UNIVERSITY OF TROMSØ UIT

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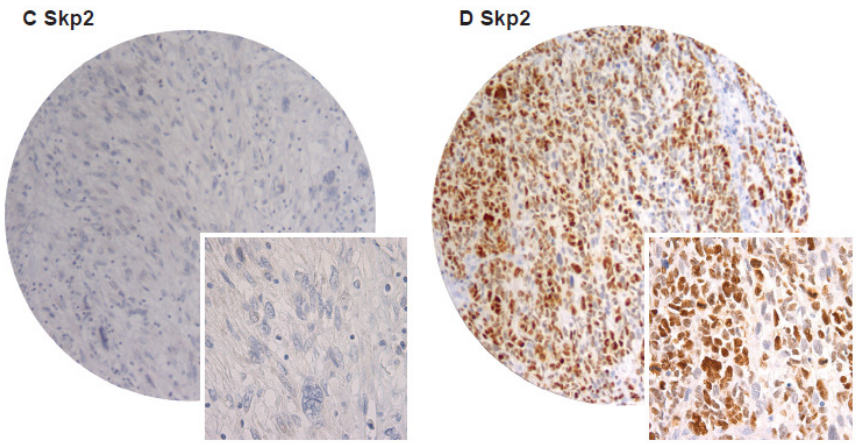
DEPARTMENT FOR CLINICAL PATHOLOGY

Prognostic value of adaptive and innate immune system in soft tissue sarcomas

A retrospective tissue microarray-based study



Sveinung Wergeland Sørbye

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Prognostic value of adaptive and innate immune system in soft tissue sarcomas

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by

Sveinung Wergeland Sørbye

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The work presented in this thesis was conducted in the period from spring 2005 to spring 2013. I began collecting material in spring 2005. In 2009 I started the PhD program and became a member of translational cancer research at the University of Tromsø. In addition to research, I have worked in the Department of Clinical Pathology at the University Hospital of North Norway, where I have been employed as a consultant since 2002. The project is funded by the Northern Norway Regional Health Authority.

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LIST OF PAPERS

I. **Sorbye SW, Kilvaer T, Valkov A, Donnem T, Smeland E, Al-Shibli K, Bremnes RM, Busund LT.** Prognostic impact of lymphocytes in soft tissue sarcomas. *PLoS One.* 2011 Jan 27;6(1):e14611. doi: 10.1371/journal.pone.0014611.

II. **Sorbye SW, Kilvaer TK, Valkov A, Donnem T, Smeland E, Al-Shibli K, Bremnes RM, Busund LT.** Prognostic impact of CD57, CD68, M-CSF, CSF-1R, Ki67 and TGF-beta in soft tissue sarcomas. *BMC Clin Pathol.* 2012 May 3;12:7. doi: 10.1186/1472-6890-12-7.

III. **Sorbye SW, Kilvaer TK, Valkov A, Donnem T, Smeland E, Al-Shibli K, Bremnes RM, Busund LT.** Prognostic impact of Jab1, p16, p21, p62, Ki67 and Skp2 in soft tissue sarcomas. *PLoS One.* 2012;7(10):e47068. doi: 10.1371/journal.pone.0047068.

IV. **Sorbye SW, Kilvaer TK, Valkov A, Donnem T, Smeland E, Al-Shibli K, Bremnes RM, Busund LT.** Prognostic impact of Skp2, ER and PGR in male and female patients with soft tissue sarcomas. *BMC Clin Pathol.* 2013

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
BAD	Bcl-2-associated death promoter
CDK	Cyclin-dependent kinase
CD	Cluster of differentiation
CK	Cytokeratin
CT	Computer tomography
DAB	Diaminobenzydin
DAKO	Dakota Manufacturing Company
DFSP	Dermatofibrosarcoma protuburens
DSS	Disease-specific survival
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ESMO	European Society for Medical Oncology
EWSR1-ETS	Ewing sarcoma breakpoint region 1-E twenty six
FAP	Familial adenomatous polyposis
FISH	Fluorescent *in situ* hybridization
FKHR	Forkhead homolog 1 in rhabdomyosarcoma
FNCLCC	Fédération Nationale des Centres de Lutte Contre le Cancer
GSK3	Glycogen synthase kinase 3
Gy	Grey
HHV8	Human herpes virus 8
HR	Hazard ratio
IMRT	Intensity-modulated radiation therapy
IHC	Immunohistochemistry
Mab	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MFS	Metastasis free survival
MPNST	Malignant peripheral nerve sheath tumor
MRI	Magnetic resonance imaging
MSKCC	Memorial Sloan-Kettering Cancer Center
mTOR	Mammalian target of rapamycin
m TORC	Mammalian target of rapamycin complex 2
NCI	National Cancer Institute
NF-κB	Nuclear factor-kappa B
Non-GIST STS	Non-gastrointestinal stromal tumor soft tissue sarcoma
OS	Overall survival
p-Akt Ser^{473}	Akt phosphorylated on serin 473
p-Akt Thr^{308}	Akt phosphorylated on threonin 308
Par6	Partitioning protein 6
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor

PET	Positron emission transmission
PGR	Progesterone receptor
PI3K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidylinositol trisphosphate
PKC	Protein-kinase C
PNET	Peripheral neuroectodermal tumor
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RNA	Ribonucleic acid
SIN	Size, Invasion, and Necrosis
SMA	Smooth muscle actin
SPSS	Statistical Package for the Social Sciences
SSG	Scandinavian sarcoma group
STS	Soft tissue sarcoma
TGF-beta	Transforming growth factor beta
TMA	Tissue microarray
TNGM	Tumor, nodule, grade, and metastasis
UICC	Union Internationale Contre le Cancer
WHO	World health organization

1. BACKGROUND

1.1. Epidemiology and incidence

Soft tissue sarcomas (STS) represent a heterogeneous group of tumors that arise from mesenchymal tissues and consist of 50 histologic subtypes [1]. They are malignant tumors derived from nonepithelial extraskeletal tissue (except glia, the reticuloendothelial system, and the supporting tissue of different parenchymal organs) [1]. STSs occur at diverse sites of the body, and different subgroups of STSs have very different prognoses. Seventy-five percent are located in the extremities, most common in the thigh, and 10% each in the trunk wall and peritoneum. Three quarters of all STSs are histologically classified as liposarcoma, leiomyosarcoma, high grade pleomorphic sarcoma, synovial sarcoma, and malignant peripheral nerve sheet tumors. One fifth of the patients have local recurrence and one third have distant metastases [2]; however, this occurs more frequently in high-grade tumors. Despite treatment 30–40% of these patients will die of STSs [3].

STSs are rare tumors with an estimated annual incidence of around 30 new cases per 1,000,000 of population [4–7]. They comprise only 0.5–1% of all cancer types [8]. In Norway the number of new cases per year (incidence 2006−2010) was 81 males and 68 females. The proportion related to all cancers was 0.6% for males and 0.5% for females. The number of deaths per year (2006−2010) was 24 males and 25 females [9].

In children the incidence of STSs is relatively higher, at 1–3%, but cancer is not a childhood common disease. Like other malignancies, STSs become more common with increasing age, with 65 years being the median age of diagnosis [4, 5, 8]. The age-related incidence varies among the different histological subtypes. Embryonal rhabdomyosarcoma is found mostly in children; synovial sarcoma is more common in young adults. Liposarcoma, pleomorphic high-grade sarcoma, and leiomyosarcoma dominate in the elderly (Figure 1) [1].

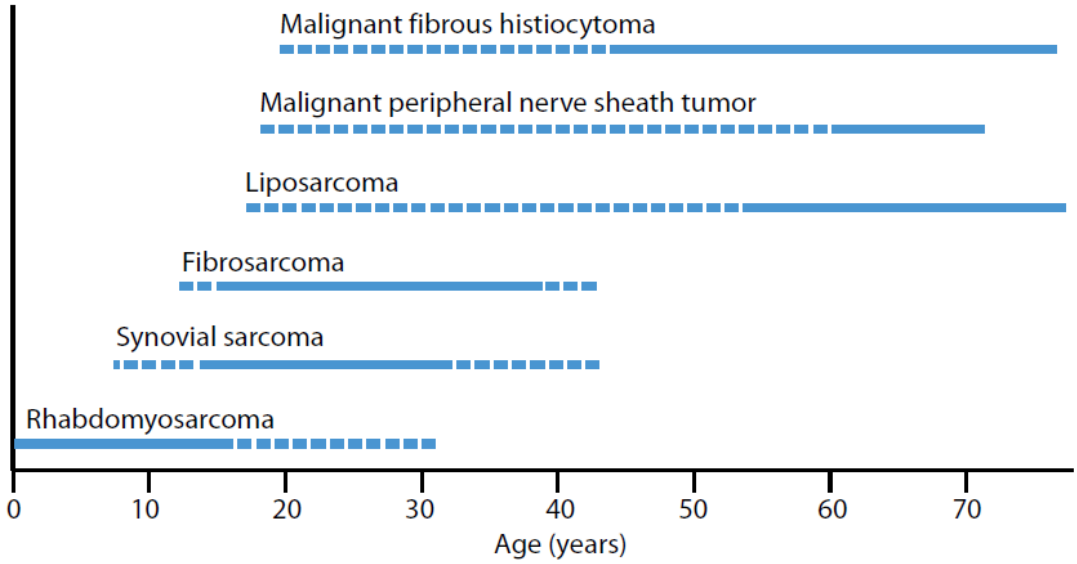

Figure 1. Approximate relation of age to incidence of various types of sarcoma. *Modified from Weiss SW, Goldblum R: Enzinger & Weis's Soft Tissue Tumors, 5th edn. Philadelphia: Mosby, Elseiver Inc; 2008[1] Permission obtained from Elseiver Inc.*

The age-adjusted incidence rates of STSs in Norway have shown a slight increase in the last 50 years (Figure 2), recorded at January 2013 [6].

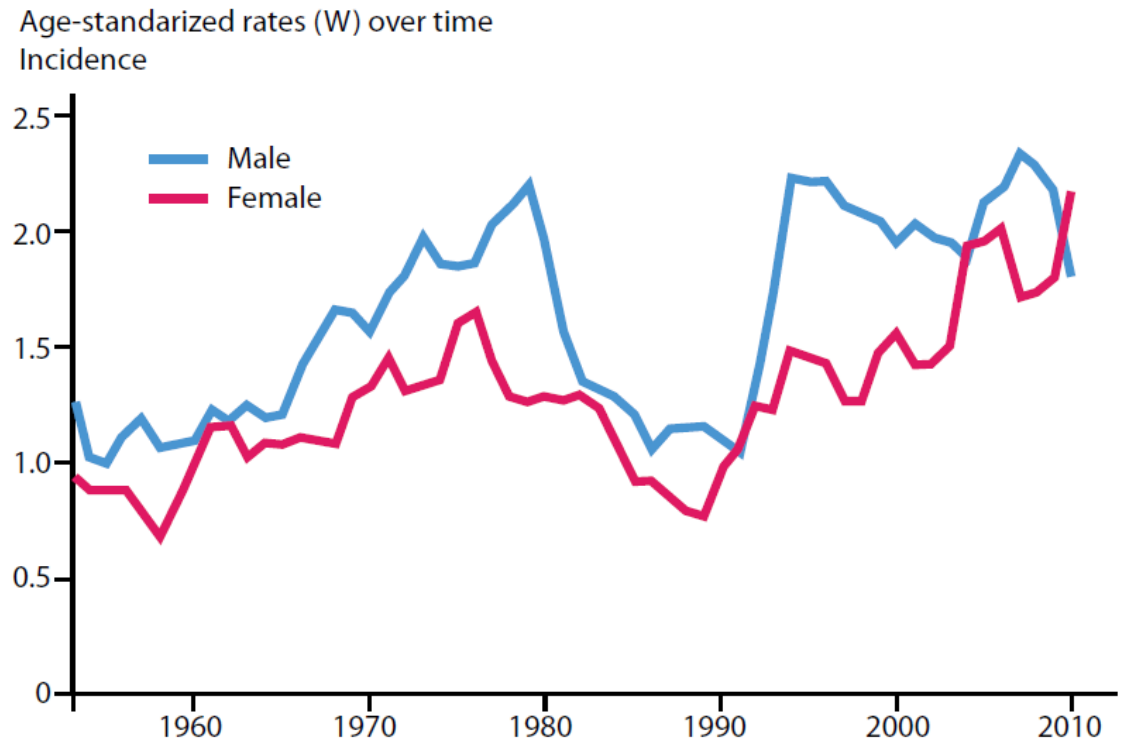

Figure 2. Age-adjusted incidence rates of STS in Norway, 1954 to 2010. *Modified from NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 5.0. Association of the Nordic Cancer Registries [9]. Permission obtained from The Cancer Registry of Norway.*

For the Russian Federation, this figure was 2.3 per 100,000 in 2007, but specifically in the Arkhangelsk region, where our research material was partly gathered from, it was 3.6 per 100,000 [5].

The incidence of STSs increases with advancing age and is approximately the same for male and female patients, with the exception of a drop in incidence in females during the age range 65–70 (Figure 3).

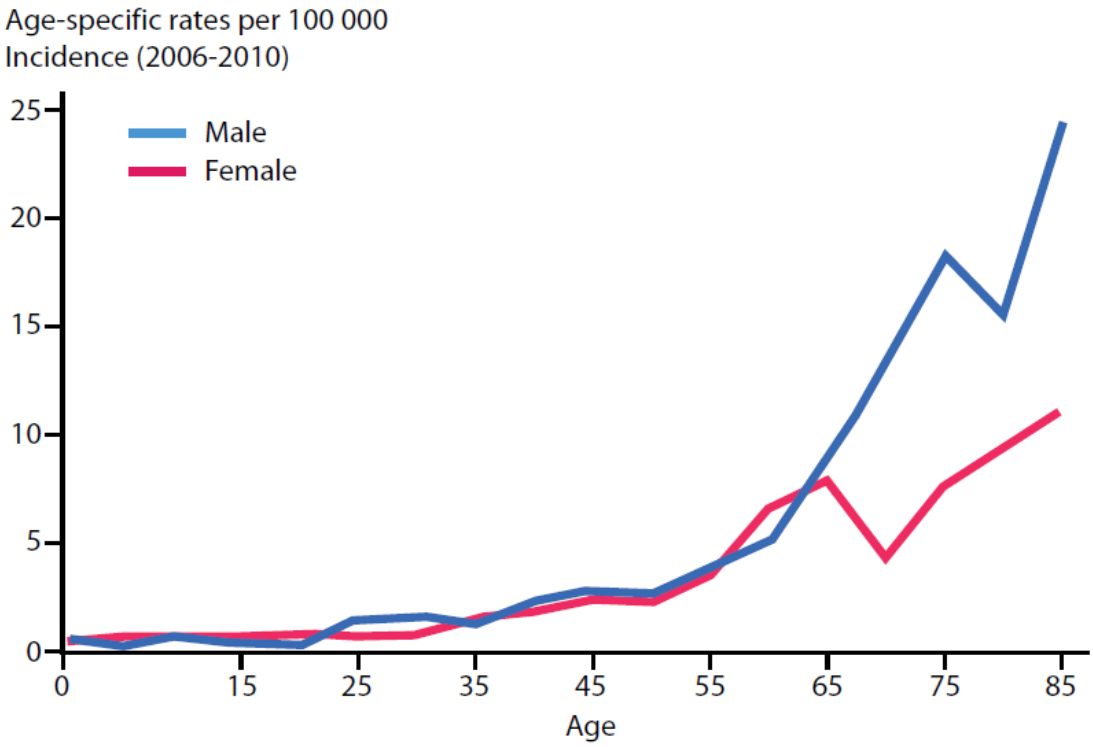

Figure 3. Age-specific incidence rates of STS in Norway per 100,000, 2006 to 2010. *Modified from NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 5.0. Association of the Nordic Cancer Registries [9]. Permission obtained from The Cancer Registry of Norway.*

Mortality due to STSs remains high at 30–40%, making STSs, prognostically speaking, one of the more unfavorable forms of cancer [3–5]. In Norway the survival has gradually increased during the last 50 years, from a 30–40% five-year survival during the 1960s to a 60–70% survival after 1990 (Figure 4). The relative five-year survival (1999−2003) was 66% for males and 68% for females (Nordcan 2013). This increase in survival rate is partially due to new and better treatment protocols for childhood STSs, giving the younger age groups a better overall prognosis [10]. Even so, the prognosis in the adult population has also improved, due to multidisciplinary teams with optimized diagnostic and treatment protocols (Figure 4) [11].

