancer, glioma and melanoma [102-105]. Concerning mesenchymal tumors, involvement of ezrin has been discussed for gastrointestinal stromal tumors [106], osteosarcoma [96, 107], and rhabdomyosarcoma [97]. In this thesis the impact of ezrin expression as a prognostic marker in STS was further evaluated.

3.3 Genetic markers

Genetic markers represent a field of increasing importance in STS diagnostics and prognostics. In general, the oncogenes, which can induce malignant transformation and cell proliferation, have been implicated in the development of STSs. In the majority of cases, oncogene activation result from chromosomal rearrangements or gene amplifications. Changes of the microenvironment of the gene, for example following epigenetic modifications, must also be considered. Examples of oncogenes linked to STSs (Figure 4) are *C-KIT* and *PDGFRA* mutations in GIST, and fusion oncogenes such as *SS18-SSX* in 90% of synovial sarcoma. In contrast, the tumor suppressor genes (TSG) play a critical role in cell growth and dictate the cell program

program to apoptosis. In contrast to oncogene activation, loss or change of the TSG function commonly result from deletions or discrete mutations. Two major TSGs that are relevant to STS are the *RB1* and the *TP53* genes. Approximately 30%-60% of STS have been reported to harbour aberrations of the *TP53* gene, including a subset of patients with germ-line mutations i.e. the Li-Fraumeni syndrome [108, 109].

Table 2. Prognostic markers in soft tissue sarcoma

Parameter		
Clinical and Histopathological		
tumor size		
malignancy grade		
high mitotic rate		
tumor necrosis		
surgical margin		
tumor depth		
proliferation		
growth pattern		
vascular invasion		
metastasis		
local recurrence		
Immunohistochemical	**Targets of detection**	
Factor VIII	vascular density	
CD44	adhesion protein	
ezrin	adhesion protein	
Ki-67	nuclear proliferation marker	
cyclin A	proliferation	
IGF-1R	proliferation, differentiation	
bcl-2	oncoprotein, anti-apoptosis	
p53	cell growth, differentiation	
p27	regular of G1-S transition of the cell cycle	
Pgp	multidrug resistance gene product	
MDR1	multidrug resistance gene product	
MRP1	multidrug resistance gene product	
Genetic	**Tumor type**	**Genes involved**
mutation of *C-KIT*	GIST	*C-KIT*
mutation of *PDGFRA*	GIST	*PDGFRA*
Gain of 12q13-15	LPS	*HHMGA2*
Gain of 1q21-q23	LPS	*COAS*
Gain of 8p23.1	MFH	*MASL1*
Gain of 17q	MFH	
fusion genes	several STSs	fusion oncogene (Figure 4)
DNA ploidy	several STSs	

4. Aims of the study

The overall goal of this study was to determine specific genetic alterations involved in the development or progression of soft tissue sarcomas, with the aims to identify specific genetic events associated with clinical outcome, drug resistance and tumor progression.

Specifically, we proposed to:

1. Determine the specific recurrent DNA copy number changes in MFHs that might be related to the clinical outcome of this disease.

2. Investigate the prognostic impact of ezrin immunohistochemical expression in primary highly malignant STSs.

3. Identify the genetic alterations associated with the development of IGF-1R inhibitor resistance.

4. Identify the genetic compositions of giant rod chromosome markers in a case of MFH.

5. Assess the genetic alterations in intra-abdominal sarcomas in relation to the tumor development or progression.

6. Determine the genetic changes in a child with bifocal synovial sarcomas.

5. Materials and methods

5.1 Patients and samples

In this thesis, a series of 92 patients diagnosed with soft tissue sarcomas were included. All tumor samples were collected at the Karolinska University Hospital-Solna, except a single case with bifocal synovial sarcomas that was recruited from University of Kiel, Germany. Of these, 65 cases were selected from all patients treated for primary soft tissue sarcoma of high malignancy grade, whereas 26 patients were diagnosed with malignant sarcomas in the abdominal cavity. The first group of patients was collected for the identification and evaluation of prognostic markers (Papers I and II), whereas the latter group was used to identify the genetic events in relation to the tumor development or progression (Paper IV and V). In addition, two established cancer cell lines and their resistance derivatives were genetically characterized for the identification of genetic alterations associated with the development of IGF-1R inhibitor resistance (Paper III).

5.1.1 Primary highly malignant soft tissue sarcomas (Papers I and II)

A total of 65 primary soft tissue sarcomas with high malignancy grade (i.e. III or IV) were collected from patients operated for primary STSs during 1986 to 1993 at the Orthopaedics clinic of the Karolinska University Hospital [87]. All patients were retrospectively followed up from the time of surgery until October 2001 or until the patient's death, whereby survival and the occurrence of metastasis and/or local recurrence were recorded. None of the patients had received preoperative or postoperative chemotherapy or radiation treatments, and all were without local or distance metastases at the time of the initial surgery.

The 65 patients include 32 men (49%) and 33 women (51%) with a mean age at diagnosis of 61 years (range 20-82 years). The primary tumors were located in the upper extremities (n=8), in the lower extremities (n=42), in the pelvic area (n=7), or in the trunk or abdominal wall (n=8). The histopathological diagnoses were re-evaluated, the classification followed established histopathological criteria, and

malignancy grading was according to a four-graded scale [110]. The material consisted of the following entities: malignant fibrous histiocytoma (MFH; n=49), liposarcoma (LPS; n=7), malignant peripheral nerve sheath tumor (MPNST; n=2), synovial sarcoma (SS; n=1), fibrosarcoma (FBS; n=1), alveolar soft tissue sarcoma (ASTS; n=2), mesenchymoma (MNS; n=1), angiosarcoma (AGS; n=1), and high grade neurofibrosarcoma (NOS; n=1).

Of the 49 MFHs, frozen tumor samples were available from 39 cases, which were included for CGH analyses (Paper I). For these cases, the diagnosis of MFH was based on: 1) exclusion of other types of sarcomas; 2) demonstration of typical heterogeneous morphology with myxoid, pleomorphic or storiform growth pattern or the presence of giant cells; and 3) supportive results from immunostainings when necessary. A total of 50 cases including 24 MFHs from the CGH study, an additional 10 MFHs and 16 cases of different sarcoma entities were evaluated for ezrin expression by immunohistochemistry (Paper II).

5.1.2 Intra-abdominal STSs (Papers IV and *V)*

A total of 32 intra-abdominal malignant STSs from 26 different patients were included in Papers IV and V. Two to three different tumors were obtained from subsequent surgeries from four of the patients and two samples from opposite sides of the same tumor were taken from one patient (3 and 15; 6 and 26; 12 and 17; 24 and 27; 25, 30 and 31). None of the patients had received preoperative irradiation or chemotherapy. The tumors included were evaluated by routine histopathology, and when necessary supported by immunohistochemistry. The tumors had thus been diagnosed as leiomyosarcoma (LMS; n=11), liposarcoma (LPS; n=7), gastrointestinal stromal tumors (GIST; n=10), malignant fibrous histiocytoma (MFH; n=2), fibrosarcoma (FBS n=1), or unclassified sarcoma (n=1).

Tumor 18 (from Paper V) was also characterized comprehensively both regarding histopathology and using multiple genetic approaches in Paper IV. For this purpose the diagnosis of MFH was reviewed and substantiated with additional immunostaining markers. Tumor 18 thus showed a varied morphology from cellular areas of spindle cells, sometimes in storiform pattern, to less cellular areas of shorter cells with oval nuclei. Some larger cells with multiple or multilobulated nuclei were

seen, and single fat cells were noted in a few areas. The tumor was positive for vimentin, smooth muscle actin, desmin and CD34, but negative for S-100, CD68, and CD117. Approximately 20%–25% of the cells expressed the proliferation marker Ki-67. Based on these findings, the tumor was diagnosed as MFH, of malignancy grade III on a IV-tiered scale. The lack of lipoblasts and areas of well-differentiated LPS excluded the differential diagnosis of dedifferentiated LPS.

5.1.3 Synovial sarcoma (Paper VI)

Synovial sarcomas are highly aggressive mesenchymal tumors with distinct morphological, clinical and genetic characteristics. The two main histopathological subtypes are composed of spindle cells only (monophasic type) or in combination with epitheloid cells (biphasic type) [110]. The development of synovial sarcoma is strongly linked to the translocation t(X;18)(p11.2;q11.2), through which the SS18 gene on chromosome 18 is fused with a member of the SSX gene family on the X chromosome. Synovial sarcomas of biphasic type almost exclusively express a SS18-SSX1 fusion transcript, while in the monophasic type SS18-SSX1 and SS18-SSX2 are equally frequent [111].

The unusual patient presented in Paper VI was diagnosed with synchronous appearance of synovial sarcoma in both feet. No other disease manifestations were detected at diagnosis or follow-up, and the family and personal history of neoplasia was negative. Histopathological examination showed that both lesions were highly malignant synovial sarcomas with typical biphasic morphology. The diagnosis was further supported by immunohistochemistry, demonstrating positivity for vimentin (a mesenchymal marker), and the pancytokeratin markers MNF116 and KL1 (epithelial markers). The unusual features of this patient included multifocality and atypical location of synovial sarcoma at a young age, which prompted us to investigate the genetic changes in these tumors. Therefore, we screened for SS18-SSX fusion genes in both tumors obtained from paraffin-embedded specimens. In addition, we also collected the peripheral blood sample for both cytogenetic and molecular studies to determine the possible genetic events at the constitutional level.

5.1.4 Cell lines (Paper III)

Several lines of evidence implicate the IGF-1R in tumor development, and many tumor types including highly malignant soft tissue sarcomas were previously shown to frequently express the IGF-1R. This has motivated the application of small molecular inhibitors of IGF-1R for cancer treatment, such as the cyclolignan PPP. In this study we aimed to establish human malignant cells with PPP resistance, and to investigate whether and how resistance is developed in PPP treated cells. Four established human cancer cell lines (BE=Line 1, DFB=Line 2, ES1=Line 3 and RD-ES=Line 4) with documented IGF-1R function and responsiveness to PPP treatment [112], were exposed to increasing concentrations of PPP for up to 80 weeks. Only two lines survived the selection process and therefore all analyses of intracellular events in relationship to resistance development were carried out only on these two cell lines (Line 2 and Line 3) and their resistance derivatives (Line 2Res and Line 3Res).

5.2 Methods

In the papers presented in this thesis, multiple cytogenetic and molecular techniques were applied. Genome-wide screenings were performed to characterize the specific DNA copy number changes in the tumor samples and cell lines using the comparative genomic hybridization method (Papers I, and III-V). G-banding, spectral karyotyping and/or fluorescence *in situ* hybdridization were applied to identify specific chromosomal rearrangements in two cell lines (Paper III), three of the tumor samples (Papers IV and V) and peripheral leukocytes (Paper VI). Short tandem repeat profiling was used to genotype the two cell lines and their resistant derivatives (Paper III). DNA sequencing analyses were performed to screen for *C-KIT* mutation in 32 intra-abdominal sarcomas (Paper V), as well as to determine the *SS18-SSX* fusion gene in the bifocal synovial sarcomas (Paper VI). The 13q deletions were further assessed by Southern analysis (Paper V). In addition, immunohistochemical staining was applied to determine the ezrin expression in 50 highly malignant STSs (Paper II).

5.2.1 Comparative genomic hybridization (CGH) (Papers I and III-V)

CGH allows positional identification of gains and losses of DNA sequences in the entire tumor genome [113]. The method involves competitive hybridization of differentially labelled total genomic DNA from appropriate control tissue and tumor DNA to normal chromosome spreads. The ratio of the fluorescence intensities generated by two different fluorochromes incorporated into tumor and control DNA, is used to differentiate chromosomal regions altered between the normal and tumor DNA (Figure 6). Chromosomal sequences present in additional copies within the tumor DNA result in higher green-to-red ratio intensity (i.e. appear more green) at the corresponding chromosomal target sequences, compared with the normal genomic content. Conversely, losses of chromosomal sequences result in lower green-to-red ratio intensity (i.e. appear more red) than in the normal control.

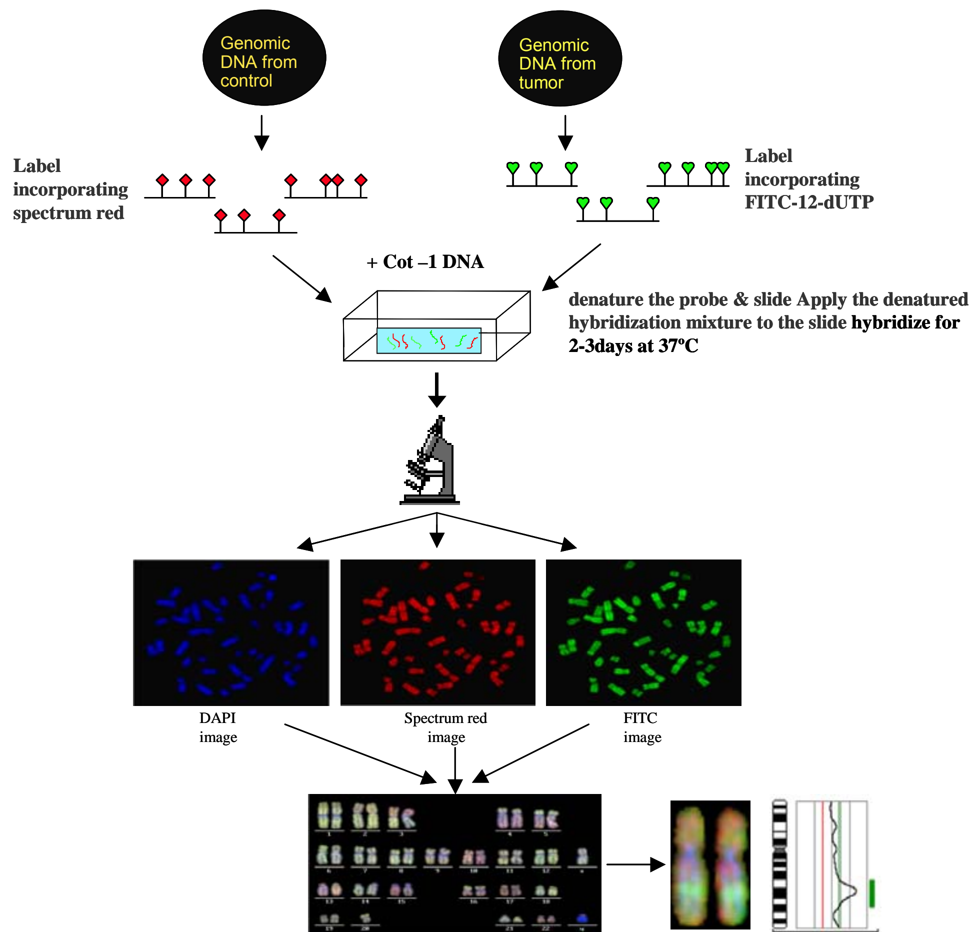

Figure 6. Comparative genomic hybridization. Differentially labelled tumor and reference DNAs are co-hybridized onto normal chromosome spreads; gains and losses are identified from the green-to-red ratio intensities. In the example (from Paper IV) shown, a high-level amplification at chromosome 6q21-23 was detected where the ratio exceeded 2.0.

This method is useful in cases with complex karyotypes containing double minutes, homogeneously staining regions, and addition of unknown genetic materials, for example, the giant rod chromosome markers from an intra-abdominal sarcoma in Paper IV. However, CGH only detects imbalances that are present in the majority of tumor cells, but is unable to detect balanced chromosomal rearrangements, such as the t(12;16) in tumor 1 in Paper V. CGH does not reliably detect gains or losses in the heterochromatic regions, the short arm of the acrocentric chromosomes, or the Y chromosome. The possibility of using genomic DNA extracted from archival material (after tissue micro-dissection and DNA amplification by universal primer polymerase

chain reaction), allows the analysis of minute subregions of tumors or even single cells, making possible the comparison of different stages of tumor progression [114]. CGH analysis has been widely used as an important research tool in the identification of genetic imbalances associated with histopathologic subtype, progression, prognosis, and clinical outcome of human malignancies [115, 116].

Here we have applied the CGH method to determine specific chromosomal imbalances in 71 STSs (Papers I, IV and V), as well as in two cell lines and their derivatives during the development of IGF-1R inhibitor resistance (Paper III). In all cases, at least 10 ratio profiles were combined to reduce noise. Ratios above 1.2 were described as gains, ratios below 0.8 were interpreted as losses, and high-level amplifications were indicated when the ratios exceeded 2.0. In the evaluation, heterochromatic regions, the short arm of the acrocentric chromosomes and Y chromosome were excluded. Similarly, the profiles were interpreted with caution for certain GC-rich regions that are known to give false positive results.

5.2.2 array-CGH (Paper IV)

In conventional CGH, comparative hybridization is applied to normal metaphase chromosomes, restricting the level of resolution to approximately 20 Mb. Within the last years, several studies developed a high-resolution CGH approach in which hybridization is performed on a matrix or microarray containing thousands of genomic DNAs (eg, from BAC or P1 clones) or cDNAs instead of metaphase chromosomes [117, 118]. This technical advances provide a locus-by-locus measure of DNA copy number changes, thereby significantly overcoming some of the limitations of metaphase CGH. The cDNA array CGH was first developed by Pollack *et al*. in 1999 [117], and subsequently several studies have applied similar approaches to detect genome-wide copy number changes in the tumor genome [119-121]. In addition to its higher resolution, cDNA array CGH has an advantage in allowing parallel analysis of DNA copy number and mRNA expression in the same sample, which may facilitate comprehensive characterizations of genetic alterations in tumors and aid in the identification of candidate genes for tumor development or progression.

In Paper IV, cDNA array-CGH was performed to identify specific copy number alterations in a MFH and the findings were compared with the imbalances detected by metaphase CGH. The procedure of this method is illustrated in Figure 7.

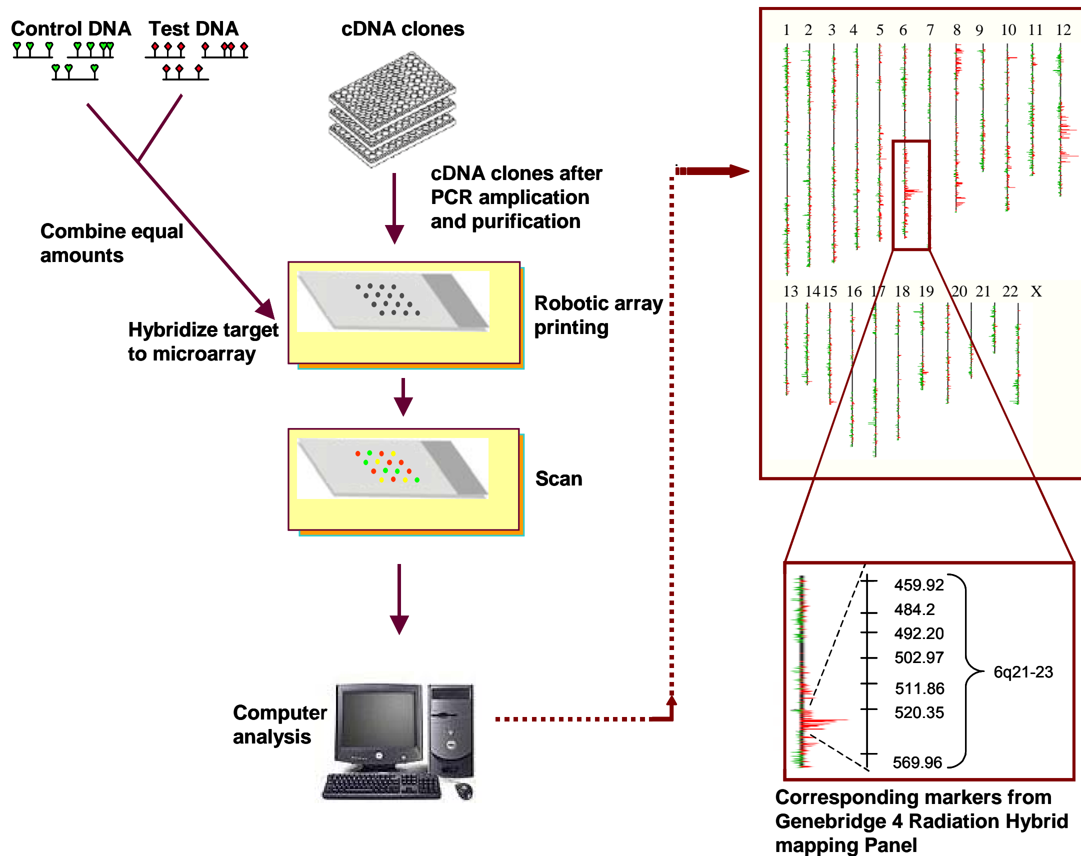

Figure 7. A schematic diagram illustrating the method of cDNA array CGH. Differentially labelled DNAs are hybridized onto defined spotted cDNAs. The resulting relative fluorescence intensities are measured by a confocal scanner. In this example (from Paper IV), a high-level amplification at the approximate location 460-570 cR of chromosome 6q21-23 was detected.

The ratio between fluorescent intensities (from Cy5 and Cy3) represents DNA copy numbers in the tumor DNA relative to the normal control DNA. DNA copy number profiles that deviated significantly >1.20 were interpreted as gains, <0.80 as deletions, and >1.50 as high-level amplifications.