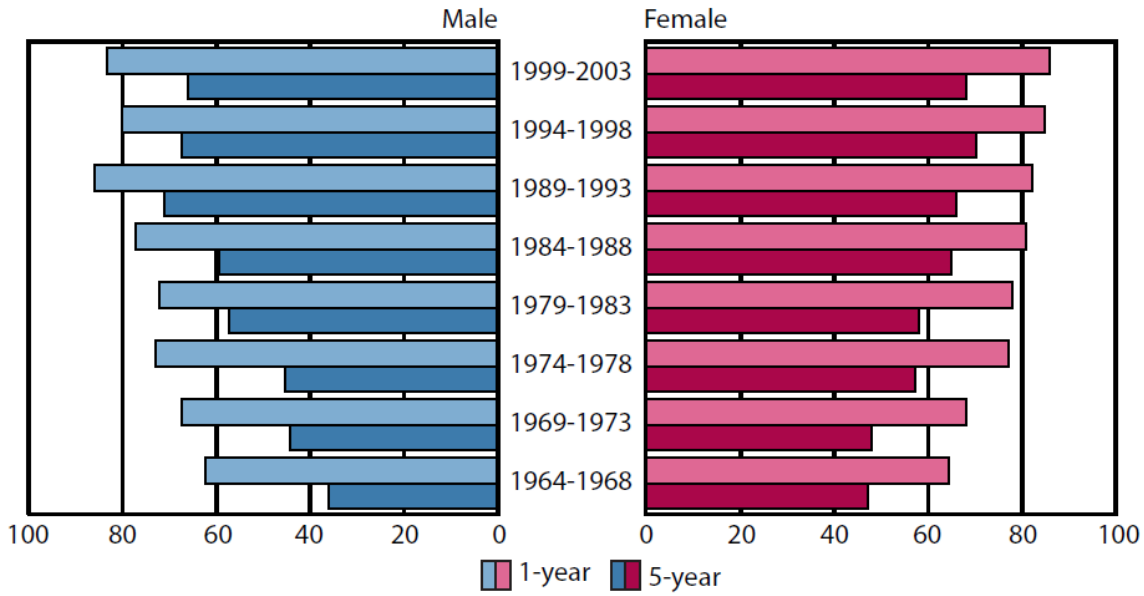

Figure 4. Age-standardized relative survival of STS in Norway, all ages. *Modified from NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 5.0. Association of the Nordic Cancer Registries [9]. Permission obtained from The Cancer Registry of Norway.*

1.2. Histopathology

STSs are usually classified according to their similarity to normal mature mesenchymal tissues [1]. However, high-grade lesions gradually lose resemblance to their tissue of origin. Moreover, some sarcomas have no obvious normal counterpart and therefore belong to a class of tumors of uncertain differentiation. Taking into consideration the rarity and variability of sarcomas, these tumors often represent a diagnostic challenge for a pathologist, who in many cases has to give a pathologic diagnosis based on small-sized biopsy specimens [12].

According to the current World Health Organization's classification of tumors of soft tissue and bone, there are nine main groups of STSs [12]. Some examples of major STS types are demonstrated in Figure 5.

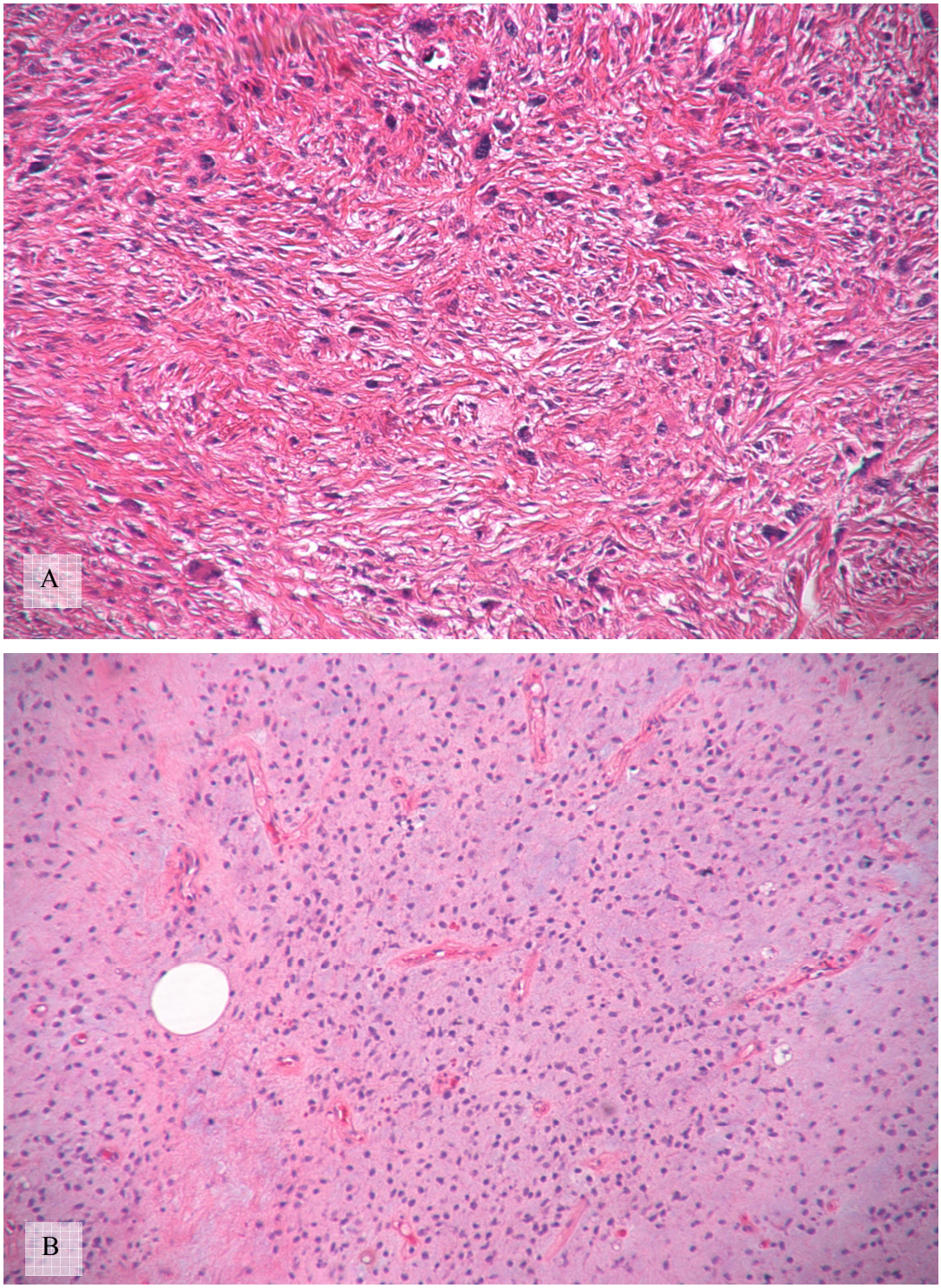

Figure 5. Examples of major STS types. A, Undifferentiated pleomorphic sarcoma; B, Round cell/myxoid liposarcoma. *Unpublished data. Valkov A.*

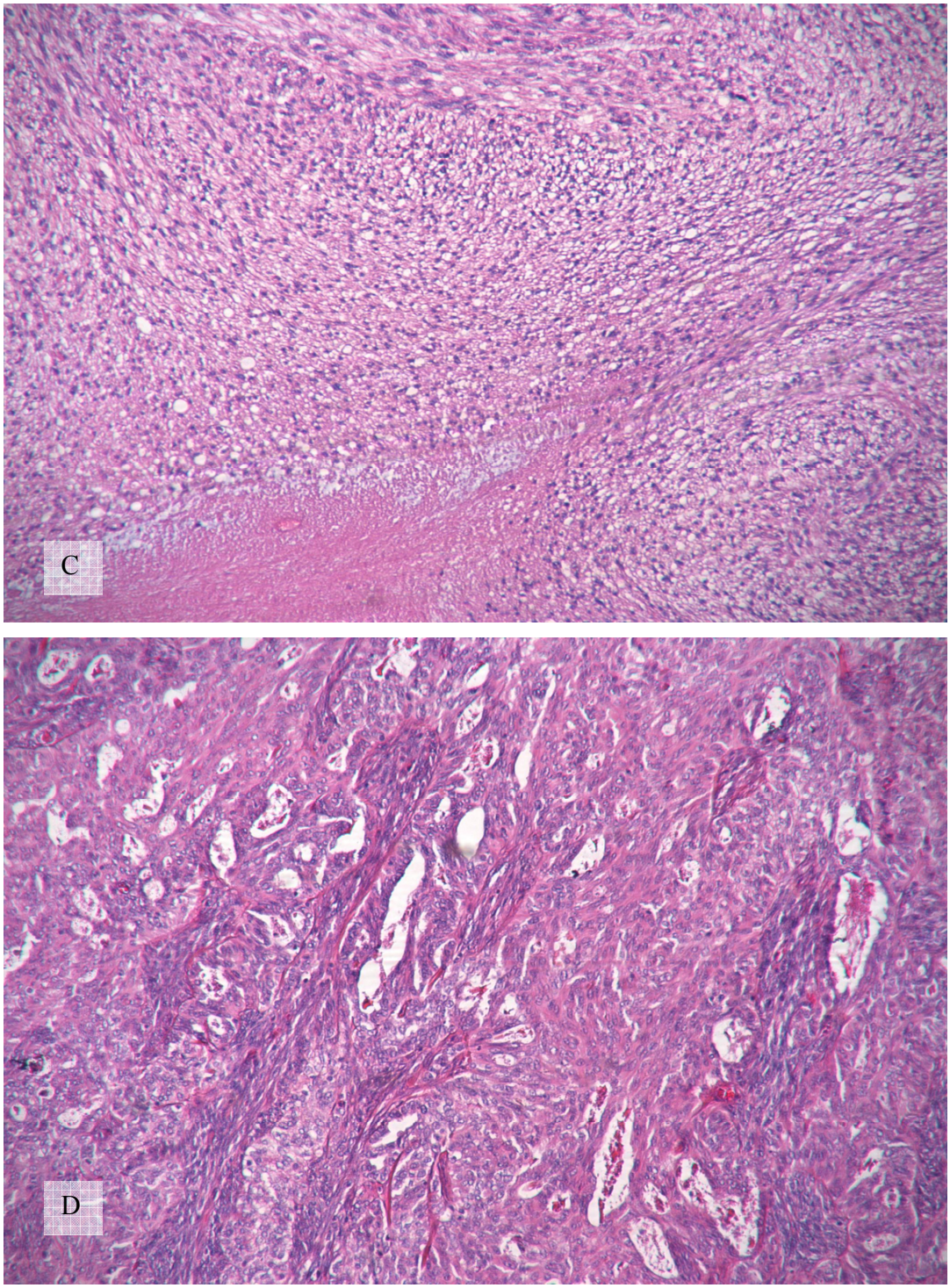

Figure 5 (continued). Examples of major STS types. C, Leiomyosarcoma; D, Biphasic synovial sarcoma. *Unpublished data. Valkov A.*

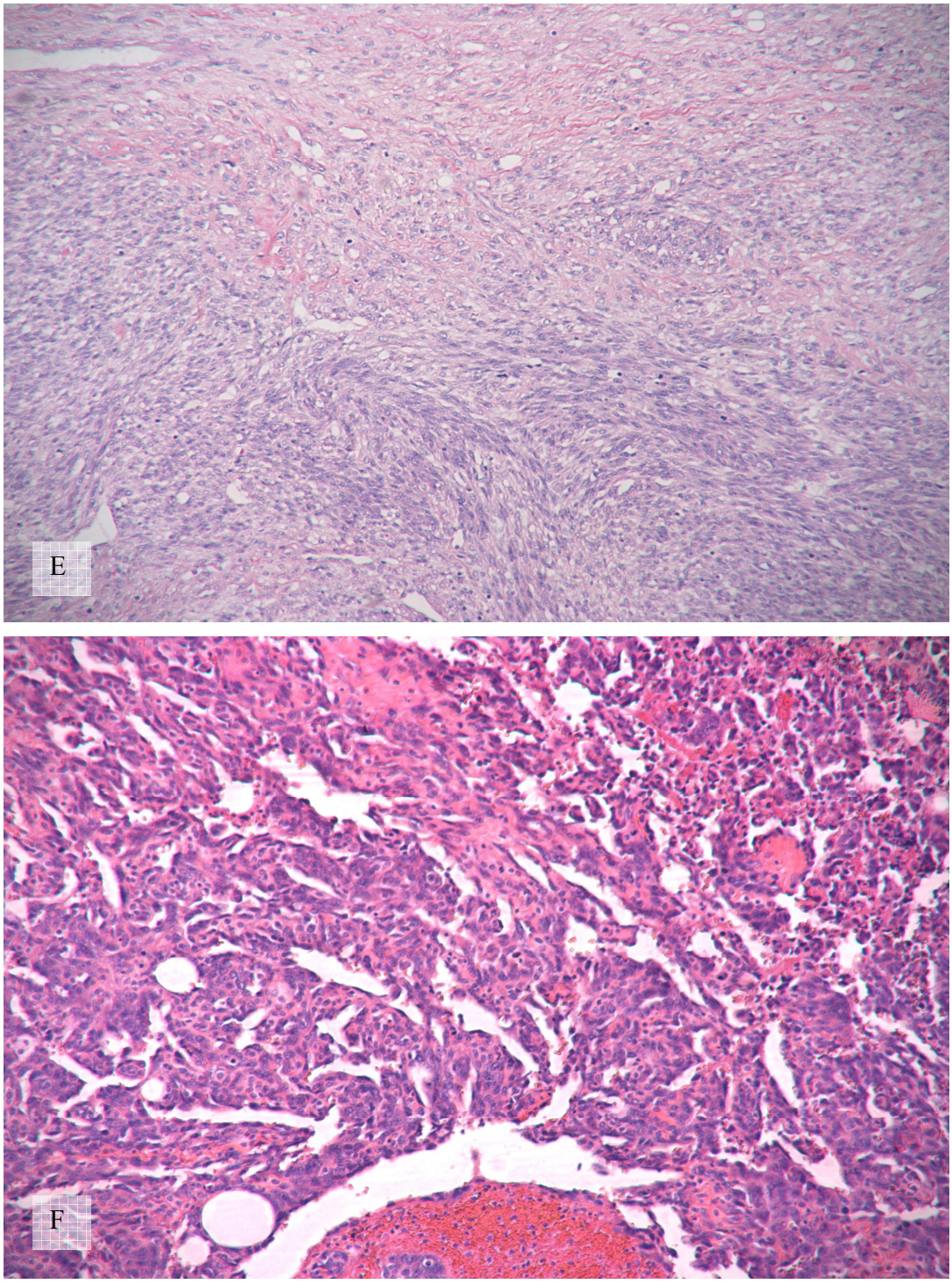

Figure 5 (continued). Examples of major STS types. E, Malignant peripheral nerve sheath tumor (MPNST); F, Angiosarcoma. *Unpublished data. Valkov A.*

When conducting studies on STSs it appears that some specific sarcomas differ greatly from others and should be investigated separately. This is particularly the case for skin sarcomas, gastrointestinal stromal tumors (GISTs), rhabdomyosarcomas, and Ewing/peripheral neuroectodermal tumor (PNET) sarcomas, as these have their own tailored treatments [10, 13, 14].

1.3. Pathogenesis

The pathogenesis of most STSs is still unknown [1]. Nevertheless, there are some recognized causes, which are listed below.

1.4. Hereditary sarcoma

A number of syndromes are associated with STS development. Syndromes with the ability to induce STSs are most often due to mutations in tumor suppressor-, growth factor-, and growth factor receptor genes and translocations forming new potent fusion-genes and proteins [15]. The list of the most common cancer syndromes leading to STSs includes Li Fraumeni, neurofibromatosis type I (Von Recklinghausen's) and type II, familial adenomatous polyposis (FAP)/Gardner, Retinoblastoma, Werner, Lynch syndromes, and tuberous sclerosis/Burneville disease, among others [15]. This list will undoubtedly lengthen with an increased understanding of the molecular underpinnings of mesenchymal neoplasia [1].

1.5. Environmental factors

Among the environmental factors implicated in the development of STSs, trauma is most frequently mentioned. It is now clear, however, that trauma often seems to be an event that merely calls attention to the underlying neoplasm. But there are several well-documented reports of STS plainly linked to trauma [1, 16]. Radiation exposure can result in radiation-induced sarcomas, which in the majority of cases is represented by pleomorphic undifferentiated sarcoma [17]. In addition, there is an increased risk of subsequent sarcoma in survivors of childhood

cancers such as leukemia, retinoblastoma, Wilms's tumor, Hodgkin's lymphoma, and neuroblastoma [18, 19].

1.6. Oncogenic viruses and immunologic factors

Kaposi's sarcoma is closely linked to human herpes virus 8 (HHV8) infection. However, very few healthy individuals infected with HHV8 develop Kaposi's sarcoma, but in immunocompromised individuals many of those with HHV8 infection will develop Kaposi's sarcoma [20, 21]. There is also a large body of literature supporting the role of Epstein-Barr virus in the pathogenesis of leiomyosarcoma in patients with suppressed immunity [22, 23]. In Stewart-Treves syndrome, angiosarcomas can arise in the setting of chronic lymphedema secondary to radical mastectomy [24, 25], which is often explained by the loss of regional immunosurveillance.

1.7. Diagnostics

Most patients with suspected sarcoma present with a growing, painless extremity lump. Pain is reported in only about one third of the cases. Because of the mostly painless presentation, the diagnosis of STSs is often delayed. Late diagnosis of patients with retroperitoneal sarcomas is especially common because of the large retroperitoneal space, generally slow growth rate, and the tendency of sarcomas to gradually displace rather than to invade adjacent tissues [26].

In Scandinavia, patients presenting with a superficial tumor or lump > 5 cm in greatest diameter or deep tumor irrespective of size should be referred to a sarcoma center as soon as possible and prior to any surgical intervention [27]. This is extremely important, as initial inadequate surgery leads to an unfavorable clinical course [28]. All patients with a suspected sarcoma are subjected to imaging procedures in order to establish the extent of the tumor (and eventual metastases) and hence determine the type of surgical procedure needed. Normal skeletal x-ray, CT, and MRI are used, although MRI gives the best impression of the soft tissues and therefore is the imaging modality of choice [29, 30]. In recent years positron emission tomography (PET) scans have become popular and have been implemented in the diagnostics for many types of cancer. The role of PET in STS diagnostics is yet to be elucidated and its use is

recommended only as a supplement to MRI [31]. PET scans are, as of today, more efficiently used to detect local recurrence after the completed therapy [31].

The necessity of pretreatment biopsy is a topic of discussion due to the risk of possible tumor contamination with further possible recurrence in the needle track after a core biopsy [32]. In Norway, a biopsy is recommended only in cases where initial wide resection is not feasible. The biopsy is used to determine the histological type and malignancy grade, and together with imaging procedures, also the stage of the tumor.

1.8. Prognostic factors

1.8.1. Grading

Since the first grading system for sarcomas was introduced by Broders et al. in 1939, a number of systems have been utilized in sarcoma diagnostics [33]. Several parameters have been used to grade sarcomas, such as cellular pleomorphism, cellularity, mitotic index, vascular invasion, tumor necrosis, surgical site, nuclear atypia, histologic type and subtype, tumor size, and tumor differentiation [34, 35]. The WHO manual on the Pathology and Genetics of Tumors of Soft Tissues and Bone recognizes two grading systems used on STSs: the FNCLCC and the NCI grading systems [12].

The FNCLCC grading system, reviewed by Coindre 2006 [34], is calculated from tumor differentiation, mitotic count, and tumor necrosis. Tumor differentiation and mitotic count are given a score from 1–3 and tumor necrosis is scored as 0–2 [1, 12, 33–36]. The histologic grade is derived from the total score, with 2–3 being grade 1, 4–5 being grade 2, and 6–8 being grade 3 (Table 1).

Table 1. Definitions of grading parameters for the FNCLCC system.

Parameter	Criterion
Tumor differentiation	
Score 1	**Sarcoma closely resembling normal adult mesenchmal tissue (e.g., well-differentiated liposarcoma)**
Score 2	**Sarcomas for which histologic typing is certain (e.g., myxoid liposarcoma)**
Score 3	**Embryonal and undifferentiated sarcomas; sarcoma of uncertain type**
Mitosis count	
Score 1	**0-9/10 HPF**
Score 2	**10-19/10 HPF**
Score 3	**≥20/10 HPF**
Tumor necrosis (microscopic)	
Score 0	**No necrosis**
Score 1	**≤50% tumor necrosis**
Score 2	**>50% tumor necrosis**
Histologic grade	
Grade 1	**Total score 2, 3**
Grade 2	**Total score 4, 5**
Grade 3	**Total score 6, 7, 8**

Adapted from *Weiss SW, Goldblum R: Enzinger & Weis's Soft Tissue Tumors, 5th edn. Philadelphia: Mosby, Elseiver Inc; 2008[1]. Permission obtained from Elseiver Inc.*

The NCI grade is derived from the histologic type or subtype and histopathological parameters, including necrosis (the most important), cellularity, pleomorphism, and mitosis, as described by Costa et al. in 1984 and modified in 1990 [37, 38].

In a comparative study of 410 patients diagnosed with STSs, Guillou et al. found the FNCLCC grading system to be marginally better at predicting metastasis and disease-specific survival (DSS) compared to the NCI grading system [1, 35]. However, both systems yielded prognostic groups and are recognized in the WHO manual as suitable for grading STS [12].

In addition to these well-recognized systems, both two-, and four-tiered (as for SSG) systems exist [35]. A proposed conversion between two-, three-, and four-tiered grading systems for STSs is presented in Table 2.

Table 2. Conversion table between different grading systems for soft tissue sarcomas

Two-tiered system	Three-tiered systems	Four-tiered systems
Low grade	**Grade 1**	**Grade 1**
		Grade 2
High grade	**Grade 2**	**Grade 3**
	Grade 3	**Grade 4**

Adapted from *The WHO Classification of Tumors: Pathology and Genetics oft Tumors of Soft Tissue and Bone [12]. Permission obtained from WHO IARC.*

The three-tiered systems are considered most suitable for predicting survival and likelihood of treatment response, since they are able to predict the behavior of both low-grade, intermediate-grade, and high-grade tumors, which seem to be well-defined categories of STSs. Nevertheless, the recently proposed system, termed SIN by the SSG group, anticipated promising binary stratification that would help to simplify treatment strategy schemes [35, 39]. The system uses three factors, namely size, vascular invasion, and necrosis in a dichotomous fashion (size < or > 8 cm, and +/– vascular invasion and necrosis). The low-risk group (score 0–1) had an 81% five-year survival compared to the high-risk group (score 2–3) with a five-year survival of 32%.

1.8.2. Staging

STSs are typically staged according to the tumor, nodule, grade, and metastasis (TNGM) system developed by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC), as devised by Russell et al. in 1977 (later revised and recently

published in the AJCC Cancer Staging Manual 7th edition [40, 41]). The TNGM system for STS includes tumor size, nodal metastasis, malignancy grade, and distant metastasis, yielding a stage ranging from I–IV. The system is designed to include two-, three-, and four-tiered grading systems using a conversion table (Table 2). Table 3 summarizes the current TNGM stages based on grades derived from a three-tiered grading system.

Table 3. Clinical staging and survival of soft tissue sarcoma according to the tumor, node, grade, and metastasis system

Stage	Tumor	Node	Metastasis	Grade	Definition
Ia	**T1a**	**N0**	**M0**	**G1, GX**	**T1: Tumor ≤5cm in greatest**
	T1b	**N0**	**M0**	**G1, GX**	**dimension**
Ib	**T2a**	**N0**	**M0**	**G1, GX**	**T1a: Superficial tumor**
	T2b	**N0**	**M0**	**G1, GX**	**T1b: Deep tumor**
IIa	**T1a**	**N0**	**M0**	**G2, G3**	**T2: Tumor>5cm in greatest**
	T1b	**N0**	**M0**	**G2, G3**	**dimension**
IIb	**T2a**	**N0**	**M0**	**G2**	**T2a: Superficial tumor**
	T2b	**N0**	**M0**	**G2**	**T2b: Deep tumor**
III	**T2a, T2b**	**N0**	**M0**	**G3**	**N1: Regional lymph node**
	Any T	**N1**	**M0**	**Any G**	**metastasis**
IV	**Any T**	**Any N**	**M1**	**Any G**	**M1: Distant metastasis**
					G: Histological grade

Adapted from *AJCC: Soft tissue sarcoma. In: Edge SB, Byrd DR, Compton CC, et al., eds.: AJCC Cancer Staging Manual. 7th edn. New York, NY: Springer, 2010, pp. 291-8 [41].* *Permission obtained from Springer.*

In 2002, Kattan et al. published the Memorial Sloan-Kettering Cancer Center (MSKCC) nomogram for twelve-year sarcoma-specific deaths in which they utilized a subset of independent prognostic markers to predict the clinical cancer development [42, 43]. This approach was later adapted for several clinical situations (pre-/postoperative, after recurrence, etc.) and for specific

subsets of patients (specific sites and histology, etc.) [44–46]. If developed and used correctly, these nomograms seem to give a better prediction of the prognosis of each patient than the conventional staging systems [47].

1.8.3. Other prognosticators in STS

Primary tumor location has been previously reported as an important prognostic marker in STSs, with head and neck as well as retroperitoneal location greatly increasing STS-specific mortality [43, 48].

Traditionally, the specific histopathologic subtype has been considered to be of secondary importance since individual histologic subtypes of comparable histologic grade appear to behave similarly [48, 49]. However, several reports have established the independent adverse prognostic significance of specific histologic subtypes [50, 51]. Our data could not support the observation that different high-grade sarcomas possess discrepant biological behaviors.

Several studies suggest that margin positivity is a marker of adverse prognosis. For instance, the MSKCC group reported in 2002 [52] that a positive microscopic margin was correlated with a 1.6-fold increase in disease-specific survival. Our current data further support these observations; in the multivariate analysis, margin positivity was associated with a 2.9-fold increase in STS-related death ($P < 0.001$). Other clinical factors reported as a prognosticator in STSs include local and distant recurrence [42] and nodal status [53, 54].

Specific molecular prognostic markers may be particularly useful in this epoch of new insight into the molecular biology of cancer. The detection of such markers may be based on high-throughput assays. The main aim of this project is to investigate the prognostic impact of molecular markers of the innate and the adaptive immune system as well as cell cycle regulatory proteins in patients with STSs.

1.9. Treatment

1.9.1. Surgery

Surgery with wide resection margins is the main choice for treating STS patients [30]. Several studies show that surgery should be planned and implemented at a center with expertise in sarcoma surgery. Patients requiring re-excision, due to poorly planned surgery or when malignancy is found in lesions that were perceived as benign before surgery, have a greater risk of recurrence than patients with a well-planned primary surgery [55, 56].

Previously, amputation was perceived as necessary to obtain adequate resection margins when STSs is in the extremities, but in the last twenty years, limb-conserving surgery has become a good alternative to amputation and involves significantly less morbidity [57, 58]. A recently published study on the treatment of STSs of the extremities suggests that for tumors ≤ 3 cm in greatest diameter, surgery alone is adequate treatment [59]. For larger tumors and small tumors with marginal or uncertain resection margins, the recommended treatment is surgery in combination with radiotherapy and/or chemotherapy [30].

For STSs of the trunk, head, and neck, as well as visceral and retroperitoneal sites, the recommendation is surgery with wide resection margins. However, it is often a challenge to obtain wide resection margins for these places, and combinations with other treatment methods are often required [60, 61].

1.9.2. Chemotherapy

Pre- and postoperative chemotherapy is broadly used in treatment of bone sarcomas [62] and rhabdomyosarcomas. In STSs its usage is controversial as there have been conflicting reports regarding the treatment's effects [63]. The ESMO clinical recommendations for STS diagnosis, treatment, and follow-up assess adjuvant chemotherapy as an option in cases of large or high-grade tumors rather than as a standard treatment [29].

Doxorubicin and Ifosfamide containing regimes are used both for adjuvant and for neoadjuvant treatment of advanced STSs [64–66]. Novel drugs such as gemcitabine and taxans, among others, are also used [11, 67]. Additionally, Trabectidin® was recently approved by the FDA for palliative STS treatment [68].

Neoadjuvant chemotherapy is used for primary inoperable STSs in order to shrink the tumor and facilitate wide resection and elimination of subclinical disease [69]. Isolated limb perfusion and hyperthermic isolated limb perfusion are novel techniques available in some cancer centers for the treatment of primary unresectable extremity STSs. These techniques render the tumors operable in up to 40% of the cases, although often at the cost of considerable toxicity [70–72].

1.9.3. Radiotherapy

Primary radiotherapy is mostly used in cases where surgery is not possible, and the specific effect of this therapy is difficult to assess, as these tumors often have a dismal prognosis [73]. Intensity-modulated radiation therapy (IMRT) is a modern type of high-precision radiotherapy. Using computer technology, linear accelerators deliver defined radiation doses to a malignant tumor or specific areas within the tumor. Several studies recently demonstrated that IMRT can be administered safely and with promising efficacy, especially in patients with locally advanced STSs [74, 75].

Adjuvant radiotherapy is warranted for limb STSs where initial resection yields uncertain, marginal, or intralesional resection margins [76, 77]. The dosages are typically between 50 and 75 Gy, with higher radiation doses (63 Gy or more) yielding much better tumor control and survival [78]. The therapeutic window is between 63 and 68 Gy. An increase in complications occurs in patients that are given doses of 68 Gy or more. [78].

During the last 20–30 years, adjuvant radiotherapy has become more and more commonly used in the treatment of localized STSs. In a study of 1,093 patients with STSs in an extremity or trunk wall, adjuvant radiotherapy was shown to prevent local recurrence regardless of the malignancy grade, tumor depth, and surgical margin status. The effect was seen more clearly in deep-seated, high-grade tumors and in tumors treated with surgery with wide resection margins [79]. For STSs of other sites, adjuvant radiotherapy remains controversial [60, 74].