5.2.3 G-banding and spectral karyotyping (SKY) (Papers III- VI)

G-banding is the most commonly used banding technique for conventional karyotyping analysis, because of its unique chromosome landmarks, simplicity, and

robustness. After trypsin treatment and Giemsa staining, unique characteristic and stable G-banding patterns can be generated along the chromosome arms. This method provides the most fundamental analysis of chromosome composition in neoplasia and is used most commonly in routine cytogenetic laboratories to assist in diagnosis, prognosis, and therapeutic evaluation of cancer cases. It therefore remains the technique of choice as the initial screening method for chromosomal abnormalities given that metaphases from cultured cells are available. However, its limited chromosome-specific banding resolution makes the recognition and interpretation of masked or cryptic chromosome aberrations difficult to ascertain. With the advances in molecular cytogenetic techniques, which are based on fluorescence *in situ* hybridization (FISH), this issue can be improved by the combination of both classical and molecular cytogenetic techniques (e.g. spectral karyotyping), such as the examples provided in Papers III- VI.

Spectral karyotyping (SKY) allows the simultaneous visualization of all human (and mouse) chromosomes in different colours because of the different (or combination of several) fluorescent dyes assigned to each pair of chromosomes [122, 123]. This technique is a powerful screening tool that has improved the detection of subtle chromosomal translocations, which may not be easily revealed by classical karyotyping methods (due to lower resolution). Chromosome-specific probe pools are generated from flow-sorted chromosomes, amplified, and fluorescently labelled by DOP-PCR with different combinations of five fluorochromes (rhodamine, Texas Red, FITC, Cy5 and Cy5.5) to create a unique spectral color for each chromosome pair. The repetitive sequences within these chromosome-specific probe pools are then suppressed by the addition of excess Cot-1 DNA before hybridization. After hybridization, images are acquired through fluorescent microscopy, CCD imaging, and Fourier spectroscopy, allowing the measurement of the entire emission spectrum at all image points with a single exposure. Dedicated software then classifies the image by identifying pixels with identical spectral. A schematic diagram illustrating the method of SKY is shown in Figure 8.

In this study, karyotyping was performed on metaphases prepared from two established cell lines and their derivatives (Paper III), short-term cultures of a MFH and two myxoid liposarcomas (Papers IV and V) and PHA-stimulated peripheral

leukocytes from a child with bifocal synovial sarcomas (VI). After G-banding, at least 10 metaphases were analyzed in each case. The clonality criteria and the description of karyotypes followed the recommendations of the International Systems for Human Cytogenetic Nomenclature, 1995 [124].

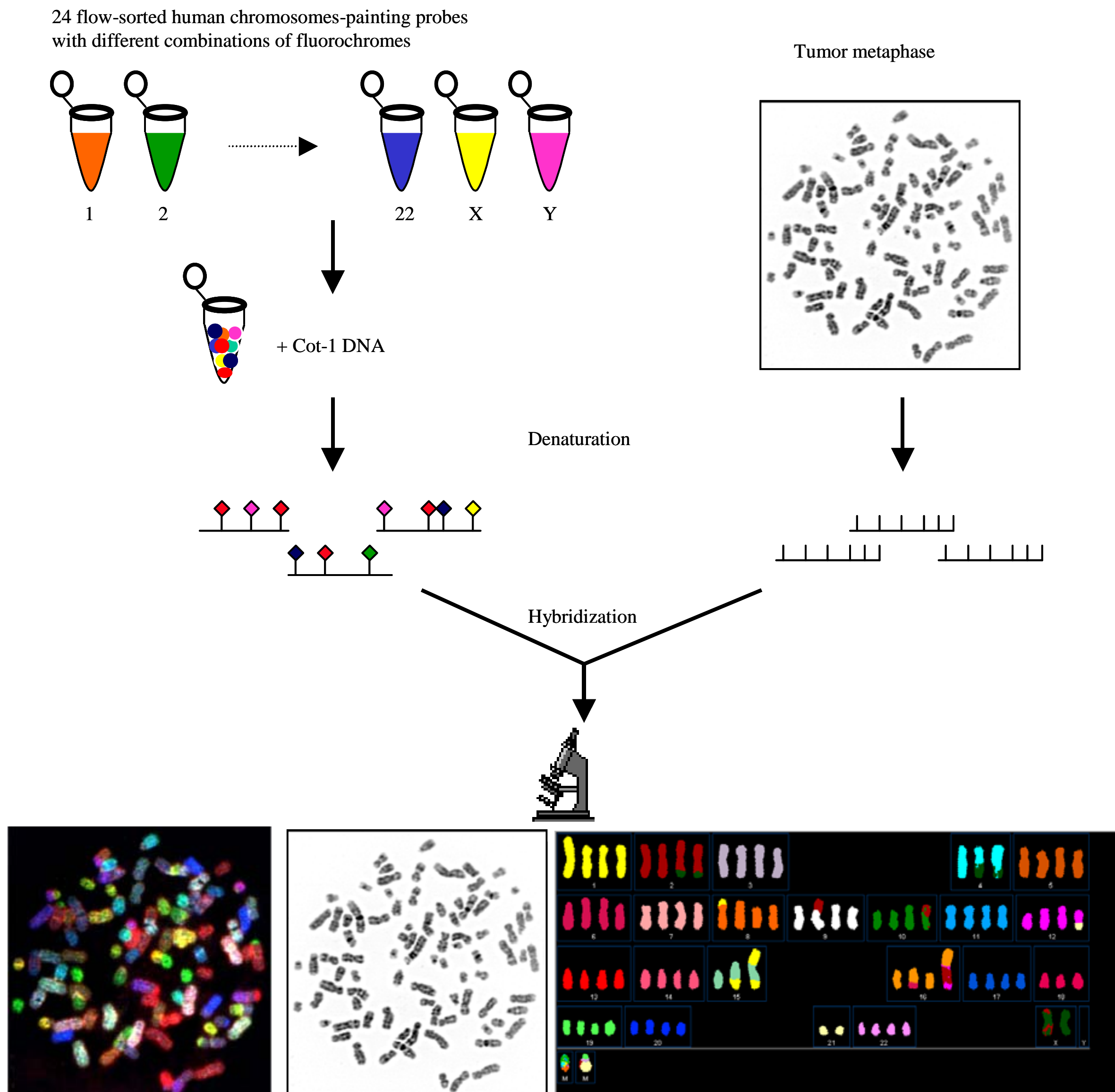

Figure 8. Spectral karyotyping (SKY). Chromosome-specific probe pools are generated from flow-sorted chromosomes, amplified and fluorescently labelled by DOP-PCR with different combinations of five fluorescent dyes (Rhodamine, Texas Red, FITC, Cy5 and Cy 5.5). After hybridization of these differentially labelled probe pools, each chromosome can be recognized based on its unique spectral signature.

5.2.4 DNA sequencing (Papers V and VI)

Cycle sequencing (or linear sequencing) is the method that exactly and directly detects the mutated nucleotide sequence in a gene. The principle of this method is

built on a thermocycling reaction, which incorporates fluorescently labelled dideoxynucleotides (ddNTP) chain terminator in the PCR reaction. The products are separated by gel electrophoresis, when the DNA passes through a fixed point in the gel, the fluorescent signals of the nucleotide sequence can be detected and recorded as chromatograms (Figure 9).

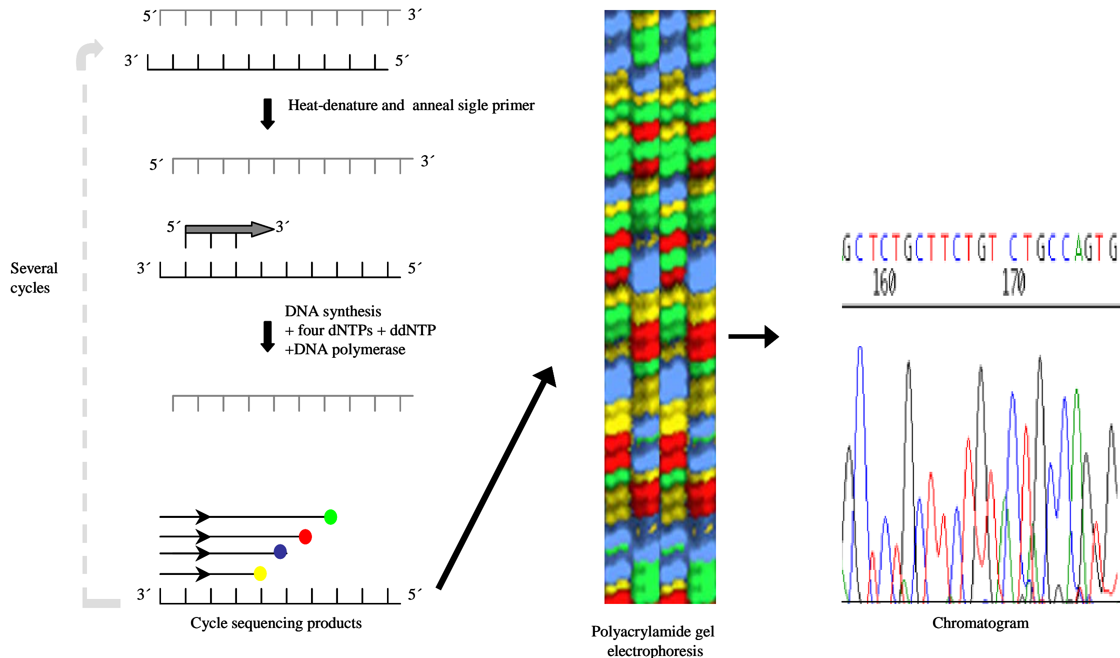

Figure 9. Schematic illustration of the principle of cycle sequencing.
The sequencing reaction is set up using a mixture of all four dNTPs plus one of the four labelled ddNTPs, and one single primer. The final result is visualized as chromatograms.

In Paper V, direct sequencing of the *C-KIT* gene (three hot-spot exons 9, 11, and 13) was performed on tumor DNA samples from 32 intra-abdominal sarcomas. In Paper VI, cDNAs from the two tumors were sequenced for *SS18-SSX2* fusion gene. The PCR products were run in cycle sequencing reactions and the products were run and analyzed in an automated sequencer. The resulting sequences were then visualized as chromatograms with alternating peaks in four different colours for each nucleotide base.

5.2.5 Southern blot analysis (Paper V)

Southern analysis is commonly used to detect the DNA copy number of a gene or nucleotide sequences. DNA from a sample is cleaved by a restriction enzyme,

separated by electrophoresis, transferred onto a nylon (or nitrocellulose) filter, and hybridized with complementary labelled single-stranded DNA. The results are analyzed by autoradiography where the hybridized fragments are detected as bands on an X-ray film.