1.10. Molecular-genetic abnormalities in sarcomas

The molecular-genetic background of cancer in general is a hotspot in today's research. Most STSs carry complex, but non-specific karyotypes, with numerous gains and losses [80], while ~ 15–20% of them—namely synovial sarcoma, Ewing sarcoma, and myxoid/round cell liposarcoma—have specific translocations and relatively simple karyotypes [81]. In addition, a minority of tumors have specific mutations, like c-kit mutation in GIST. The essential mechanisms of carcinogenesis were proposed in 2000 and considerably upgraded in 2011 by Hanahan and Weinberg [82, 83]. Each of these mechanisms is regulated by several intracellular signaling pathways that further interact in a complicated, cross-talk network. There is, however, growing evidence that certain molecular aberrations are more likely to influence the clinical behavior of a malignant tumor, including invasion and metastasis.

1.11. Tumor proliferation and growth

Tumor proliferation can be defined as an increase in tumor cell number due to altered balance between growth–antigrowth signaling and/or resistance to apoptosis and differentiation. Tumorigenesis is caused by abnormal cell proliferation. The rate of tumor cell proliferation depends on the rate of cell division, the growth fraction, and the rate of cell loss due to apoptosis or terminal differentiation. This is important since the aim of most cancer therapy strategies is to kill or reduce the growth of tumor cells.

The growth fraction of a tumor can be registered by several techniques. The easiest and most frequently used method is the mitotic count under light microscopy, which is incorporated in several STS grading systems, including the FNCLCC system [12, 35]. Alongside the advantages, this method has some drawbacks such as high intra- and interobserver variability and subjective estimation. This can be avoided by using immunohistochemical markers of proliferation, like Ki-67 or MIB-1 [84, 85]. Other methods of measuring the proliferation rate are identification of cells with active DNA synthesis [86], flow cytometry to find the approximate percentage of cells in S-phase, and the detection of cycle-linked markers.

The transition between cell cycle phases is regulated by checkpoints that, in turn, require an expression of a variety of proteins. These include regulating cyclin-dependent kinases

(CDKs), regulatory proteins, and transcription factors like Ras oncogene, retinoblastoma tumor-suppressor protein (Rb), transforming growth factor beta (TGF-beta), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-1), and a host of others [87–90]. Many of these are known molecular biomarkers and current objects for research both in epithelial tumors and in STSs.

1.12. Molecular markers

Molecular markers are biological molecules found in blood, other bodily fluids, or tumor tissue [91–93]. They can be classified as those that can establish more accurate and definitive diagnoses, those that can predict responses to specific therapies, and those that can give a survival prognosis. [94–100].

There can be considerable overlap for a marker's role across functional categories. For instance, an immunohistochemical testing of tumor tissue for female steroid hormone receptors can be used both as a diagnostic procedure in differential diagnostics of metastasis and as a predictor of tamoxifen or aromatase inhibitor therapy success in breast cancer [101, 102]. In addition, some prognostic value of these receptors has also been reported in gynecological cancers [103, 104]. The evidence for the efficacy of anti-estrogens in desmoid tumor growth is based on non-placebo-controlled trials. Tamoxifen is the most common antiestrogen agent used for treating desmoid tumors [105]. Molecular markers may offer great promise in the care of cancer patients, especially with respect to individual, tailored cancer treatment [106, 107].

1.12.1. Markers of tumor growth, proliferation, and differentiation

Several studies show a close interaction between the malignant tumor cells and cells in the tumor stroma (see Figure 6 below). Here we investigate the expression profiles of STS tumor cells and the surrounding stoma.

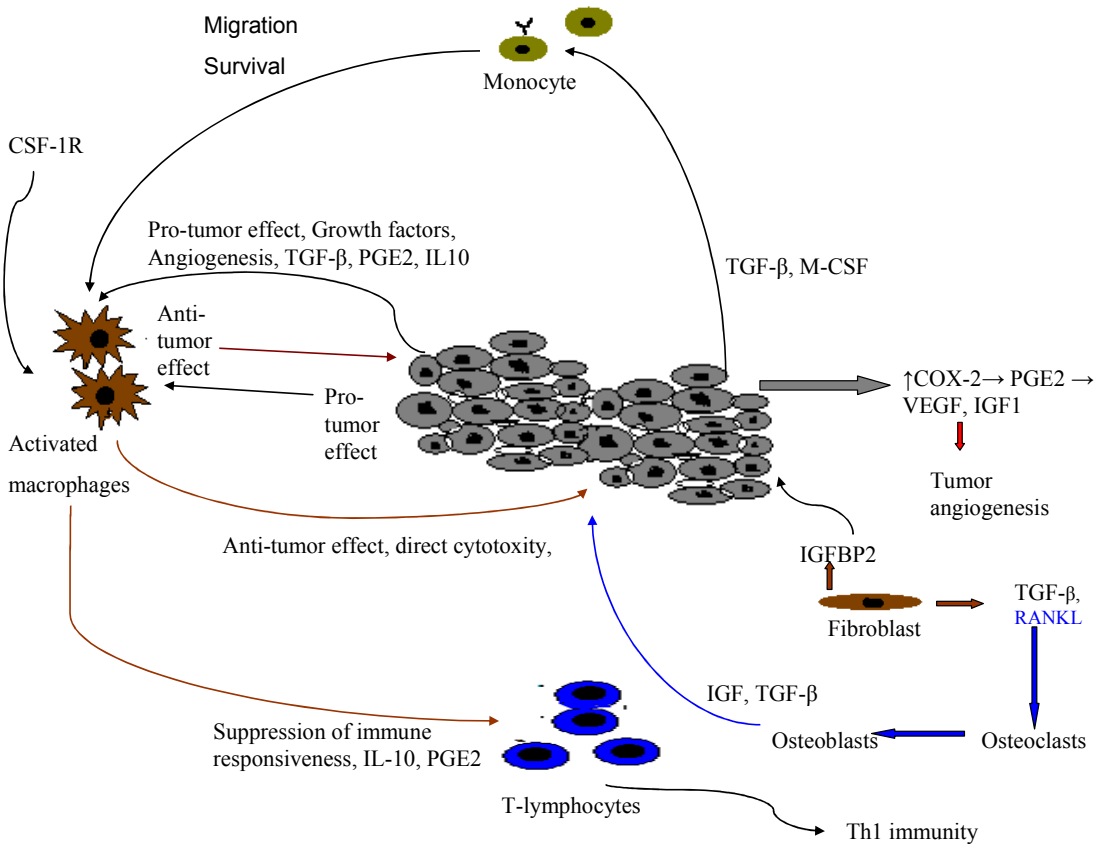

Figure 6. Paracrine interactions between neoplastic cancer cells and supporting cells in tumor stroma (E. Richardsen, 2008).

1.12.2. The adaptive and the innate immune system in STSs

Tumor-infiltrating lymphocytes (TIL) are often found in tumors, indicating that cancer triggers an immune response.

The growth of malignant cells can be suppressed by activation of the adaptive immune system, or tumor growth may be promoted by a variety of innate immune cells [108]. The adaptive immunity consists of antigen-specific T- and B-lymphocytes and can inhibit tumor growth by a combination of cytokine-mediated and antibody-mediated tumor cell lysis or direct killing by cytotoxic T-lymphocytes [108]. Recently, it became clear that it is important to study the anti-tumor effects of the innate system in the tumor stroma [109]. Efficient tumor eradication requires cancer infiltration by tumor-reactive T-lymphocytes [110]. However, there are many

mechanisms by which cancer cells can escape the immune surveillance, such as accumulation of myeloid suppressor cells and suppression of cytotoxic T-cells by regulatory T-cells [110–112].

In general we can divide tumor-infiltrating lymphocytes into three groups: a) epithelial lymphocytes, b) stromal lymphocytes, and c) peritumoral lymphocytes [113]. Infiltration of CD8+ lymphocytes in malignant tumors is associated with improved survival in different types of cancer [116–123]. The role of CD8+ cells in soft tissue sarcomas is controversial, and many publications either have a small number of cases and/or neglect the stromal component. In addition, CD4+ T- and B-lymphocytes may both promote or inhibit tumor growth [124], and their role is controversial in many cancers, including STSs [125, 126].

The most important components of the innate immune system are macrophages, granulocytes, dendritic cells (DCs), NK-cells, their receptors, and growth factors [108]. In contrast to the adaptive immune system, the innate immune system lacks “memory” when re-exposed to the same antigen. The innate immune system is important in the limitation and elimination of foreign threats to the host [108, 127].

The NK-cell plays a major role in tumor rejection in many different types of cancers [128–130]. The way these immune cells identify tumor cells has provided valuable information on tumor immunosurveillance. Based on this insight new strategies in the treatment of human cancer have been developed [131, 132].

DCs represent the most potent antigen-presenting cells and are important in the activation, recruitment, and stimulation of T-lymphocytes [133]. CD1 + DC is one of the major steps in the innate immune response against cancers. A high number of DCs in the tumoral or peritumoral area have been shown to correlate with better survival for patients with various solid tumors [134–137] and are used in therapeutic vaccination against cancer [138].

Tumor-associated macrophages are a double-edged sword. They may help tumor eradication by production of cytotoxic cytokines (IL-1, IL-6, and TNF-α). On the other hand, macrophages may favor tumor progression by TGF-beta production and by contributing to the formation of tumor stroma and angiogenesis through the release of angiogenic factors [114]. Macrophage Colony Stimulating Factor (M-CSF) is the major regulator of the mononuclear phagocytic lineage and plays a major role in innate immunity [139]. M-CSF mediates its effect

with a high affinity trans-membrane tyrosine kinase receptor (CSF-1R). Substantial evidence exists in different cancers, especially those of the breast and female reproductive system, that overexpression of CSF-1R is associated with poor survival [140]. The expression and role of M-CSF and its receptor in both the malignant and stromal components of STSs are not well studied.

To better understand the prognostic impact of the innate immune system in soft tissue sarcomas, we will analyze the degree of infiltration of cell subsets, growth factors, and their corresponding receptors belonging to the innate immune system, both in the malignant mesenchymal compartment and the stromal compartments, and study their relations to their clinicopathological variables and survival. The figure below shows schematic interactions between cells belonging to the immune system and the neoplastic cells during cancer progression.

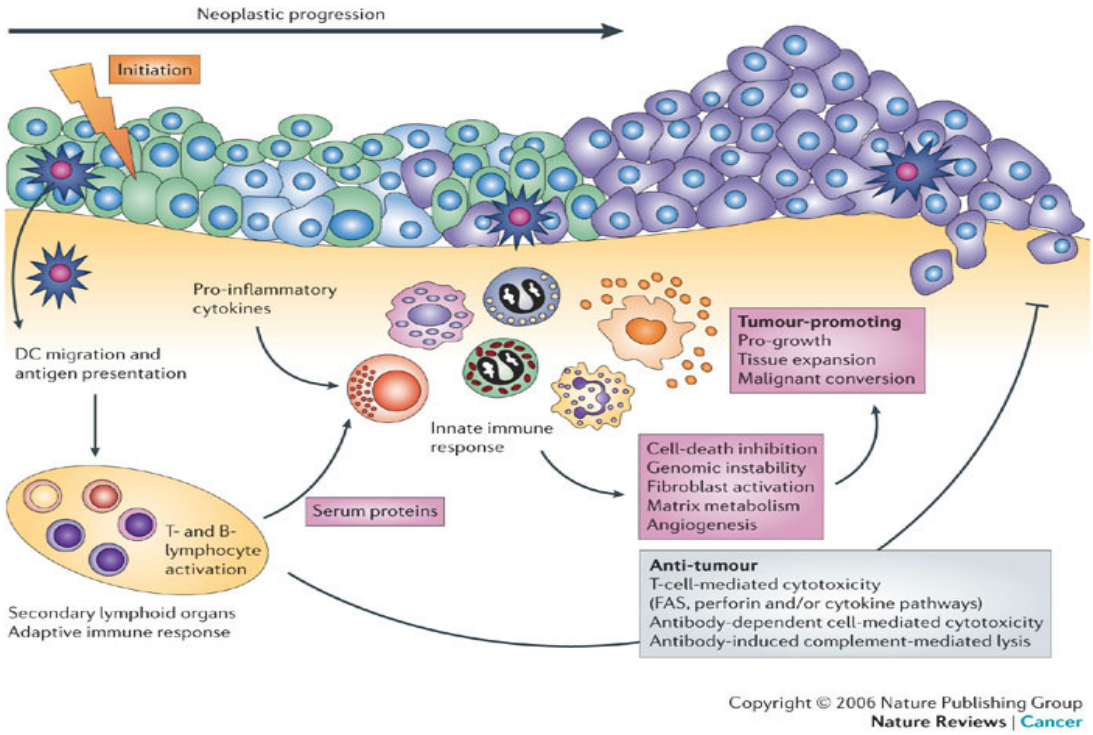

Figure 7. Visser KE et al. Nature Reviews Cancer 2006:6; 24–37.

1.12.3. Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes are considered to be an indication of the host immune reaction to tumor antigens [141], and their clinical significance has been reported in a variety of human solid tumors.

1.12.4. Cell cycle regulatory proteins

The loss of cell cycle control is a critical step in the development of neoplasia. The cell cycle is a series of carefully coordinated and regulated steps that govern cellular proliferation. Cyclin-dependent kinases (CDK) phosphorylate the retinoblastoma (Rb) protein, a classic tumor suppressor and key component of the G1/S checkpoint. This allows DNA replication to proceed. Inhibitors of CDK, such as p16(INK4A), p21, and p27, act as brakes on progression through the cell cycle.

1.12.5. Female steroid hormone receptors

Estrogen receptors (ER) are a group of mostly intranuclear receptors activated by the hormone 17beta-estradiol (estrogen). There are two separate but highly homologous isoforms of ER, ERα, and ERbeta, which have completely different tissue distributions [171]. They are encoded by two separate genes, ESR1 and ESR2. ER, mostly in α isoform, mediates the action of estrogens and is responsible for growth and differentiation of target cells.

These steroid hormone receptors act as ligand-activated transcription factors. There are several mechanisms with such action, including (1) classic, when transcription starts after receptor-ligand complex binding to the specific response element in the gene promoter, (2) response element-independent pathway via binding to a transcription factor which in turn directly contacts the target gene promoter, (3) ligand-independent genomic action, when different growth factors induce phosphorylation of the hormone receptor followed by binding to the specific response element in the gene promoter and transcription/translation/protein synthesis, and (4) non-genomic actions, involving extranuclear fraction of hormone receptors [173].

Both ER and, to a lesser degree, PGR are well known predictive markers of endocrine therapy in breast cancer [174, 175]. They are also shown to have a slight positive prognostic

effect irrespective of endocrine therapy [103]. Steroid hormone receptors are known to be expressed to some extent by soft tissue tumors. In leiomyomatous tumors of the uterus, their expression level correlates inversely with tumor malignancy grade [176, 177]. In addition, effect of hormone-ablation therapy such as tamoxifen has been a reported in aggressive intraabdominal fibromatosis [178, 179].

1.12.6. TGF-beta

TGF-beta is a family of three highly homologous proteins, called TGF-beta-1, TGF-beta-2, and TGF-beta-3, which have very similar functions. They are natural tumor-suppressive agents and induce G1 to terminate proliferation, promote apoptosis, and induce differentiation in normal cells. However, in cancer development, this mediator initiates dedifferentiation through activation of SMAD and non-SMAD (DAXX) signaling pathways [180]. The TGF-beta pathway activation is associated with poor survival in epithelial tumors [183, 184] and in mesenchymal bone [185] and soft tissue tumors [186–188].

1.13. Tissue microarray

Tissue microarrays (TMAs) represent a powerful technology tool designed to explore molecular targets, on the DNA, RNA, or protein level, from several tissue specimens assembled in a single microscope slide [194]. This method implies the extraction of small tissue cylinders from a donor tissue block to be embedded in a recipient block (Figure 7).

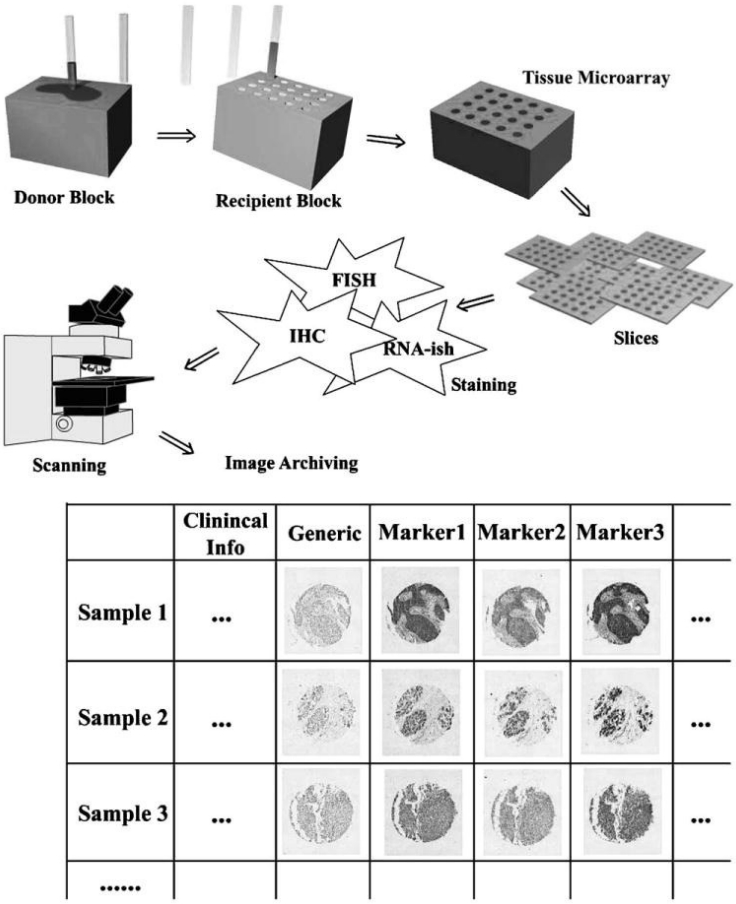

Figure 7. Tissue microarray method. Cores punched from the donor blocks and embedded into the recipient block. The TMA block can then be sectioned and used for various staining methods. Adapted from *Chen W, Foran DJ: Advances in cancer tissue microarray technology: Towards improved understanding and diagnostics. Anal Chim Acta 2006* [195]. *Permission obtained from Elseiver Inc.*

This block can then be cut into thin slices available for immunohistochemistry (IHC), *in situ* hybridization (ISH), etc. Once constructed, one block can potentially yield tissue for several hundred analyses, depending on its thickness [196, 197].

The method was first introduced by Battifora in 1986 as a so-called "multitumor (sausage) tissue block" [198] and further modified in 1990, referred to as "the checkerboard tissue block" [199]. Although offering significant benefits even at this early stage, the TMA technique was not embraced on a large scale before 1998, when Kononen et al. devised an instrument able to standardize the TMA construction process [200]. Adaptation has also allowed the use of material

other than paraffinized tissues, including frozen tissue, cell-lines, and needle biopsies. This has led to a vast increase in TMA studies, and in 2007 nearly 10% of all biomarker studies were conducted using TMA as the principal method of investigation [196].

2. AIMS OF THESIS

The aim of our study is to look into the role of different essential molecular markers of the innate and the adaptive immune system as predictors for disease-specific survival (DSS) in patients with STSs.

More specifically, the aims were to:

- ✓ explore the prognostic impact of lymphocytes in STSs by using immunohistochemistry to evaluate the expression of CD3+, CD4+, CD8+, CD20+, and CD45+ lymphocytes in tumors.

- ✓ evaluate the prognostic significance of macrophages (CD68), their growth factor macrophage colony-stimulating factor (M-CSF), its receptor colony-stimulating factor-1 receptor (CSF-1R), natural killer cells (CD57), and the general immunomodulating molecule (TGF-beta) in tumors and peritumoral capsule.

- ✓ investigate the prognostic significance of Jab1, p16, p21, p62, Ki67, and Skp2 in STSs.

- ✓ explore the prognostic significance of Skp2 related to ER and PGR in male and female patients with STSs.

3. MATERIAL AND METHODS

3.1. Study population and material

Figure 8 shows the inclusion and exclusion of patients in the different studies. We conducted a retrospective search for patients with sarcoma diagnosis in archival material at the University Hospital of North Norway (1973–2006) and hospitals in Arkhangelsk County, Russia (1993–2004). In the Russian material we searched for patients within a ten-year period, since the archival system before the selected time period was less organized. A total of 959 patients were found (Norwegian, n = 632; Russian, n = 337).

Formalin-fixed and paraffin-embedded samples from primary tumor tissues were obtained. All biopsies were re-evaluated by two experienced pathologists. The tumors were graded according the FNCLCC system and histologically subtyped according to the World Health Organization guidelines. For the Russian material new slides were made of all paraffin blocks. For the Norwegian material new slides were made when necessary. All biopsies were immunostained with actin, CK, CD34, CD117, SMA, and vimentin. Some slides were also stained with the S100 when it was necessary to exclude or verify the differential diagnosis. Other molecular methods were not used in our study, but in some cases PCR or FISH were performed at the time of the initial diagnosis. About 10% of the initial diagnoses were revised due to changes in classification systems and the creation of new entities such as GIST. Non-sarcoma, other sarcomas not classified as STSs, and GIST were excluded. Exclusions based on this were as follows: carsinosarcomas (n = 81), dermatofibrosarcoma protuberans (n = 78), GIST (n = 47), osteosarcomas (n = 42), chondrosarcoma (n = 30), Kaposi's sarcoma (n = 30), endometrial stromal tumors (n = 27), benign tumors (n = 18), malignant mesothelioma (n = 11), and other sarcomas/unknown (n = 99).

In total, 496 non-GIST STSs (Norwegian, n = 299; Russian, n = 197) were registered. However, 247 patients were excluded due to inadequate paraffin-embedded fixed-tissue blocks (n = 161) or missing clinical data (n = 86). Thus, 249 non-GIST STS patients (Norwegian, n = 167; Russian, n = 82) were eligible and included in the study.

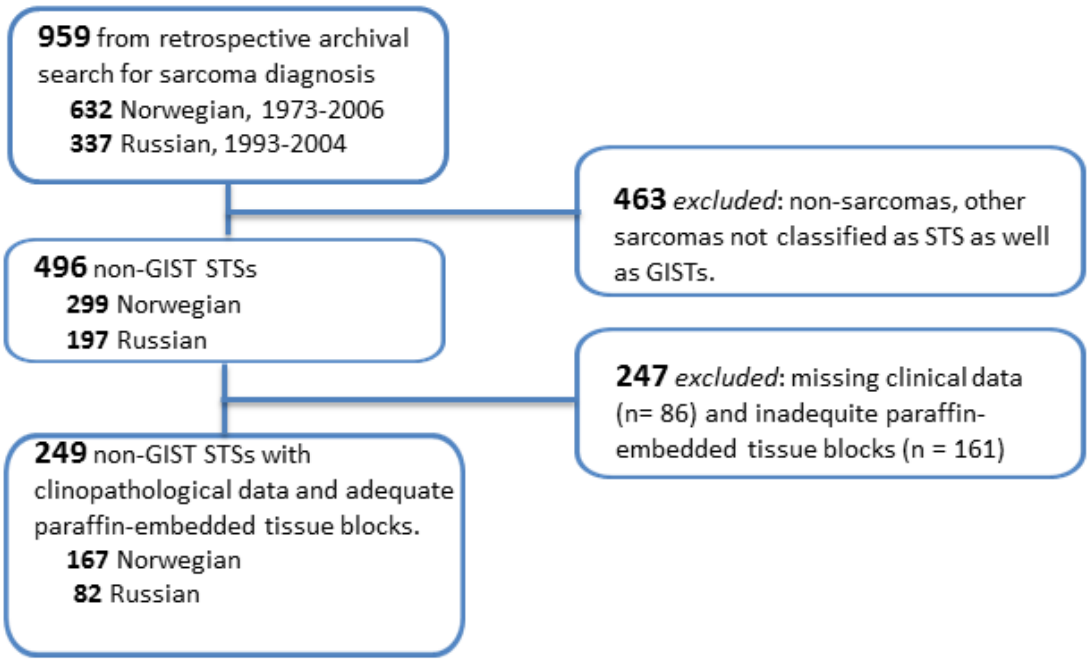

Figure 8. Flowchart visualizing inclusion and exclusion of patients in the study.

Demographic and clinical data were collected retrospectively and include follow-up data as of September 2009. The minimum follow-up for the survivors was 41 months and the median follow-up for the entire patient population was 37.6 (range 0.1–391.7) months.

3.2. Immunohistochemistry (IHC)

The applied antibodies were subjected to in-house validation by the manufacturer for IHC analysis of the paraffin-embedded material. The antibodies used in the study are summarized in Table 4. All stainings were performed in the Ventana Benchmark XT automated slide stainer (Ventana Medical System, Illkirch, France). Before staining, the sections were incubated over night at 60 degrees Celsius. Tissue sections were incubated with primary mouse monoclonal antibodies as well as rabbit polyclonal antibodies recognizing the different antigens (Table 4).

Table 4. Schematic overview of the antibodies used in the studies.

Antigen	Dilution	Antibody	Clone	Source
CD3	Prediluted	Mouse monoclonal	2GV6	Ventana Medical Systems
CD4	1:5	Mouse monoclonal	1F6	Novocastra
CD8	Prediluted	Mouse monoclonal	1A5	Ventana
CD20	Prediluted	Mouse monoclonal	L26	Ventana Medical Systems
CD45	Prediluted	Mouse monoclonal	RP2/18	Ventana Medical Systems
CD57	Prediluted	Mouse monoclonal	NK-1	Ventana Medical Systems
CD68	Prediluted	Mouse monoclonal	KP-1	Ventana Medical Systems
M-CSF	1:5	Rabbit polyclonal	H-300	Santa Cruz Biotechnology
CSF-1R	1:25	Rabbit polyclonal	C-20	Santa Cruz Biotechnology
Ki67	Prediluted	Mouse monoclonal	30-9	Ventana Medical Systems
TGF-beta-1	1:50	Rabbit polyclonal	SC-146	Santa Cruz Biotechnology
Jab1	1:50	Mouse monoclonal	4D11D8	Zymed
P16	Prediluted	Mouse monoclonal	INK4A	Ventana Medical Systems
P21	Prediluted	Mouse monoclonal	SX118	Dako
P62	Prediluted	Mouse monoclonal	LCK lig	Ventana Medical Systems
SKP2	1:10	Mouse monoclonal	IG12E9	Zymed
ERα	Prediluted	Mouse monoclonal	SP1	Ventana Medical Systems
PGR	Prediluted	Mouse monoclonal	1E2	Ventana Medical Systems

3.3. Scoring

The ARIOL imaging system (Genetix, San Jose, CA) was used to scan the slides for antibody staining of the TMAs. Representative and viable tissue sections were scored manually and semi-quantitatively on a computer screen for nuclear and/or cytoplasmic staining. (Figure 1). The number of CD3, CD4, CD8, CD45, CD57, and CD68 positive cells in tumors were scored as 0 (no cells), 1 (1–5 cells), 2 (6–19 cells), or 3 (20+ cells) per 0.6 mm core. Expressions of M-CSF,

CSR-1R, TGF-beta, Jab1, p16, p21, p62, Ki67, Skp2, ER, and PGR were scored as: 0, negative; 1, weak; 2, intermediate; 3, strong. Each patient's score was based on the mean score of cores from one or several biopsies. High expression was defined as mean score > 0 for CD57, M-CSF, CSF-1R, p21, Skp2, ER, and PGR, ≥ 0.30 for CD68, ≥ 0.33 for p62, ≥ 0.50 for CD20, ≥ 0.75 for p16 and TGF-beta, ≥ 1.00 for CD4, ≥ 1.50 for CD3 and CD8, and ≥ 2.00 for CD45, Jab1, and Ki67. All samples were anonymized and independently scored by two pathologists (AV and SWS). When disagreements occurred, the slides were re-examined and a consensus was reached by the observers. When assessing a variable for a given score, the scores of the other variables and the outcome were hidden from the observers.

3.4. Statistical analysis

For statistical analyses we used the SPSS (Chicago, IL) statistical package. The chi-square test and Fisher's exact test were used to examine the association between the expression of molecular marker and various clinicopathological parameters. Marker expression correlation was measured with the Pearson correlation (2-tailed) at the 0.05 and 0.01 levels. For univariate analyses we used the Kaplan–Meier method. Statistical significance between survival curves was assessed by the log rank test. Disease-specific survival (DSS) was determined from the date of histological-confirmed STS diagnosis.

For multivariate analysis we used the Cox proportional hazards model to assess the specific impact of each pre-treatment variable on survival in the presence of other variables. Only variables of significant value from the univariate analysis were entered into the Cox regression analysis. Probability for stepwise entry and removal was set at 0.05 and 0.10, respectively. The significance level used was $P < 0.05$. IHC scores from each observer were compared for interobserver reliability by the use of a two-way random effect model with absolute agreement definition. The intraclass correlation coefficient (reliability coefficient) was obtained from these results.

3.5. Ethical clearance

Our study was approved by the Regional Committee for Research Ethics (REK Nord) and the National Cancer Data Inspection Board.

4. MAIN RESULTS

4.1. Paper I

Tumor-infiltrating lymphocytes (TIL) are often found in tumors, which indicates that tumors trigger immune responses. The immune status at the time of the diagnosis of the tumor may be important, but the prognostic significance of TIL is controversial since the immune system may both promote and reduce tumor growth. The aim of this study was to investigate the prognostic significance of TIL in STSs. The number of tumor-infiltrating CD3+, CD4+, CD8+, CD20+, and CD45+ lymphocytes was analyzed in 249 patients with STSs in relation to other clinicopathological variables.

In univariate analyses increased numbers of CD4+ ($P = 0.008$) and CD20+ ($P = 0.006$) inflammatory cells were positively associated with a better disease-specific survival (DSS) in patients with wide resection margins ($n = 108$). For patients with non-wide resection margins ($n = 141$), increased numbers of CD3+ ($P = 0.028$) lymphocytes in a tumor was negatively associated with DSS. In multivariate analyses a high number of CD20+ lymphocytes (HR = 5.5, CI 95% = 1.6–18.6, $P = 0.006$) in the tumor was an independent, positive prognostic factor for DSS in patients with wide resections margins.

4.2. Paper II

This study was focused on exploring the prognostic impact of the presence of cells and growth factors belonging to the innate immune system in STSs. In univariate analyses high expressions of M-CSF ($P = 0.034$), Ki67 ($P < 0.001$), and TGF-beta ($P = 0.003$) in tumor were negatively associated with DSS. An increased expression of Ki67 in the peritumoral capsule tended to correlate with a shorter DSS ($P = 0.057$). An increased expression of CD68 in tumor correlated significantly with malignancy grade ($P = 0.016$) but not DSS ($P = 0.270$). In multivariate analyses co-expressions of M-CSF and TGF-beta ($P = 0.022$) in tumor and a high expression of Ki67 ($P = 0.019$) in peritumoral capsule were independent, negative prognostic factors for DSS.