In Paper V loss of 13q was the most frequent alteration detected by CGH, which led us to assess the deleted regions using Southern analysis. Twenty-one tumors from 19 patients were analyzed with six target probes located at different loci of chromosome 13q, as well as two control probes from the distal part of chromosome 3p (in which no alteration was detected by CGH). The ratios between the signals obtained using 13q probes and control probes were determined for each of the samples at visual inspection by three independent observers.

5.2.6 Immunohistochemistry (Paper II)

To study the specific protein expression, immunohistochemistry is an efficient, simple and low-cost technique. This method does not only allow semi-quantitative analysis of expression level of a protein, but importantly also the localization of the protein in the individual cell. To detect the presence of specific proteins in cells or tissues, the method consists of the following steps: 1) primary antibody binds to specific antigen; 2) antibody-antigen complex is bound by a secondary, enzyme - conjugated, antibody; 3) in the presence of substrate and chromogen, the enzyme forms a colored deposit at the sites of antibody-antigen binding (Figure 10).

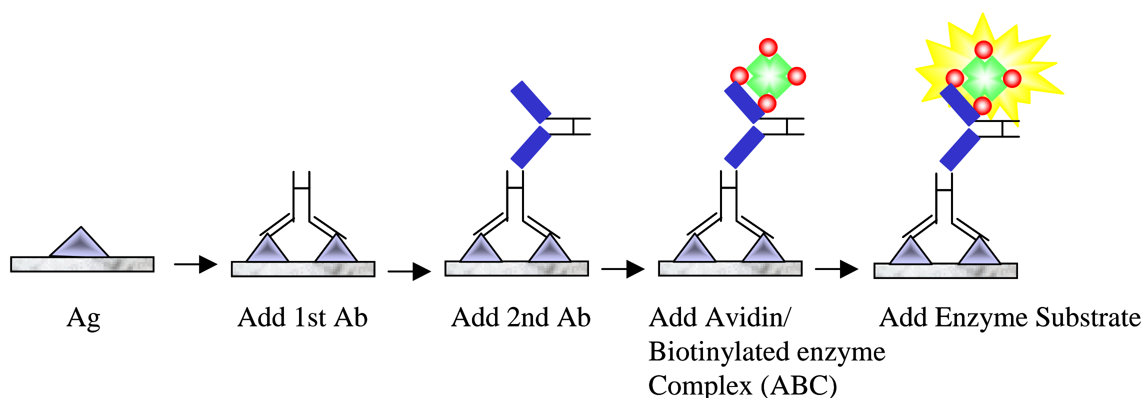

Figure 10. Major steps outlined in immunohistochemistry technique

In Paper II, paraffin-embedded sections were prepared from the primary tumors of 50 STSs. All sections were deparaffinized, rehydrated and pre-treated with citrate buffer for antigen retrieval. After quenching with hydrogen peroxidase and blocking with bovine serum albumin, the sections were stained with a monoclonal mouse antibody against ezrin, followed by detection using the ABC method, as illustrated in Figure 10. The sections were counterstained with hematoxylin. Paraffin sections from placenta collected after birth and normal mesenchymal tissues from liposarcoma patient were analyzed in parallel as positive and negative controls, respectively. The immunostaining was scored for all cases by two observers in an open discussion who were without knowledge of the clinical details. First, the ezrin expression was scored as positive or negative. Negative cases included those where no tumor cells showed cytoplasmic immunoreactivity, or where only a few single tumor cells showed immunoreactivity. All cases scored as positive showed ezrin immunoreactivity in the cytoplasm of a subset or all tumor cells. Positive cases were also evaluated concerning the proportion of positively stained cells. A semi-quantitative approach was used whereby the tumors were grouped into four classes with 1-25%; 26-50%; 51-75% or 76-100% positively stained cells. For these quantitative analyses, positive cells were only counted in areas with high proportion of tumor cell representativity, while areas with necrosis and/or lymphocyte infiltration were excluded to rule out an incorrectly high proportion of positively stained cells.

5.2.7 Short Tandem Repeat (STR) profiling analyses (Paper III)

Short tandem repeat markers are polymorphic DNA loci that contain a repeat nucleotide sequence. The STR repeat unit can be from two to seven nucleotides in length. The number of nucleotides per repeats unit is the same for a majority of repeats within an STR locus. The number of repeat unit at an STR locus may differ, so alleles of many different lengths are possible. Polymorphic STR loci are therefore very useful for human identification purposes [125]. The purpose of performing STR analyses in our study was to demonstrate the relationship between the parental lines and resistant derivatives, and at the same time provide an identification fingerprint for subsequent studies by other groups. The genomic DNA of all cell lines was isolated using a commercial DNA extraction kit. The AmpFlSTR Profiler Plus kit (Applied Biosystems, Foster City, CA) was used for DNA profiling analysis. The PCR-based kit uses primers labeled with different fluorophores (5´-FAM, JOE and NED) to

amplify nine STR markers and a gender marker in a single reaction tube. The amplicons were then analyzed on an ABI 377 DNA Automated Sequencer using GeneScan version 3.1 (Applied Biosystems).

5.2.8 Statistical analyses (Papers I and II)

To search for prognostic markers, several statistical tools were applied to evaluate different parameters in the studies. Kaplan-Meier plot is one of the most common survival tests used to evaluate the clinical outcome of the patients. This method illustrates the life table curve showing the proportion of patients free of a specific event. The criteria for this test include a certain starting-point and the time for the event of interest to occur should be recorded. In our selected samples, all cases had been followed up for at least 5 years with recording of local recurrence, metastasis, disease free survival and death. Log-rank test compares the difference between the two curves in the Kaplan-Meier plot. Cox proportional-hazards regression allows analyzing the effect of several risk factors on survival, and correlation analyses were performed with Spearman rank order test. Two-tail p-values from Chi square test were used to assess associations between categorical variables. All calculations were performed in Statistica 6.0 software or Stat View 4.02, and probabilities of less than 0.05 were accepted as significant.

6. Results and discussion

In this thesis, a total of 92 soft tissue sarcomas (STSs) as well as two malignant tumor cell lines and their derivatives were characterized using molecular and cytogenetic analyses. The sample group studied constituted of three main categories: (i) 65 primary highly malignant MFHs with a long follow-up [126]; (ii) 26 intra-abdominal STSs in which several recurrent tumors were found; (iii) two malignant tumor cell lines and their derivatives established from the development of IGF-1R inhibitor resistance. We aimed at identifying a prognostic marker(s) associated with clinical outcome in the first category and genetic alterations related to tumor progression in the second category. In the last category, we investigated the genetic alterations that may suggest the possible mechanism involved in cyclolignan PPP resistance development. In addition, we also characterized the genetics and histopathology profile of a unique case of childhood synovial sarcoma. The details are discussed in the following sections.

6.1 Identification and evaluation of prognostic markers in highly malignant STSs (Papers I and II)

6.1.1 Gain of 17q as a favourable prognostic marker in MFHs (Paper I)

Since the early 1980s, cytogenetic analyses have provided a wealth of information on the genetic constitution of some types of malignant STSs, for example, nonrandom patterns of karyotypic changes, such as the t(12;16)(q13;p11) and t(X;18)(p11;q11) in myxoid liposarcomas and synovial sarcomas, respectively, that each result in a specific clinical phenotype. However, little is known about the possible prognostic impact of acquired chromosomal rearrangements in STSs. This is especially the case for MFH, the most common type of STS [127]. MFH typically shows a clinically aggressive behavior with frequent development of distant metastases and local recurrences after surgery. Several prognostic factors have been suggested in MFH including tumor grade, size, histopathological subtype, tumor necrosis and the presence of distant metastases at the initial presentation. However, these parameters are not specific and sensitive enough to allow the identification of a patient group at

high risk of developing metastases and local recurrences. Therefore, the development of additional and objective prognostic markers would be of obvious clinical value in the treatment planning.

The cases were identified from our initial review of patients operated for a primary STS of high malignancy grade during the time period 1986-1993. In this initial series of 101 cases the prognostic impact of histopathological and immunohistochemical analyses were evaluated. A large tumor size, subcutaneous site, surgical margin, high number of mitosis (>2/HPF) and tumor necrosis were all significantly correlated to poor outcome (p=0.00007 and 0.0047, 0.026, 0.017, and 0.025; Figure 11) [44]. In addition, a series of immunohistochemical markers were evaluated, high Bcl2 expression was found to have prognostic impact (p= 0.026) [44]. In paper I we identified 65 cases diagnosed as MFH, and further proceeded to characterize the tumors for copy number alterations by CGH.

In Paper I, 39 of the high malignancy grade MFH were characterized for chromosomal alterations. DNA copy number changes detected by CGH were revealed in 37 of the 39 cases analyzed, in which at least one CGH alteration was found and the number of detected changes varied from 1 to 16 in the individual tumors. The most frequent CGH alterations, including gains of 17p, 20q, 16p, 17q, 1p31, 7q21, and 9cen-q22, and losses of 9p21-pter and 13q21-22, were then evaluated in relation to the survival and the development of metastases only or in combination with local recurrence during follow-up. Remarkably, we found that cases with four or more alterations were significantly associated with longer survival (p=0.007) and disease-free survival (p=0.018), and with lower frequency of metastases only (p=0.009) or in combination with local recurrence (p=0.036) during follow-up (Table 3). Most significantly, patients with gain of 17q in their tumors had significantly longer overall survival and disease free survival (p=0.001 and 0.004, respectively), as well as with a lower frequency of metastasis only (p=0.018), local recurrence only (p=0.011), or in combination with both (p=0.01) during follow-up (Table 3). Furthermore, gain of 17q was independent of other known prognostic factors including tumor size and mitotic index (p-values between 0.01-0.04) (data not shown). Although the findings are statistically significant, they were based on a limited number of cases, and should therefore be confirmed in a larger sample series.

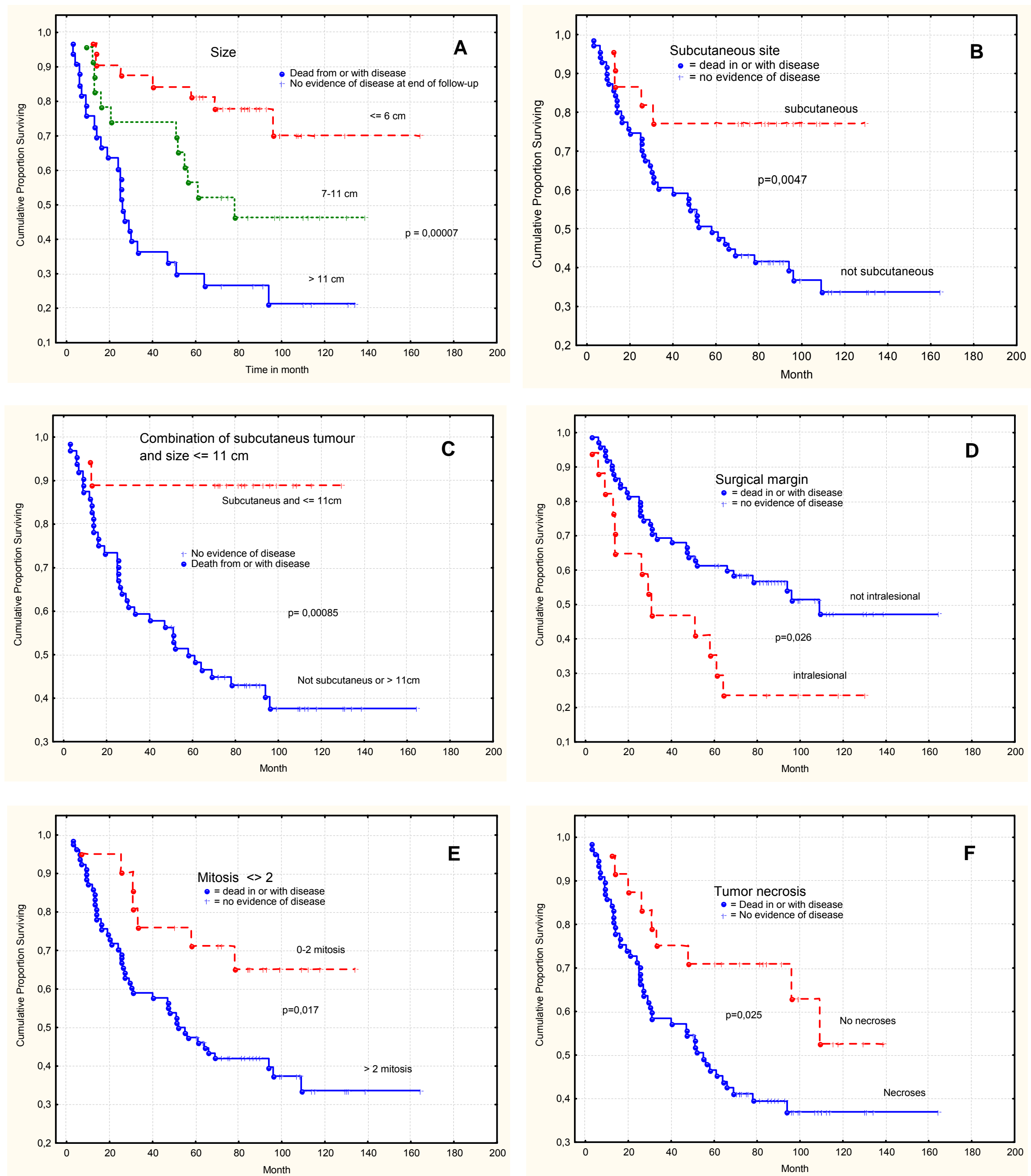

Figure 11. Kaplan-Meier plots illustrating significant association between survival and (A) tumor size; (B) tumor site; (C) combination of size and site; (D) surgical margin; (E) number of mitosis and (F) presence of necrosis in the entire series of 101 highly malignant STSs.

Table 3. Comparison between CGH alterations and clinical outcome in 39 MFH cases.

CGH alteration	Longer overall survival	Longer disease-free survival*	Lower frequency at follow-up of		
			Metastasis only*	Local recurrence only*	Metastasis + local recurrence*
Total number≥ 4	**0.007**	**0.018**	**0.009**	**0.001**	**0.036**
Gain 16p	0.10	0.06	0.11	**0.030**	**0.020**
Gain 17q	**0.001**	**0.004**	**0.018**	**0.011**	**0.010**
Gain 1p31	0.29	0.16	0.31	0.11	0.09
Gain 7q21	0.88	0.80	0.81	0.16	0.13
Gain 9cen-q22	0.91	0.34	0.13	0.45	0.38
Gain 17p	0.21	0.19	0.27	0.27	**0.048**
Gain 20q	0.16	0.11	0.13	0.43	**0.020**
Any other gain	0.14	0.07	**0.006**	0.14	**0.026**
Any loss	0.93	0.56	0.56	0.22	0.68

* The comparisons were done against patients without evidence of disease at the end of follow-up;
P-values were determined by Kaplan Meier survival test and log rank test;
Significant P-values (<0.05) are marked in bold.

Nonetheless, the findings suggest that the clinical outcome of MFH is associated with the genetic profiles of the primary tumors. We speculate that one MFH subgroup is genetically characterized by gain of 17q, and clinically by a less aggressive course second subgroup would be clinically characterized by an aggressive course with frequent development of distant metastases and local recurrences. The lack of recurrent genetic abnormality by CGH in this latter group would then imply a structural abnormality in the etiology. In further support of this hypothesis, recurrent structural alterations involving chromosomes 1, 3, 11, 17, and 19, have been reported in MFH, some of which have been associated with a high risk of developing recurrence and metastasis [128-130]. Another possibility could be that the high- and low-risk groups could have common unidentified initiating genetic events, and that the varying clinical course result from secondary chromosomal imbalances such as gain of 17q in the low-risk group. Regardless of the mechanism involved, detailed elucidation of the structural abnormalities in cultured MFH tumors would be worthwhile to proceed using a molecular cytogenetic approach. The exact identification of the putative oncogene activation driving the 17q gain is expected to be valuable for genetic dissection of the MFH entity, and in addition for the development of additional prognostic markers for clinical practice.

Since a few years ago, Fletcher and co-workers doubted whether MFH was a diagnostic entity, and they emphasized that most cases initially diagnosed as the so-called MFH could be reclassified as another type of STS, with MFH only remaining in a small number of cases[52]. Therefore it is possible that the prognostic impact of 17q gain could become relevant for STSs in general rather than being restricted to MFH. Regardless of the outcome of the ongoing MFH debate a study on the prognostic impact of 17q gain in a consecutive series of STSs would be valuable.

6.1.2 Prognostic impact of ezrin expression in STSs (Paper II)

The recent identification of ezrin as a key component in the metastasis of pediatric cancers, suggests its role in late tumor progression and metastasis. Ezrin is a member of the ERM (ezrin-radixin-moesin) cytoskeletal-associated protein family, which has been demonstrated to be involved in cell adhesion functions, interactions with the Rho-associated signal transduction, and the Akt-mediated apoptotic pathway [98 95, 131]. Alterations of ezrin expression can mediate many changes in the metastasis-associated cell surface signals and intracellular signalling cascade that confer the metastatic capacity in tumor cells. Several studies demonstrate correlations between ezrin expression levels and tumor progression in both animal models and prospective human studies [132 96, 97], consistent with a crucial role for ezrin in tumor dissemination. However, data for its prognostic impact is till limited. Therefore, we evaluated the prognostic impact of ezrin immunohistochemical expression in 50 primary high grade STSs. Half of the cases showed positive ezrin immunoreactivity in the membrane and cytoplasm of the tumor cells and in positive controls. In addition, two MFH cases scored as negative were demonstrated nuclear immunoreactivity in tumor cells. Both patients did not have any metastasis and no evidence of disease at the end of follow-up was noted.

Interestingly, our findings show that high ezrin expression was strongly associated with shorter overall survival (p=0.007) and death from or with disease (p=0.014). Patients with ezrin positive tumors were found to more frequently developed metastases during follow up. This association was statistically significant both when comparing metastasis vs. no metastasis for all patients (p=0.031), and comparing metastasis vs. no recurrent disease for the 42 patients concerned (p=0.049). In multivariate analyses, ezrin

expression was found to be an independent prognostic marker for both poor survival and metastasis development. Furthermore, the association between ezrin expression and metastasis was observed both over time and irrespective of time. Of the 25 ezrin positive cases, 17 of them developed metastasis as compared to only 9 of the 25 negative cases (p=0.023).

Using correlation analyses, several clinical, histopathologic and immunohistochemical characteristics previously evaluated in these 50 primary STSs [87] were compared with ezrin immunohistochemical expression. No significant correlations were found for the clinical variables (including sex, size, site, and depth) or for the histopathological parameters (malignancy grade, necroses and mitoses). No correlations were identified to immunohistochemical expression of Ki-67, p53, p27, Bcl-2, IGF-1R or Factor VIII. However, ezrin expression was significantly correlated to infiltrative growth pattern outside the tumor capsule (r=0.31; p=0.03).

Twenty-four of this tumor series had been previously characterized for DNA copy number changes using CGH (Paper I). Interestingly, a strong correlation between ezrin expression and copy number gain of 9cen-q22 (r=0.47; p=0.02) was observed. The alteration was previously found as a late event in genetic progression of these tumors (Paper I). However, copy number alteration of the 6q25.2-26 interval encompassing the villin 2 (ezrin) gene was rarely seen and was not correlated with expression of ezrin. Similar findings were reported for prostate cancer where no amplification or deletion of villin 2 was revealed by FISH analysis in samples with strong ezrin immunoreactivity [104]. Furthermore, high ezrin expression has been observed on the RNA level in gastrointestinal stromal cell tumors by cDNA expression array [106]. This would imply a regulatory effect, an epigenetic or an activating gene mutation as underlying the increased ezrin expression.

In conclusion, we have demonstrated a significant association between ezrin immunoreactivity in primary high grade STSs and poor outcome in terms of survival and development of metastasis. The findings thus expand the spectra of sarcomas where ezrin is related to metastasis from specific pediatric sarcomas to also include the more frequent adult STSs. The relative abundance of metastasis in ezrin positive cases was observed

both over time and irrespective of time. This suggests that ezrin has a crucial role in the tumor dissemination, and the ezrin functions are good targets for new therapy strategies.

6.2 Characterization of genetic events in relation to drug resistance, tumor development or progression (Papers III–VI)

6.2.1 Genetic changes associated with the development of IGF-1R inhibitor resistance (Paper III)

IGF-1R has been demonstrated to play important roles in tumor transformation and development [112, 133-135], as well as important for maintaining chemotherapy resistance [133, 136]. Thus, IGF-1R is a candidate therapeutic target in the treatment of cancer. Significant efforts have been invested into development of drugs that can inactivate the expression and function of IGF-1R, including the development of small molecular inhibitors of IGF-1R tyrosine kinase. Cyclolignan PPP is one of these inhibitors, which specifically inhibits phosphorylation of the IGF-1R without affecting phosphorylation of the highly homologues insulin receptor. As this inhibitor is a potential drug in cancer therapy, it is important to investigate the possible resistance mechanisms that may occur after long-term treatment. Therefore, we maintained four human cancer cell lines by increasing concentrations of PPP for up to 80 weeks. Only two cell lines survived through the selection process, and in both of them the resistance development was remarkably slow and limited. During the first 40 weeks, these lines successively developed moderate increase of IGF-1R expression, both on the mRNA and protein levels, whereafter the expression returned to normal levels. The increased IGF-1R expression was overlapped by some genetic changes detected by CGH (Figure 12). Notably, Line 2 and Line 2Res 0.5µM shared several alterations. For example, the non-resistant parental Line 2 exhibited –4, +7(++q32-qter), –9q11-13, –9p22-pter, –10p12-qter, which were either lost or less pronounced after treatment with PPP. However, the initial profile was essentially restored in the Line 2Res 0.5µM. It could be speculated that some clones of the tumor cells have been selected temporarily by PPP treatment. The parental Line 3 showed gains and losses involving five chromosomes. Some of these aberrations were retained in some or all of the resistant derivatives (Figure 11), while others were not detected after PPP treatment. On the other hand, +13q22/13-qter as well as ++11p12-q13 and –11q23-

qter were detected after treatments with PPP in all Line 3 resistant derivatives. Interestingly, gain of 11p12-q13 was found after treatment with 0.1 μM or higher concentrations of PPP. This aberration was first noted after 25-40 weeks and still remained at the end of the treatment periods. The roles of genes involved in the 11p12-q13 region remain to be investigated.