4.3. Paper III

The purpose of this study was to clarify the prognostic significance of expressions of Jab1, p16, p21, p62, Ki67, and Skp2 in STS. In univariate analyses a high expression of Skp2 (P = 0.050) and a high expression of Ki67 (P = 0.007) were negatively associated with DSS. In a subgroup analysis, a negative correlation between Skp2 and DSS was seen in patients with malignancy grade 1 or 2 (P = 0.027), tumor size >5 cm (P = 0.018), no radiotherapy given (P = 0.029), and no chemotherapy given (P = 0.017). High expression of Ki67 was strongly positively associated with high malignancy grade (P = 0.001). In multivariate analyses, Skp2 was an independent negative prognostic factor for DSS in women (P = 0.009) and in patients without administered chemotherapy or radiotherapy (P = 0.026).

4.4. Paper IV

This study focused on clarifying the prognostic significance of Skp2 expressions related to gender, estrogen receptor (ER), and progesterone receptor (PGR) in STS. In subgroup analyses expressions of PGR in males (p = 0.010) and in patients older than 60 years (p = 0.043) were negative prognostic factors for DSS. A high expression of ER in females was a positive prognostic factor for DSS (p = 0.041). In co-expression analyses of the whole cohort, a low expression of Skp2 in combination with a low expression of ER was positive for DSS (p = 0.049). In females, a high expression of Skp2 in combination with a low expression of ER was a negative prognosticator (p = 0.021). In the multivariate analyses malignancy grade (p<0.001), age (p = 0.012), wide resection margins (P = 0.010), ER negative/PGR positive co-expression profiles (p = 0.002), and ER positive/PGR negative co-expression profiles (p = 0.015) were independent, negative prognostic factors for DSS. In females expressions of Skp2 (p = 0.006) were associated with shorter DSS.

5. DISCUSSION

5.1. Methods

5.1.1. Data collection and study population

We have included patients from two countries, Norway and Russia, to achieve adequate statistical power for the analyses. The representativity of the studied population may be a problem in studies. The risk of heterogeneity in the population may also be a disadvantage. In terms of ethnicity and geography, however, northern Norway and Russia are quite close, as seen in the corresponding distribution of clinicopathological variables. Despite possible differences in diagnosis or treatment traditions, the histopathological reassessment of all tumors and the relatively limited and rough classification of treatment strategies are meaningful to study in both the Norwegian and Russian patients in a cohort. The study focuses on the natural biology of the STSs and not on treatment.

5.1.2. Representativity of Norwegian and Russian study populations

STSs represent about 0.5–1% of all cancer cases. Of the total of 21,000 cancer cases reported annually in Norway, sarcomas represents more than 160 cases, of which two thirds are STSs and third bone sarcoma. The proportion living in northern Norway accounts for about 10% of the Norwegian population. In view of this, it is estimated a total of 350 new STS patients during a period of 33 years (1973–2006), of which some had GIST and some had sarcomas of the skin (for example, dermatofibrosarcoma).

There were 299 Norwegian cases of STSs observed in our population. We excluded 132 cases due to lack of clinical data or inadequate paraffin-embedded fixed-tissue blocks (Figure 8). Since the lack of paraffin-embedded material is random, it can be argued that the patient group is representative of the population.

Nevertheless, there is more reason to question the representativeness of the Russian material. The population of Arkhangelsk Oblast is about three times larger than northern Norway.

As the number of Russian cases in our study (n = 82) is about a third of the Norwegian material, there is certainly a need for more patients in the Russian material, although the duration of enrolment period for the Russian sample (1993–2004, a total of 11 years) was a third (1973–2006, a total of 33 years) of the Norwegian. This potential selection bias should be considered when our results are analyzed. We also see that the subsets of the Russian patients have significantly poorer prognoses than the Norwegian cohort. However, when comparing the clinicopathological variables, only the distribution of histologic grade is significantly different in the Russian versus the Norwegian material. A larger proportion of Russian patients with malignancy grade 3 may partially explain the reduced survival of the Russian population. A significant part of the Russian material was from Arkhangelsk Regional Oncology Centre, while patients with less aggressive tumors can potentially be cured locally by the local district hospitals. In short, we cannot rule out a selection bias in the Russian material.

5.1.3. Separate investigation of differently located sarcomas

Sarcomas located on extremities and trunk (ET), versus retroperitoneal and visceral tumors (VR), may be regarded as distinct STS entities based on clinical and prognostic data. Metastases are the main cause of sarcoma-related death in patients with visceral tumors, while local relapse is a more common cause of sarcoma-related death in patients with STSs in extremities and trunk. We have stratified patients according to ET (n = 115) versus VR (n = 66) subgroups (patients with head and neck STS (n = 13) were excluded from these analyses). Significant differences and trends from the original papers were persistent in patients with STSs located on extremities and trunk. The number of patients with visceral tumors was insufficient for conducting reliable analyses.

5.1.4. Heterogeneity of histological entities in the study population

Heterogeneity with regard to the histological units included in the analyses may be a problem. It is possible that different subtypes of STSs have different expressions of prognostic molecules. We conducted subgroup analyses of the histological units in terms of expression of

different markers, and we found the same trends in the major subgroups compared to the smaller subgroups.

5.1.5. Conclusion on material representativity

STSs are rare tumors, and there are many sarcoma subtypes. In our study, it was difficult to collect a sufficient number of similar patients with similar tumors that have received the same treatment. This is a known issue in the implementation of patients in STS studies. But our study is largely focused on generating hypotheses rather than testing them, so patient similarities are less crucial. To be more conclusive, future STS studies should be based on large, multi-institutional and multi-national studies designed to collect the highest possible number of STS patients to ensure a sufficient number in each subgroup. At the same time, all tumors we examined were of mesenchymal differentiation and they belong to the same generic group. Moreover, we examined the role of some important molecular markers of the innate and the adaptive immune system as predictors for DSS in patients with STSs. Similar findings are found in many different epithelial and non-epithelial malignant tumors of diverse histological locations and devices and do not seem to depend on the tumor type.

5.1.6. Tissue microarray

A tissue microarray (TMA) assembles on a single histologic slide several small representative tumor cores from many different patients, thus making it possible to analyze multiple specimens in one staining [201]. Two pathologists (AV and SWS) reviewed the histology of all STSs. TMAs were constructed for high-throughput molecular pathology research [10]. The most representative areas of viable tumor cells were carefully selected and marked on the hematoxylin and eosin (HE) slides for the corresponding donor blocks and sampled for the TMA collector blocks. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments).

TMA is a valuable tool for high-throughput analyses of tissues to identify prognostic markers and possible targets for therapy in human cancers [201]. Obvious advantages of the TMA technique, versus whole slide assessments, include the high throughput, robust benefits at a lower cost, the possibility for large cohorts simultaneously, supreme staining standardization,

reproducibility, and relative simplicity. It is also possible to use the donor specimens for further analysis and to share the material between institutions.

Along with these apparent benefits, there are some disadvantages often discussed with regard to the use of TMAs. A common question is whether a few core samples are representative for large tumor specimens. Instead of 0.6 mm cores, some investigators have used larger cores (2–4 mm or more) to increase the representativity [202–204]. Others suggest that multiple, small cores from different regions offer better coverage of tumor heterogeneity [194]. After reviewing all the original tumor sections and taking heterogeneity into consideration, we decided to use duplicate 0.6 mm cores that were selected to maximize representativeness. Studies reveal a 95% correlation when comparing evaluations of tumors in duplicate 0.6 mm cores versus the whole slide [194]. To include all core samples, we constructed 12 tissue array blocks.

Another often mentioned drawback is that TMAs are not suitable for individual diagnosis of patients. In the involved institutions, all diagnostic procedures were performed using full slides before construction of TMAs for marker studies.

5.1.7. Immunohistochemistry

Immunohistochemistry is one of many techniques used to analyze the tissue for expressions of proteins and other molecules. In addition to standard HE staining, immunohistochemistry is one of the most widely used techniques in routine diagnosis of pathological laboratories. Immunohistochemistry is also commonly used in research. It is reliable, well developed and familiar, easy to interpret, and widely available. Unlike a number of more modern techniques, immunohistochemistry visualizes the final protein product, localization of protein, and not just an up or down regulated gene, etc.

5.1.8. Antibodies

Choosing antibodies is one of the major steps in conducting a study using immunohistochemistry. When available, commercial antibodies are the best choice, as they have data leaflets with rigorous specifications and are easily available for conformational studies. The next step is choosing between monoclonal and polyclonal antibodies. Monoclonal antibodies all target one epitope on the antigen, thus providing excellent specificities. In addition, they are

homogenous from production lot to production lot, making conformational studies easier to conduct. The drawback of monoclonal antibodies is the chance that post-processing of the tissue could conceal the targeted epitope and lead to a type II error. Polyclonal antibodies target several epitopes on the same antigen, resulting in a more robust antigen binding. The robust antigen binding happens at the cost of a risk of cross reaction with other antigens and an increased risk of a type I error.

The Sarcoma Study Group in Tromsø is a part of a larger Translational Cancer Research Group. All the immunomarkers we used were chosen from published literature and validated by the manufacturer and by the group's previous studies of lung cancer [205, 206].

A common concern is whether improper tissue storage over years may affect the results of immunohistochemistry. To address this question we used the date of diagnosis to divide the total material (n = 194) into three categories (1973–1989, n = 48; 1990–1999, n = 97; 2000–2006, n = 49) and two categories (1973–1996, n = 101; 1997–2006, n = 93). There were no significant differences (defined as $r > 0.2$, $P < 0.01$ due to multiple testing) in any of the marker expressions with regard to time period.

5.1.9. Controls

The principle of immunochemical staining is that a specific antibody will combine with its specific antigen, making a unique antibody–antigen complex. Antibody specificity was ensured by a western blot showing binding of a protein of the expected size. In the case of the antibodies used in our studies, this was done by the manufacturer and presented in the data leaflets of the antibodies.

The use of staining controls helps to reduce false positive and false negative results and make it easier to read the results of the immunochemical staining. Negative controls are conducted by replacing the primary antibody with a primary antibody diluent to check for unspecific staining in the absence of the antibody. Negative controls could be made even more stringent by introducing isotype controls to check for unspecific binding. A positive control may be any tissue that contains the antigen of interest. We used tissue controls with other tumor groups and normal tissue on each TMA slide, representing both positive and negative controls.

5.1.10. Statistics

Almost every time we make a decision based on data, there is some chance we will make an error. There are many approaches to statistical analysis of survival data, and no optimal method of analysis exists. In order not to over- or under-interpret the significance of their data, investigators have to be vigilant when choosing an approach. The objective of the hypothesis test will be to make a decision about the null and alternate hypothesis statements. The possibility of error comes in because we make this decision regardless of whether the null hypothesis is actually true or false. We believe that we in our analyses have found a reasonable balance between type I and type II errors. A short discussion of the statistical methods used in our studies is presented below.

5.1.11. Significance level

Type I errors occur in a situation where the null hypothesis is true but our statistical test rejects it anyway. This is a situation when inappropriate significance levels are used. Type II errors occur in a situation where the null hypothesis is a false statement and we should reject it. However our hypothesis test fails to reject the null. In biological studies it has become a norm to use $P < 0.05$ as the cut-off point where a difference is considered significant. This shows that one in twenty tests for the same difference will be a type I error. When conducting a large number of tests the chance of an erroneous positive result thus increases. Several approaches have been developed for reducing the chance of a type I error in the setting of multiple testing. The drawback of these approaches is the increased chance of a type II error. There is no consensus whether such methods should be used in prognostic studies. We chose not to conduct a correction of multiple testing, as we see our studies as hypothesis generating. This increased the risk of type I errors but decreased the chance of type II errors.

5.1.12. Cut-off values

In our study we explored the prognostic value of the adaptive and innate immune system in soft tissue sarcomas. In prospective studies of clinical and biological prognostic factors the cut-off values are meant to divide the subjects under investigation into diagnostic groups based

on the relative expression of molecular markers. As biological values are continuous scales, this produces a skewed view of reality, and the results must be interpreted in that context. The most common approach is to dichotomize the material, but sometimes several groups give a better picture. When selecting the cut-off values the researchers must choose between using a predefined value either based on previous research, the mean or median, percentiles, standard deviations, etc., or based on finding the cut-off value that yields the two groups with the largest possible difference in the end-point under investigation. There are drawbacks and advantages to both approaches. When using a predefined cut-off value the chance of type I errors decreases at the cost of type II errors. In many cases it is also difficult to find meaningful previous studies that suggest a usable cut-off value. In the case of a conformational study, using a predefined value makes sense since a cut-off is already established. In the case of a novel study, choosing the mean, median, or percentiles as cut-off values makes sense in that it increases the reproducibility of the cut-offs and therefore will be easier to evaluate in a conformational study. When choosing the cut-off that yields the two groups with the largest possible difference in prognosis, the chance of type II errors decreases at the cost of type I errors. This approach makes sense in novel hypothesis-generating studies where there are no predefined values to help in selecting the appropriate cut-offs. Such studies could be the basis of further research into novel fields, and their results should be interpreted in this light. We have used the latter approach in our studies, and we regard our findings as hypothesis generating. Hence, our results should be confirmed in other prognostic studies before being incorporated into clinical practice.

5.1.13. Survival analysis

Survival analysis is used to analyze datasets in which the response variable denotes time until an event occurs. These events often refer to time between diagnosis and death or time until relapse and recovery [207]. There are several different statistical methods for analyzing survival data. One well-proven method is the Kaplan–Meier (KM) analysis, which tests the difference between groups in time to event data. However, the KM method does not adjust for the presence of other clinical variables. To address this point we used the Cox proportional hazards method to adjust for clinical variables found to be significant when using the KM method. This stringent method works to ensure that the variables found in our studies are in fact independent of known

demographic, clinical, or pathological variables and could therefore contribute when calculating the prognosis of STS patients.

An issue is which endpoint to use. In prognostic studies there are a variety of endpoints, such as overall survival (OS), metastasis-free survival (MFS), time to recurrence (TTR), time to progression (TTP), and, as we have chosen, disease-specific survival. DSS is a well-established endpoint. In our study we excluded from the survival analysis patients with non-sarcoma-related deaths.

5.2. Discussion of the results

There are few studies with large cohorts of STS patients because sarcomas are rare tumors. Our study population is quite large compared to similar studies. Fully reassessed histology, scrutinized staining, visualizing and scoring processes, as well as comprehensive clinical data for each patient and rather long follow-ups ensure objectivity to the study performance and assessment. Our research group has investigated the prognostic impact of several families of proteins that are responsible not only for tumor growth, proliferation, and differentiation, but also for angiogenesis and local immunity, and estimate possible co-expressions within and between these marker families.

Although the total amount of patients in our studies is rather large, the histological subgroups are not numerous enough to conduct meaningful subgroup analyses, which is a common problem in sarcoma-related research. Among other possible concerns are differences in treatment over time and between Norwegian and Russian patients, and challenges regarding immunohistochemistry. Nevertheless, the results of the univariate and multivariate analyses of the clinicopathological variables in the present cohort are in accordance with the published literature indicating a representative patient population and a good basis for marker analyses. An important exception is the varying malignancy grade rate between the Norwegian and Russian populations.

In summary, the results of our studies suggest the involvement of these molecular markers in the innate and the adaptive immune system, as well as cell cycle regulatory proteins as predictors for treatment response, metastasis, and treatment strategies within subgroups of STSs. The exact mechanisms of such involvement are, however, yet to be elucidated.

5.2.1. Paper I

TILs are considered to be a response of the host immune reaction to tumor antigens [141], and their clinical significance has been reported in several different cancer subgroups. The purpose of this study was to explore the prognostic significance of TILs in STSs. We used immunohistochemistry to evaluate the CD3+, CD4+, CD8+, CD20+, and CD45+ TIL. In univariate analyses, high numbers of CD4+ (P = 0.008) and CD20+ (P = 0.006) TIL were positively associated with DSS in patients with wide resection margins (n = 108). High numbers of CD3+ (P = 0.028) TIL were negatively associated with DSS in patients with non-wide resection margins (n = 141). In multivariate analyses, a high number of CD20+ TIL (HR = 5.5, CI 95% = 1.6–18.6, P = 0.006) was an independent, positive prognosticator for DSS in patients with wide resections margins. In conclusion high density of CD20+ TIL is an independent, positive prognostic indicator for STS patients with wide resection margins.

5.2.1.1. CD20 positive tumor-infiltrating cells

CD20+ TILs are associated with a better survival in lung cancer, cervical cancer, prostate cancer, and ovarian cancer [208–212]. CD20+ cells in metastatic lymph nodes are positively correlated with better prognosis in patients with oro- and hypopharyngeal carcinoma [213]. A high density of CD20+ cells was associated with a good clinical outcome prognostic factor for stage IIIa gastric cancer [214]. The presence of both CD20+ and CD8+ tumor-infiltrating lymphocytes correlated with increased patient survival compared with CD8+ TIL alone [215]. In contrast, using flowcytometry with CD19, high B-cell infiltration was correlated with poor prognosis in metastatic ovarian carcinoma [216]. In a series of 3,261 prostate cancers, the number of CD20+ cells per tissue spot was not associated with other clinical and histopathological parameters [217]. In our material a high number of CD20+ TILs was an independent, positive prognostic indicator.

5.2.1.2. CD3 positive tumor-infiltrating cells

Several studies show that tumor-infiltrating CD3+ lymphocytes are strongly correlated with improved survival in epithelial tumors [218–222]. Our study did not uncover any such association in the mesenchymal tumors in patients with wide resection margins, but a high number of CD3+ TILs was correlated with reduced DSS in patients with non-wide resection margins. Combining both patients with wide and non-wide resection margins, the results were not statistically significant. Our results were included in forest plots in a meta-analysis of various cancer types [223] (Figure 9).

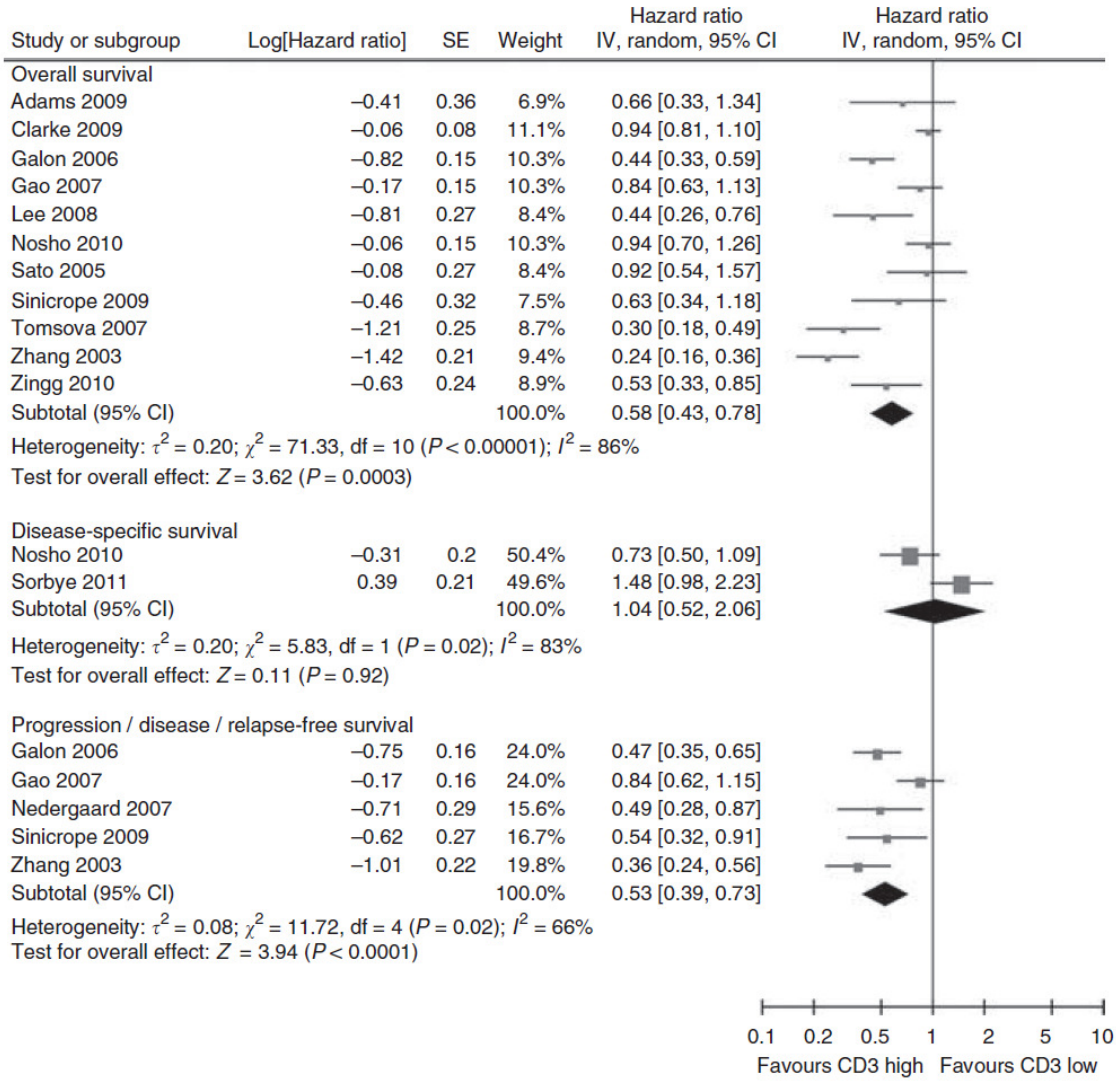

Figure 9. Forest plots of studies on CD3+ TILs. Hazard ratios and 95% confidence intervals from individual studies are depicted as squares and horizontal lines, respectively. The pooled estimate is shown as a diamond shape, where the center represents the pooled HR and the horizontal borders represent the 95% CI. Hazard ratios are defined as high CD3 versus low CD3 counts; therefore a hazard ratio < 1 represents a lower risk of death or progression associated with high CD3 counts [223]. *Permission obtained from British Journal of Cancer.*

In Figure 9 only two studies used disease-specific survival as the main endpoint, but with opposing results. Nosho et al. studied tumor-infiltrating CD3+ T-cells in colorectal cancer [224]. We studied tumor-infiltrating CD3+ T-cells in STSs. Different tumor biology in epithelial and mesenchymal tumors may explain the differences. However, the two studies had overlapping 95% confidence intervals, and both confidence intervals included the number 1.

5.2.1.3. CD4 positive tumor-infiltrating cells

CD4 is a glycoprotein that is expressed on the surface of regulatory T-cells, T helper cells, macrophages, monocytes, and dendritic cells. CD4+ T helper lymphocytes (Th) are a heterogeneous cytokine-secreting class of T-lymphocytes. T helper type 1 lymphocytes (Th1) have a crucial role in activating cytotoxic T-lymphocytes (CTL). T helper type 2 lymphocytes activate eosinophils and stimulate humoral immunity. In terms of antitumor immunity, Th1 activation is more effective than Th2 activation [225]. In cancer, Tregs preferentially move to the tumor by chemotaxis because of chemokines from tumor cells and microenvironmental macrophages [226].

The role of CD4+ T- and B-lymphocytes is controversial in many cancers including STS; CD4+ cells in the absence of the CD8+ cytotoxic T-cells are critical and sufficient for NKT cell-dependent rejection of experimental tumors [227]. In lung cancer the prognostic impact of CD4 is controversial [208, 228], but in our material CD4+ cells were a positive prognostic factor in univariate analyses.

In a meta-analysis of six publications from different cancers studying overall survival in CD4+, the pooled HR is 0.82 (95% CI: 0.69–0.98), which is statistically significant (P = 0.03). In

a pooled analysis, disease-specific survival [208, 232] and progression-free survival [229–231] were not influenced by CD4+ TIL [223].

5.2.1.4. CD8 positive tumor-infiltrating cells

CD8+ TIL has been positively correlated with better survival in a variety of cancers, including carcinomas of the bile duct, colon, endometrium, esophagus, follicular lymphoma, lung, urothelium, and uveal melanoma [116–122, 208, 233]. The prognostic impact of CD8+ TIL in sarcomas is controversial. Most of these studies are based on relatively few cases. There was an association between a high number of stromal CD4+ and CD8+ lymphocytes and favorable prognosis in non-small-cell lung cancer [208]. In our material CD8 was not a significant prognostic factor (P = 0.15). Gooden et al. included 23 studies in the meta-analysis below [223]. Here, the presence of CD8+ results in better prognosis for all survival endpoints tested (Figure 10).

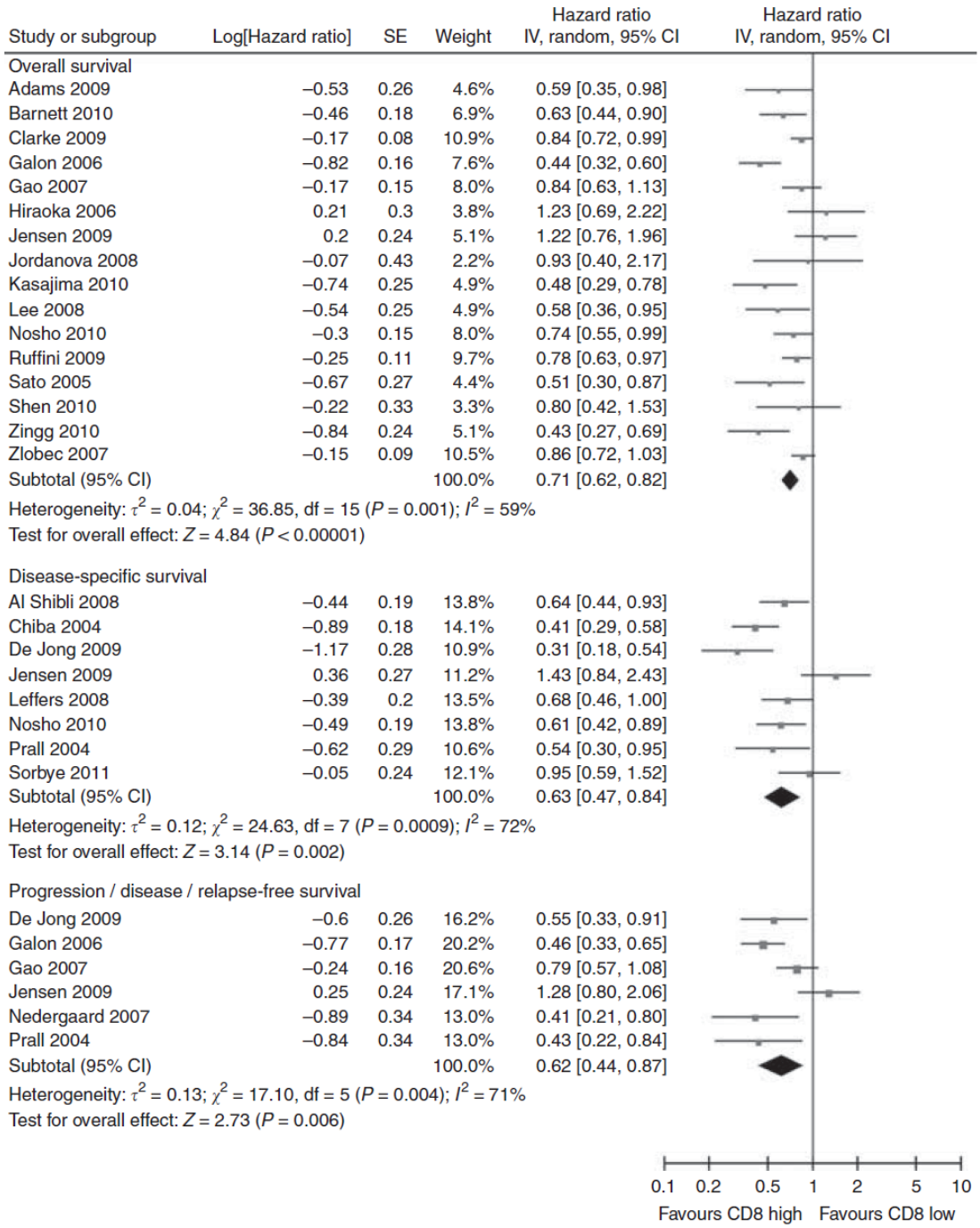

Figure 10. Forest plots of studies on CD8+ TILs. Hazard ratios and 95% confidence intervals for death or progression associated with high versus low CD8 counts [223]. *Permission obtained from British Journal of Cancer.*

5.2.2. Paper II

The purpose of this study was to evaluate the innate immune system in STS. We used immunohistochemistry to study the expression of CD68, M-CSF, CSF-1R, CD57, TGF-beta, and Ki67 in tumor and peritumoral capsule. High co-expressions of M-CSF and TGF-beta in tumor and a high expression of Ki67 in the peritumoral capsule were associated with poor DSS.

5.2.2.1. CD68

Macrophages are the first line of defense against pathogens and are frequently present in the tumor stroma of carcinomas [139, 234, 235]. The majority of tumor-associated macrophages (TAM) produces anti-inflammatory factors and promotes tumor growth. TAMs have a major impact in cancer development because they can adopt tropic roles and are educated by the tumor microenvironment to facilitate tumor cell motility, matrix breakdown, and angiogenesis [236]. Altogether, this gives malignant tumors the capacity to infiltrate the surrounding normal tissues and metastasize to other parts of the body [236, 237]. The pan-macrophage marker CD68 is commonly used to identify TAMs in diagnostic biopsies. Several studies show a negative correlation between high density of TAMs and survival in women with breast cancers [238]. The same is found in thyroid cancer, liver cancer, and non-gynecological leiomyosarcomas [239–241]. No such relationship was observed in malignant melanoma or prostate cancer [242, 243]. High influx of CD68+ TAM improved survival in colon cancer and lung cancer [244, 245]. In our material a high number of CD68+ cells was positivity associated with malignancy grade ($P = 0.016$) but showed no correlation with disease-specific survival ($P = 0.270$).

5.2.2.2. M-CSF

The proliferation and differentiation of monocytes to macrophages is regulated by the hematopoietic growth factor macrophage stimulation factor (M-CSF). In inflammation M-CSF induces macrophages to produce proteases and cytokines, thereby enhancing the macrophages' ability to combat microbial infections [246]. In our study a high M-CSF expression in tumor was positively associated with a high malignancy grade, increased Ki67, and DSS in univariate

analyses. However, the expression of M-CSF in the peritumoral capsule was not correlated with DSS.

5.2.2.3. CSF-1R

The macrophage colony stimulation factor 1 receptor (CSF-1R) is one of the growth factor receptors that regulate proliferation and differentiation of monocytes [253]. The expression of CSF-1R and/or CSF-1 is strongly associated with poor survival in several epithelial tumors, such as breast, ovarian, and prostate cancer [140, 254–256]. A high expression of CSF-1R in peritumoral liver tissue is correlated with poor prognosis in hepatocellular carcinoma [257]. Expression of CSF-1R is associated with histological grade in STSs [252]. In our material the expression of CSF-1R did not correlate with histological grade or DSS, but high CSF-1R was correlated with a high expression of Ki67 ($p = 0.001$, data not shown).

5.2.2.4. CD57

CD57 positive cells are important in the defense against malignant and virally infected cells. Presence of these cells is a positive prognostic marker for DSS in lung cancer [258][259], as well as in other malignant tumors like gastric and colon cancers [128, 129]. A high number of stromal CD57+ cells was positively associated with DSS in patients with lung cancer, whereas a high density of CD57 positive cells within the epithelial tumor cell was not [249].