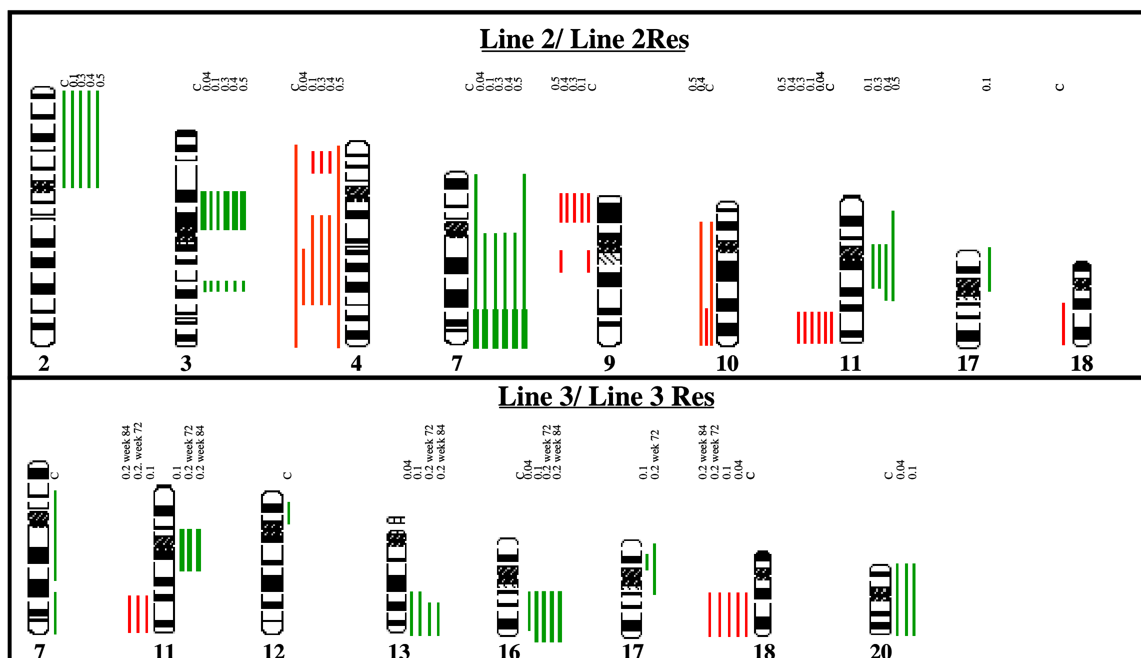

Figure 12. A summary of DNA sequence number alteration detected by CGH in Line 2, Line 3 and their resistance derivatives (indicated as C and the PPP concentrations respectively). One alteration identified in one sample is represented by one line, with losses indicated to the left and gains to the right of the ideograms. High-level amplifications of subchromosomal regions are marked with thick lines.

Among the genes residing in this region, CCND1 is the most interesting candidate. Cyclin D1 encodes the regulatory subunit of a holoenzyme that phosphorylates and inactivates the retinoblastoma protein and promotes progression through the G1-S phase of the cell cycle. Amplification or over expression of cyclin D1 plays pivotal roles in the development of a subset of human cancers including parathyroid adenoma, breast cancer, colon cancer, lymphoma, melanoma, and prostate cancer [137-142]. Interestingly, several studies have demonstrated the effect of CCND1 overexpression on drug sensitivity [143, 144]. In a human fibrosarcoma cell line model, alterations in the expression of cyclin D1 led to increase resistance in methotrexate

treatment [143]. More importantly, Kalish et al. recently showed that head and neck squamous cell carcinoma cell lines with CCND1 amplification and/or overexpression were resistant to gefitinib, which is an EGFR tyrosine kinase inhibitor [144]. The IGF-1R and EGFR are closely related members of the receptor tyrosine kinase superfamily, and a number of studies have highlighted the interactions between these two receptors. This implies that the cross-talk between the IGF and EGF receptors might play a significant role in affecting the intracellular signalling. Therefore, the roles of CCND1 in IGF signalling as well as its effect on the IGF-1R inhibitor treatment are warranted for investigations.

6.2.2 Characterization of large chromosome markers in a MFH (Paper IV)

The study performed in Paper IV was undertaken because of the identification of large chromosome markers in an intra-abdominal STS. Large chromosome markers and ring chromosomes are highly related to chromosomal aberrations that are revealed as breakage-fusion-bridge events during the anaphase of cell division [145]. However, the exact mechanism by which these special types of marker chromosomes occur is still unknown. Large chromosome and ring markers have been recurrently found in mesenchymal tumors, preferentially in low grade/well-differentiated LPSs and these cases are commonly characterized by lower metastasis rate and better prognosis [146]. In MFH, large chromosome markers and rings have only been reported in a few cases, preferentially of pleomorphic subtype. The present study supports that large chromosome markers are a recurrent observation in MFH and its chromosomal compositions were comprehensively examined in this study.

The karyotyping analyses were successfully performed on tumor metaphases of the MFH, which revealed a complex composite karyotype consisting of near tetraploid or hexaploid cells, with 3-13 large chromosome markers in addition to multiple numerical and structural aberrations. Large chromosome markers were found in all cells analyzed and each was composed of different chromosome components, although chromosomes 6 and 8 were clearly over-represented.

By combining metaphase and array CGH, we demonstrated that the most likely amplified regions in the large chromosome markers were 6q21-23, 8p21-pter and 8q24-qter. Amplification of 8p has been previously shown in MFHs using

conventional CGH, and *MASL1* in 8p23.1 has been suggested as a likely candidate gene[78]. Notably, the high-level amplification at 8q24-qter suggests the possibly involvement of the *C-MYC* oncogene. The *C-MYC* gene has been previously associated with development, differentiation, and malignancy of MFH and LPS [147-149]. Moreover, high-level amplification of 6q23 has also been described previously in MFH and LPS [150]. Intriguingly, two homologous proto-oncogenes, i.e. connective tissue growth factor (*CTGF)* and *novH*, have been mapped to 6q23.1 and 8q24.1, respectively [151]. It has been shown that rhabdomyosarcoma cells are dependent on autocrine *CTGF* for *in vitro* growth. Therefore, it would be of interest to study whether these two genes are of importance in the growth of other sarcomas, such as MFH and LPS.

In addition to the chromosomal segments included in the large chromosome markers, we also found high-level amplification at 12q13-21. The region involved includes several genes that are commonly amplified in well-differentiated LPS such as *SAS*, *MDM2*, *CDK4* and *GLI*. The *CHOP* gene located in the same interval is rarely amplified but instead translocated in myxoid LPS [25]. The finding of similar 12q amplifications in the present MFH as reported for well-differentiated LPS could implicate a relationship between these types of tumors. Indeed, from previous CGH analyses, it was suggested that the undifferentiated status of these two tumor types is closely related to the amplification of specific loci including 12q [152-154].

6.2.3 Distinct patterns of chromosomal imbalances in intra-abdominal sarcomas (Paper V)

Soft tissue sarcomas arising in the abdomen constitute a group of highly aggressive tumors, typically of very large size and with a high recurrence rate in the affected patients. While some distinct genetic etiologies have been described, the genetic background of this tumor group is not well characterized. Here, we determined gross chromosomal alterations of 32 such tumors from 26 patients by CGH analysis. This revealed copy number imbalances in 28 of the 32 tumors (88%). The most common losses were found in 1p21-22, 13q21, 14q13-24 and Xp22; while gains were mainly revealed in 9q34, 12q13, 17p, 17q and 20q13. High-level amplifications vvxczgfinvolving eight different subchromosomal regions were detected in 11 tumors from eight patients, with the most frequent sites being 12q13 and 17p. The CGH

alterations were then evaluated in relation to three aspects: (1) sex of the patient; (2) involvement of 12q13; and (3) *C-KIT* mutations.

The CGH alterations detected in the intra-abdominal sarcomas were found to vary in relation to the sex of the patient. While the most common losses (i.e. –1p21-22, –13q21 and –14q13-24) and gains (i.e. +9q34, +12q13, +17p, +17q and +20q13) were found in comparable frequencies in tumors from both men and women, loss of Xp22 was revealed in nine tumors from six patients who were all women (Table 4).

Table 4. Summary of the most common genetic alterations in relation to different variables.

Variable		Total number (%)
All STSs studied		
Loss of Xp22 in relation to sex		
	Female (n=14)	**6 (43%)**
	Male (n=12)	0 (– %)
Loss of 1p21-22 in relation to *C-KIT* mutation		
	C-KIT $mutation_{pos}$ (n=4)	**3 (75%)**
	C-KIT $mutation_{neg}$ (n=22)	4 (18%)
	Total (n=26)	7 (27%)
Loss of 13q21		
	Total (n=26)	**12 (46%)**
Gain of 20q13		
	Total (n=26)	**10 (38%)**
STSs with gain/translocation of 12q13		
Loss of 1p21-22		
	$12q13_{pos}$ (n= 8)	0 (– %)
	$12q13_{neg}$ (n=18)	**7 (41%)**
Loss of 14q13-24		
	$12q13_{pos}$ (n= 8)	0 (– %)
	$12q13_{neg}$ (n=18)	**7 (39%)**
Loss of Xp22		
	$12q13_{pos}$ (n= 8)	0 (– %)
	$12q13_{neg}$ (n=18)	**6 (33%)**
Gain of 17p		
	$12q13_{pos}$ (n= 8)	0 (– %)
	$12q13_{neg}$ (n=18)	**8 (44%)**

"n" denotes number of patients; Bold: show the higher percentage in each group.

Strikingly, two distinct groups, with or without involvement of the 12q13 region were found (Table 5). The first group includes the nine tumors (from eight patients), which showed either a t(12;16)(q13;p11) by SKY or a gain involving 12q13 by CGH. Within this group the two tumors with t(12;16)(q13;p11) had been diagnosed as myxoid LPS as expected, four cases were classified as LPS, and two cases as MFH. In contrast, none of the 23 tumors (from 18 patients) without demonstrated involvement of 12q13 had been classified as LPS or MFH. In both groups, highly variable numbers of CGH alterations were seen, varying between 0-17 in those tumors involving 12q13 (mean 5.9) and between 0-16 in those tumors without 12q13 involvement (mean 7). However, distinctly different profiles were revealed between the two groups. The common losses of 1p21-22, 14q13-24, Xp22 and gain of 17p were not seen in the tumor group with gain/translocation of 12q13, while gain of 20q13 and 9q34, and loss of 13q21 were commonly detected. High-level amplifications of different chromosomal regions were seen in the two groups. In the $12q13_{pos}$ tumors, which involved chromosomal regions of 6q, 8p and 8q in addition to 12q; while in the $12q13_{neg}$ group, high-level amplifications were found in 5q, 17p and 17q. In addition, +12q13 was detected as a single event in the $12q13_{pos}$ group. However, no CGH alteration was detected as a single event in the $12q13_{neg}$ group, suggesting that those specific common alterations (i.e. –1p21-22, –14q13-24, –Xp22 and +17p) in the $12q13_{neg}$ group do not represent tumor initiating events.

Mutations were found in exon 11 of *C-KIT* in five tumors from four patients. Several of the most common CGH alterations detected in the 32 tumors were also demonstrated in the five tumors with *C-KIT* mutation. The total number of CGH alterations varied from 0-12, however none of the *C-KIT* mutated tumors showed gain/translocation of 12q13. The *C-KIT* mutated tumors displayed –1p21-22, –13q21, –14q13-24, –Xp22, +17q and +20q13, which were also found in the *C-KIT* negative tumors. However, it can be noted that neither +9q34, +12q13 nor +17p were detected in the *C-KIT* mutated cases. Notably. High-level amplifications at 5q33-qter was found in one of the *C-KIT* mutated tumors.

Table 5. CGH results from 32 intra-abdominal STSs in relation to the total copy number changes.

Tumor no.	No of changes	Most common alterations -1p21-22	-14q13-24	-Xp22	+17p	+ 12q13	+17q	-13q21	+20q13	+9q34	Other losses	Other gains and high level amplifications
Tumors without gain/translocation of 12q13												
2	0											
3	0											
4[b]	0											
6[b]	2						17q22-qter					**5q33-qter**
7[b]	3	1p21-22										5p15-pter, 8q22-qter
8	3		14q								3q22-qter, 22q11	
9[b]	4	1p21-32						13q21-22			15q14-24	21q21-qter
13	5		14q11-24	Xp							2q22-24, 9p21-pter, 10p14-pter	
14	5				17		17		20			1p, 16, 18p
15	5	1cen-p31	14q12-24		17p-q21		17p-q21			9q	21q21	
16	6									9q		3q13-qter, 4q12-21, 5p14-pter, 5q15-qter, 7
19	7				17p-q21		17p-q21	13q	20q			12q23-qter, 14q24-qter, 15, 22
20	8				17		17	13q14-31	20q12-qter		3q12-13, 6q13-22	1p33-pter, 16p, 22q13
21	9			Xp11-pter	17p			13q14-qter			2p16-pter, 4q13-qter, 10, 11p, 12q14-21, 18q	
22	10			X	17		17			9q34	4q13-qter, 9p, 10q, 11q, 22q13	1p33-36, 16p
24	12	1p13-31	14q11-24	Xp22-q26					20	9q34	9p11-21, 15q11-25, 16q11, 18	2q22-qter, 5, 7
25	12			Xp22-pter	**17p**		17q11-22			9p11-q34	16q11-13	1p32-36, 1q41, 3p21, 3q26-28, 7p13-21,10p12, 15
26[b]	12	1p	14q13-qter	Xp22-pter			17q11-qter	13q14-qter	20p12-q13		2p, 9p21-pter, 15q15-qter, 18	1q, 16p-q22
27	14	1p13-31	14q11-24	Xp22-q26				13q21-22	20	9q33-qter	6q11-22, 9p, 15q11-25, 18	2q22-qter, 5, 7, 16p
28	15	1p21-31	14q13-qter				17q	13q14-21	20	9q	2q22-32, 4, 6q16-22, 12p, 12q15-21, 15q12	16p, 21, 22
29	15	1p13-22	14q11-24		17(**q23-qter**)		17(**q23-qter**)	13q12-22	20q		4q, 6q, 8q22-23, 11p11-14, Xq21-25	1p33-pter, 2p22-pter, 4p15-pter, 7p-q11, 22
30	16			X	**17p-q21**		**17p-q21**	13q14-qter		9q	4, 6p21-pter, 11q13-qter, 12, 16q, 18, 21q11-21	1p31-q32, 7, 14, 15, 22p-q13
31	16			X	**17p**			13q21-qter	20	9q	4, 6p21-pter, 8p, 10q21-qter, 11q13-qter, 12q14-qter, 16q, 21q	5p-q23, 14p-q21, 15
Tumors with gain/translocation of 12q13												
1[a]	0											
5	1					12q12-23(**q13-21**)						
10	4					12q13-23						7p21-qter(**p21**), 9q13-31,10q21-23
11[a]	4										7p	7q, 8, 13
12	5					12q12-23(**q13**)		13q14-qter			1q32-42	1cen-q25, 8q21
17	6					12q12-23(**q13**)		13q14-qter			1q32-42, 9q12	1cen-q25, 8q21
18	6					**12q13-21**		13q21			18q	**6q21-23, 8p21-pter, 8q24-qter**
23	10					12q(**q13-15**)	17q	13q14-31	20q	9q33-qter	2q23-34, 9p23-pter	1p34-pter, 1cen-q25, **6q23-26**
32	17					12cen-q23			20q12-qter		6p23-pter, 9p21, 9q31-33,10p15, 10cen-q22	2q11-24, 4p14-pter, 6p21, 6q21-24, 8p23, 8q21-22, 11p11-12,15q21-qter, 16p, Xp22-pter

-: loss by CGH; +: gain by CGH; high level amplications are marked in bold;[a]: carry a t(12;16)(q13;p11) as determined by SKY and G-banding; [b]: cases with *C-KIT* mutations.

The patterns of genetic alterations found in the intra-abdominal sarcomas suggested a progression of genetic events. When the most common CGH alterations were analyzed in relation to the total number of imbalances, the patterns of CGH alterations in the individual tumors were found to vary depending on the total number of detected alterations. Gain of 12q13 was the only CGH alteration that was detected as a single aberration, suggesting that it is a relatively early event. *C-KIT* mutation and t(12;16) were also each found in tumors without detectable CGH alterations, supporting their roles as initiating or early events in the tumor development. The other frequent aberrations, such as –1p, –13q, –14q, –Xp, +9q, +17p, +17q and +20q were only seen in tumors with at least three or more aberrations, suggesting that they are acquired during progression.

Loss of 13q was the most frequent CGH alteration detected in the whole series. This finding prompted an independent assessment of the deleted regions by Southern analysis for: (i) verifying the 13q loss detected by CGH and; (ii) refining the target deleted region. Twenty-one tumors from 19 patients were analyzed with six probes located at different loci of chromosome 13q [126]. Homozygous loss of one or more markers was found in 10 of the 21 tumors. In six tumors, all six 13q markers were lost, while in the other four tumors, some markers were lost and others were retained, thus permitting further localization of the target regions at D13S25 locus in 13q14.3-21.1 and the *LIG4* locus in 13q34. The target areas for 13q losses assigned by CGH were clustered at 13q21, which overlaps with the D13S25 locus mapped by Southern blot analyses. However, the *LIG4* locus represents a novel finding from the Southern analyses.

Loss of 13q was only found in tumors with four or more CGH aberrations, and when different tumors were compared from the same patients; –13q was found as the acquired event in the subsequent relapse in three out of four patients. This supports –13q is acquired during tumor progression. Furthermore, tumors with –13q14-21 were found in both *C-KIT* mutated and *C-KIT* negative tumors, as well as in both the $12q13_{pos}$ and $12q13_{neg}$ groups. Tumors from both men and women showed –13q, and the sarcoma subtypes LPS, MFH, GIST and LMS were all represented. Hence loss of 13q appears to be a common alteration in intra-abdominal STSs in general.

Several CGH studies have been previously published for STSs. We selected the most commonly altered regions in soft tissue sarcomas from the most comprehensive CGH database, progenetix, (http://www.progenetix.net/) and combined with data from the literature that were not included in the database [155-159]. We then compared our own findings with results from other studies that had focused on the MFH, LMS, LPS or GIST entities (Table 6). Taken together the CGH findings show that the highest frequency of loss occurred on chromosome 13q and the most common gain occurred on chromosomal region 1q21-23. However, in our studies the most frequent gains were found on 17q. This difference is most probably due to the smaller number of cases examined in our study, and the different selections of STS in the different reports. Other common alterations were chromosomal alterations preferentially found in a specific subtype of STSs, such as gain of 12q13-15 that was not very frequent in the whole group, but was detected in 44% of LPS. Therefore gain of 12q13-15 can be a specific event for this subtype of tumor, and the genes located in this region could play a main role in the tumorigenesis of this tumor type.

Table 6. Frequency of recurrent copy number changes in previous studies

Tumors (Number)	Most common alterations							
	Gains					Losses		
	17q	1q21-23	8q	6q	12q14-15	13q	14q	10q
Present studies (71)	**32%**	13%	10%	10%	4.2%	**25%**	11%	8%
Other studies* (456)	10%	**21%**	16%	7%	16%	**29%**	8%	3%
LMS (101)	18%	27%	36%	11%	<1%	**54%**	3%	6%
GIST (87)	12%	8%	18%	1%	2%	17%	**32%**	5%
LPS (91)	4%	24%	13%	14%	**44%**	19%	5%	0
MFH (177)	8%	23%	11%	4%	15%	25%	2%	1%

Bold: show the highest percentage in each group; *: data collected from http://www.progenetix.net/ and reference [155-159].

6.2.4 SS18-SSX2 fusion gene in synovial sarcoma (Paper VI)

Synovial sarcoma accounts for 5-10% of STSs [49], which shows several distinct characteristics including 1) Histopathologically, synovial sarcoma with mesenchymal spindle cells only can be defined as monophasic, while biphasic synovial sarcoma has both spindle cell and epithelial cell components; 2) Clinically, the most common tumor sites are next to the large joints, for example the knee, or adjacent to joint or tendon sheaths; 3) Genetically, a specific chromosome alteration t(X;18)(p11.2;q11.2) can be detected in more than 90% of synovial sarcomas, which is tightly linked to the tumorigenesis [160, 161]. There are three different fusion genes associated with malignant synovial sarcoma [111, 162], all involving replacement of the C-terminal 8 amino acids of *SS18* gene by several members of the *SSX* gene family (*SSX1*, *SSX2* or *SSX4*)[163, 164] (Figure 4; Figure 13). The SSX proteins have two transcriptional repression domains, the Krüpple associated box (KRAB) repression domain and a novel repression domain (SSXRD). Through the fusions to *SS18*, the KRAB domain is removed while the SSXRD domain is retained [165, 166]. The chromosomal changes associated with the SS18-SSX fusion are illustrated in Figure 13. While biphasic synovial sarcomas almost exclusively express a *SS18-SSX1* fusion transcript, *SS18-SSX1* and *SS18-SSX2* are equally frequent in the monophasic type [167-169]. Notably, fusion genes can be seen in a small proportion of synovial sarcomas [111], suggesting that this situation may represent a recurrent event of relevance for the tumor development. In addition to the regular *SS18-SSX* fusion, a case with an alternative fusion *SS18L1-SSX1* involving chromosomes X and 20 has been reported [170].

In this study we reported a case of bipedal malignant synovial sarcoma with typical biphasic morphology in a 10-year old girl. The patient presented with several unusual features, including the multifocality and the location of one of the two tumors in the first toe. This led us to investigate the genetic changes in both tumors in details. The presence of multiple unusual tumors at a comparatively young age for this tumor type generally suggests a genetic predisposition for the disease. In the absence of a family history for cancer, such a predisposition can constitute of a de novo mutation that can be present in germ line (and thus heritable) or be present in a subset of cells only (i.e. somatic mosaicism). Hence, the peripheral blood of the patient was also included for

both molecular and cytogenetic analyses. A *SS18-SSX2* fusion was detected by both RT-PCR and FISH assays in both tumor samples but not in the blood sample. In the FISH analyses of the two tumors, a mixture of fusion positive and fusion negative cells were observed, implying somatic mosaicism for the *SS18-SSX2* fusion highly unlikely. G-banding and SKY analyses revealed an apparently normal 46,XX karyotype in all metaphases analyzed without detection of any recurrent chromosomal abnormality. However, a possibly aberrant appearance of distal 20p was noted in three metaphases, which was further evaluated by FISH analyses with probes specific for the chromosome 20 telomeric regions. Out of 200 interphase nuclei and 30 metaphases analyzed, no evidence of terminal chromosome 20 deletion or rearrangement was found.

In summary, the *SS18-SSX2* fusion identified in both tumors occurs somatically, however, this fusion was not the first step in the development of synovial sarcoma in this patient. Instead, a yet unidentified genetic alteration could predispose to the translocation event observed in the two lesions.

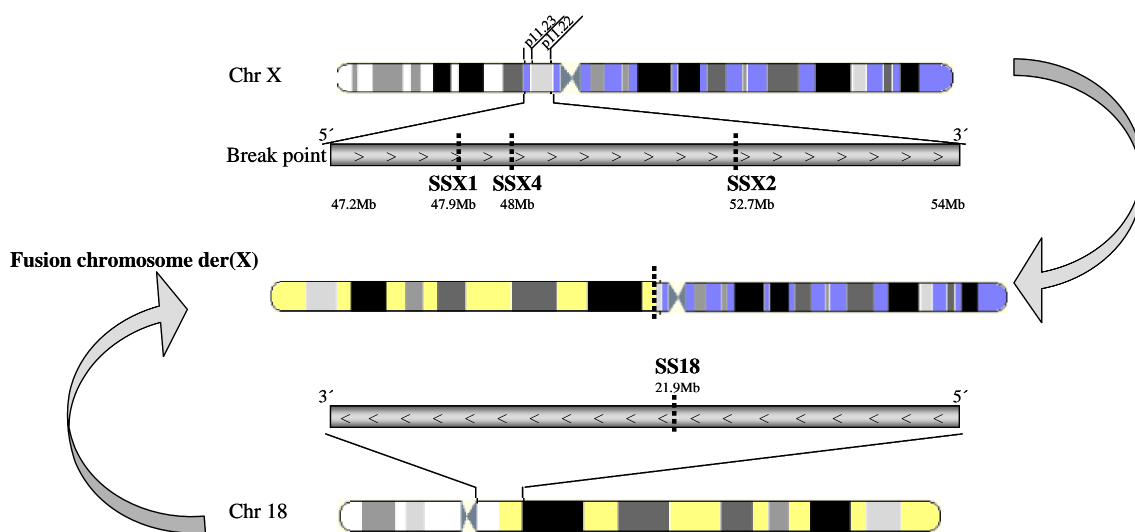

Figure 13. The *SS18-SSX* fusion gene structure.

7. Conclusions

In this study, we applied different genetic approaches to determine specific chromosomal imbalances and molecular changes of 92 soft tissue sarcomas and PPP resistant cell lines. Based on the findings, we can conclude that:

1. CGH is a good tool to assess specific genetic alterations that may relate to prognostic factor(s). Here, we demonstrated that gain of 17q is likely to be a favorable prognostic marker in MFH, and is associated with a low risk of developing metastasis and local recurrence, and hence a better survival.

2. Ezrin expression is significantly associated with poor survival and development of metastasis, suggesting its value as an additional prognostic marker in primary highly malignant STSs.

3. A recurrent acquired chromosomal alteration, gain of 11p12-q13 was identified during the establishment of IGF-1R inhibitor resistant cell lines, which points to the possibility that proteins encoded by genes in this region may be involved in the slow development of this specific drug resistance.

4. By combining SKY and CGH methods, we characterized the genetic compositions of large chromosome markers in a MFH. In addition, high-level amplification of 12q13-21 detected by CGH in the MFH is similar to the 12q amplified region reported in majority of well-differentiated LPS, suggesting that these two tumor types could be closely related.

5. In the intra-abdominal sarcomas analysed, we demonstrated that distinct CGH alterations were related to the sex of the patients, involvement of 12q13 by gain or translocation, and *C-KIT* mutations. The 12q13 gain/ translocation and *C-KIT* mutation could be early events in the tumor progression of intra-abdominal sarcomas. Furthermore, we also

demonstrated that loss of 13q is a general and late genetic event in the genetic progression of intra-abdominal sarcomas. The 13q deleted region was further refined to two target regions by Southern analysis, which provides starting points for identification of candidate gene(s) responsible for the tumor progression of this disease.

6. The case report represents the first description on synchronous appearance of a synovial sarcoma. Small size, localization and distance between both tumors, argue in favor of existence of a synchronous sarcoma. Multifocality has not been previously described in synovial sarcomas, and the identical *SS18-SSX2* fusion genes identified in both tumors underlines the uniqueness of this case.

8. Acknowledgments

I would like to express my deep and sincere gratitude to all friends and colleagues, who had supported and encouraged me, in particular to:

Professor ***Catharina Larsson,*** my excellent supervisor, for giving me an opportunity to accept me as her student, and sharing her wealth of knowledge and guidance throughout the thesis. Practically, I greatly appreciate for her quick and open mindedness to get things done, and for giving generous and well-adjusted support to my growing independence. I have learnt a lot and realized that science can be a lot of fun.

Dr. ***Weng-Onn Lui***, my co-supervisor, for his effort in bringing me to the lab and introducing me into the amazing world of molecular cytogenetics, for sharing his excellent skill in the lab, wide knowledge in cytogenetics and teaching me computer skills.

Dr. ***Jan Åhlén***, my co-author, for a lot of valuable discussions and all tumor samples collection, as well as for sharing his vast knowledge about sarcomas.

Drs. ***Svetlana Lagercrantz*** and ***Soili Kytölä*** for teaching me SKY analysis.
All the co-authors who have contributed to this work especially ***Otte Brosjö, Anette von Rosen, Gert Auer, Olle Larsson, Kristina Åström, Johan Wejde, See-Tong Pang, Michael Lerner, Dan Grandér, Daiana Vasilcanu, Ada Girnita, Magnus Axelson***, and ***Leonard Girnita.***

Dr. ***Anders Höög*** and ***Lisa Ånfalk*** for their excellent help with the samples from the tumor bank. ***Margareta Rodensjö*** for teaching me immunohistochemistry.

All the former and present members in Catharina's group: especially ***Brita Forsberg*** and ***Ann Svensson***, for their countless kindly help in the lab at the beginning of my studies, ***Trisha Dwight***, ***Srinivasan Thoppe*** and ***JiaJing Lee*** for proofreading my

thesis, ***Andrea Villablanca*** for being a nice roommate and solving many Swedish questions***,*** ***Janos Geli*** for many wonderful weekend dinners, ***Lars Forsberg***, ***Tony Frisk***, ***Fangyuan Li***, ***Emma Flordal Thelander***, ***Theodoros Foukakis***, ***Jamileh Hashemi****,* ***Stiina Välimäki***, ***Mattias Berglund****,* ***Petra Kjellman****,* ***Andrei Alimov****,* ***Declan Donnelly, Junko Takagi, Sha Sha Lu, Zhao Chen*** for creating a warm working atmosphere and providing lots of kind help in many different ways.

All the other friends and colleagues in the groups of ***Tomas Ekström, Gunnar Norstedt, Georgy Bakalkin, Mats Persson*** and ***Lars Terenius*** at CMM L8:01.

All friends in the labs at CMM L8:02: ***Günther Weber***, ***Xiaolei Zhou, Biying Zheng, Chengyun Zheng*** and ***Fengqing Xiang*** for friendship and many helps in different ways. Associate Professor ***Keng-Ling Wallin*** and ***Eric Wallin***, for inviting me to their summer house and spending the midsummer (2001) together.

Yvonne Cowan and Gunilla Risberg, for LADOK and administrative issues; ***Britt-Marie Witasp*** for financial issues, ***Delphi Post***, and the staff in IT department.

All friends outside CMM: The family of ***Yuan-Hwa Chou*** for all help and guidance during initial stay in Sweden, the family of ***Xiaolei Fang*** and ***Yu Ming*** for being great skiing and trips companions and supporters during the period when I was alone in Sweden, and especially ***Bee-Hoon Goh*** and ***Sheau-Yun Chin*** for being a good baby-sitter whenever I needed it.thank you so much.

Taipei Mission in Sweden- the representative Mr. ***Chiu Jong-jen*** and family, for their hospitality.

Lastly, but with priority in my mind, I want to thank my family, ***Jacob*** and our two little angels, ***Deborah*** and ***Benjamin*** for so many beautiful memories in my life and always encouraging me that I can make it. My parents in-law, sisters in-law ***Lin-Too Pang*** and ***Swee-Yan Pang*** for never ending support, blessings and help. My parents, borthers and sisters in-law, for their endless love and confidence in me. With their continued trust in me I could be where I am today.

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