The location of infiltrating inflammatory cells may be important. There are major differences between 1) CD57 positive cells within epithelial cancer cell nests in carcinomas; 2) CD57 positive cells present in the stroma of epithelial tumors, 3) peritumoral CD57 positive cells present along the invasive margins, and 4) CD57 positive cells in the peritumoral capsule of stromal tumors such as STSs. Even though CD57 may have a favorable impact on DSS in carcinomas, this may not be the case in STSs. In our material there was no such correlation in tumor or peritumoral capsule.

5.2.2.5. TGF-beta

TGF-beta belongs to a TGF-beta/BMP family of growth factors and is basically a tumor-suppressive agent whose functions include proliferation hampering and promotion of apoptosis in both normal and tumor cells. There is, however, broad evidence of its negative influence on prognosis, described mostly in epithelial [183, 184] but also in mesenchymal tumors [185–188]. The possible mechanisms of such pro-neoplastic action include receptor-inactivating mutations, selective inactivation of the tumor-inhibiting arm of this pathway [181], and TGF-beta induced systemic immune suppression [182]. Other proposed modulators of TGF-beta function are factors in the tumor microenvironment, particularly inflammatory cells, cancer-associated fibroblasts [182], and angiogenetic factors [193]. We found TGF-beta to be an important prognostic marker. High TGF-beta expression was negatively associated with DSS in STSs [260]. In our study co-expression of M-CSF and TGF-beta was an even stronger negative prognostic factor.

5.2.2.6. Ki67

Ki67 expression increases with increasing malignancy grade in many different cancers [165–169]. In Ewing's sarcoma, high Ki67 expression was negatively associated with progression-free survival and overall survival [170]. For patients with STSs of the extremity and trunk wall, tumor proliferation can be assessed by Ki67 expression and used in statistical decision-tree models that give prognostic information [261]. In our study high expression of Ki67 in tumor was negatively associated with DSS in patients with STSs, but Ki67 expression was dependent on malignancy grade. Ki67 did not appear as an independent prognosticator in the multivariate analysis.

Ki67 as a predictive and prognostic biomarker has been extensively studied in breast cancer [264]. An expression level of Ki67 above 10%–14% defines a group of women with aggressive breast tumors. Using this definition in future studies may make for more reliable comparisons [265]. In 2009 the panel of experts at the St. Gallen Consensus conference considered the Ki67 labeling index to be imperative for selecting patients with hormone receptor-positive breast cancers for treatment with a combination of chemotherapy and endocrine therapy. The tumors were classified as low, intermediate, and highly proliferating based on the Ki67 expression [266].

In Norway Ki67 analysis has been introduced as a routine in breast cancer and is vital for therapy selection.

5.2.3. Paper III

This study sought to clarify the prognostic significance of the Jab1, p16, p21, p62, Ki67, and Skp2 expressions in STS. A high expression of Skp2 in patients with STSs is associated with poor DSS in women and in STS patients not treated with chemotherapy or radiotherapy.

5.2.3.1. Jab1

Some studies suggest that Jab1 may interact with the protein form of the CDK inhibitor 27 and shuttle p27 from the nucleus to the cytoplasm, and, moreover, Jab1 may decrease the cellular amount of p27 by accelerating p27 degradation via the ubiquitin-proteasome system [143, 144]. Other reports have shown that a high expression of Jab1 and low expression of p27 are correlated with poor survival in a variety of cancers [145–148]. Expression of Jab1 protein in epithelial ovarian borderline tumors was significantly higher than in benign tumors [267]. Overexpression of Jab1 was associated with poor survival in patients with malignant glioma [268]. Tsuchida et al. [269] suggested that Jab1 may play an important role in determining the differentiation stage of rhabdomyosarcoma cells by modulating the activity of CDK inhibitor p27. In our material Jab1 expression was not associated with malignancy grade and had no prognostic impact on DSS.

5.2.3.2. p16

Epigenetic silencing of p16 is probably an important event in the development of Ewing sarcoma [275], and p16 has been shown as a sensitive and specific marker for distinguishing atypical lipomatous tumors, well-differentiated liposarcomas, and dedifferentiated liposarcomas from benign adipocytic neoplasms [276]. In mammary phyllodes tumors high expressions of p16 and pRb are correlated with high tumor grade [167]. High expression of p16 was associated with good response of chemotherapy in osteosarcoma [277]. In a series of 38 pediatric osteosarcomas

there was an inverse correlation between pRB loss and p16 expression, where the absence of p16 expression significantly correlated with poor survival [278]. Low expression of p16 was correlated with poor survival in malignant peripheral nerve sheath tumor [279]. In our study p16 expression was not associated with malignancy grade or DSS.

5.2.3.3. p21

Using in vivo RNA interference, Young et al. implicated the p53 target gene p21 as an important factor in STS development [280]. The expression of p21 was positively correlated with tumor malignancy grade and therefore used as prognostic markers in a series of 152 patients with STSs [90]. In patients with Ewing's sarcoma the expression of p21 ($P = 0.015$) was higher in disseminated as opposed to localized disease tumors, but p21 was not correlated with progression-free or overall survival [170]. In a series of 36 patients with leiomyosarcoma p21 was not correlated with time to recurrence or overall survival [281]. In a series of 169 primary soft tissue sarcomas of the extremities and the trunk wall, expression of p21 was not associated with prognosis [261]. Similarly, in our material p21 was not correlated with malignancy grade or DSS. This can be due to other bypass molecules involved in p53 suppression functions.

5.2.3.4. p62

There are few publications regarding p62 and STSs. Rolland et al. demonstrated that high expression of p63 in breast cancer is associated with tumor progression, but not DSS [282]. In a series of 109 NSCLC, high expression of p62 was correlated with poor survival [283]. Kitamura et al. demonstrated cytosolic overexpression of p62 in prostate adenocarcinoma and high-grade PIN, suggesting that p62 might be a useful marker for prostatic malignancy [284]. In a series of 59 colorectal carcinomas, however, p62 had no prognostic value [285]. High expression of p62 in our material was positively associated with high malignancy grade, but not DSS.

5.2.3.5. Skp2

High expression of Skp2 is negatively associated with overall survival in patients with myxofibrosacroma [287, 288]. Di Vizio et al. [289] found that a high expression of Skp2 is negatively correlated with GIST survival. Oliveira found that a high expression of Skp2 is associated with high cell proliferation and poor prognosis in 182 STSs [290]. High expression of Skp2 in our material was a negative prognostic factor for DSS. Interestingly, this correlation was statistically significant in women only (P = 0.009) (men, P = 0.577). This may be related to differences in expression of sexual hormone receptors (ER and PGR) in male and female STS patients [291, 292]. An inverse correlation between Skp2 expression and the expression of ER and PGR has been reported by others investigating breast cancer [293]. Other studies suggest that Skp2B may modulate the activity of the estrogen receptor [294, 295]. High expression of Skp2 in breast cancer is correlated with p-Akt1 and associated with poor survival [296].

5.2.4. Paper IV

The purpose of this study was to clarify the prognostic significance of Skp2 expression in relation to gender, estrogen receptor (ER), and progesterone receptor (PGR) in STSs. We found diverse prognostic impacts by expression of Skp2, ER, and PGR on DSS in male and female patients with STSs. In men, but not women, an ER positive/PGR negative co-expression profile was an independent, negative prognostic factor for DSS. In women, but not men, Skp2 expression was associated with poor DSS.

Steroid hormones, and therefore their receptors too, are known to stimulate the progression of breast cancer as well as other gynecological tumors. ER served for decades as a predictor of the success of hormone-ablation therapy for ER-positive in contrast to ER-negative breast cancers [174, 175]. A diversity of soft tissue tumors expresses both ER and PGR [176, 301–303], but there is much uncertainty concerning the steroid hormone receptor expression value in the mesenchymal tumors. This is probably due to vagueness of the positivity cut-off point for non-gynecological tumors, which is as high as 10% in most of studies. We have modified the Allred score [304] for STSs and used 1% positivity as the cut-off value. The strong and moderate (score 3 and 2, respectively) hormone receptor expression occurred mostly in

uterus, pelvic, and breast sarcomas, while the weak (score 1) expression of both ER and PGR was surprisingly evenly distributed across location, gender, and age. Generally, 36% of the tumors expressed ER and 30% expressed PGR in our material.

The rate of ER and PGR expression in leiomyomatous tumors of the uterus was frequently demonstrated to rise with the grade of differentiation of malignant tumors from benign leiomyoma to high-grade malignant leiomyosarcoma [176, 177]. However, the information concerning steroid hormone receptor expression in soft tissue tumors outside the gynecological area is scarce and controversial. In our study ER expression (using a positivity threshold at 1%) had a positive impact on survival in women (univariate analysis) but failed to show any significant value in the Cox proportional-hazards analysis. PGR expression showed a clearly negative impact on DSS in men and slightly positive, but not significant influence on survival in women.

The value of ER/PGR co-expression profiles is well studied in breast carcinoma. In few words, any hormone receptor positivity gives a better prognosis for success of antihormonal therapy [305, 306]. In our study the ER-/PGR+ profile (14% of the tumors) was a significantly unfavorable factor for the whole patient cohort both in univariate and in multivariate analyses.

This study is, to our knowledge, the first to elucidate the distribution and prognostic value of steroid hormone receptors in STSs. Both ER and PGR were surprisingly frequently expressed in sarcomas irrelatively to the patient's gender and location of the tumor. Their prognostic significance is not much of a surprise, since both of them in essence are growth factors.

We found diverse prognostic DSS impacts from gender-related expression of Skp2, ER, PGR, and DSS in STSs. In men, but not women, an ER positive/PGR negative co-expression profile was an independent, negative prognostic factor for DSS. In women, but not men, high expression of Skp2 was associated with reduced DSS. High expression of ER reduced the negative impact of Skp2 in women. While women with the Skp2+/ER+ phenotype had improved survival, the Skp2+/ER- had poor survival. To the best of our knowledge, this is the first prognostic evaluation of Skp2 related to the female hormone receptors ER and PGR in STSs.

6. CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

We have investigated markers of the adaptive and the innate immune system and cell cycle regulatory proteins in STS patients. Several markers and interesting co-expressions proved to be independent prognostic factors. Although the precise molecular interactions in STSs are still unclear, our findings may help to identify a subgroup of patients with aggressive tumors that require adjuvant therapy. Moreover, the biomarkers indicating such aggressiveness can represent molecular targets with the future development of small-molecule targeted therapy.

Adjuvant chemotherapy for patients with STSs remains controversial, while improvement in survival has never been conclusively demonstrated for metastatic STSs. In a series of 2,382 patients with resected STS, 106 (4.5%) received chemotherapy. High tumor grade, larger tumor size, and malignant fibrous histiocytoma subtype were associated with chemotherapy receipt [307]. In our material ER and PGR positivity, found to be surprisingly common in STSs, could possibly identify patients who may benefit from endocrine therapy. Among STS patients who have had wide resection margins, it will be essential to identify those who will relapse and succumb to this disease, as these patients may benefit from adjuvant therapy, including immunotherapy. Patients with the ER negative/PGR positive phenotype have especially poor DSS, while men with the ER negative/PGR negative phenotype have better DSS. Women with the ER positive/PGR positive phenotype also have favorable prognosis.

In our material Skp2 was an independent, negative prognostic factor for DSS in women and in patients without administered chemotherapy or radiotherapy. Further studies are warranted to explore if adjuvant chemotherapy or radiotherapy improve the poor prognosis of STSs with high Skp2 expression.

The human immune system contains specialized cells that are able to eliminate cancer cells [110], and tumor-infiltrating B-cells are able to produce tumor-specific antibodies [308]. Through external stimulation of the immune response, these cells may have the potential to aid the immune system in destroying single tumor cells and micro-metastases after surgery. This topic is investigated in the ongoing international osteosarcoma protocol EURAMOS, where those who respond well to chemotherapy are randomized to receive interferon or no interferon, in an attempt to improve the immune response.

TMA and IHC have proven to be reliable and feasible methods for biomarker studies on tissues. While these methods might not be the most novel, they are well proven and highly reliable when one takes into account their limitations. Our group will continue to conduct TMA and IHC studies on STSs. We would particularly like to explore factors responsible for TGF-beta modulation, such as matrix metalloproteinases, integrins, angiogenic and inflammatory agents, as well as the isoforms and specific receptor of this enigmatic growth factor. This also concerns ER and PGR isotypes.

In addition, we have started to measure proliferation-related micro-RNAs by *in situ* hybridization in paraffinized tissue from STS patients. We hope to further clarify prognostic factors in STS patients and to explore the impact of immune system, cycle regulatory proteins, and other prognostic markers in this patient group.

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Prognostic Impact of Lymphocytes in Soft Tissue Sarcomas

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Abstract

Purpose: The purpose of this study was to clarify the prognostic significance of lymphocyte infiltration in soft tissue sarcomas (STS). Prognostic markers in potentially curable STS should guide therapy after surgical resection. The immune status at the time of resection may be important, but the prognostic significance of tumor infiltrating lymphocytes is controversial as the immune system has conflicting roles during cancer development.

Experimental Design: Tissue microarrays from 249 patients with STS were constructed from duplicate cores of viable and representative neoplastic tumor areas. Immunohistochemistry was used to evaluate the CD3+, CD4+, CD8+, CD20+ and CD45+ lymphocytes in tumors.

Results: In univariate analyses, increased numbers of CD4+ (P = 0.008) and CD20+ (P = 0.006) lymphocytes in tumor correlated significantly with an improved disease-specific survival (DSS) in patients with wide resection margins (n = 108). In patients with non-wide resection margins (n = 141) increased numbers of CD3+ (P = 0.028) lymphocytes in tumor correlated significantly with shorter DSS. In multivariate analyses, a high number of CD20+ lymphocytes (HR = 5.5, CI 95% = 1.6–18.6, P = 0.006) in the tumor was an independent positive prognostic factor for DSS in patients with wide resections margins.

Conclusions: High density of CD20+ lymphocytes in STS with wide resection margins is an independent positive prognostic indicator for these patients. Further research is needed to define if CD20+ cells can modify tumors in a way that reduces disease progression and metastatic potential.

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Introduction

Soft tissue sarcomas (STS) are relatively rare, heterogeneous malignancies of mesenchymal origin with a high mortality rate. They comprise less than 1% of adult malignancies[1] and approximately 50% of the STS patients will succumb to their disease because of metastasis or local relapse[2]. There are several prognostic factors which determine tumour progression, and ultimately the patient's outcome, including positive resection margins; presence of local recurrence; and tumour grade, size, location, depth and histological entity[3–9].

Many studies have been designed to investigate the prognostic factors of STS by using immuno-histochemical methods[10]. Most of the published data have focused on the expression of markers for cell kinetics and regulatory proteins of the cell cycle.

Tumor infiltrating lymphocytes are considered to be an indication of the host immune reaction to tumor antigens[11], and their clinical significance has been reported in a variety of human solid tumors.

CD3 is a part of the T cell receptor (TCR) complex on a mature T lymphocyte. CD4 is a glycoprotein expressed on the surface of T helper cells, regulatory T cells, monocytes, macrophages, and dendritic cells. CD8 is a transmembrane glycoprotein that serves as a co-receptor for the T cell receptor (TCR). Like the TCR, CD8 binds to a major histocompatibility complex (MHC) molecule, but is specific for the class I MHC protein. CD20 is a non-glycosylated phosphoprotein expressed on the surface of all mature B-cells. CD20 is expressed on all stages of B cell development except the first and last; it is present from pre-pre B cells through memory cells, but not on either pro-B cells or plasma cells. The CD45 antigen was originally called leukocyte common antigen. The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. This gene is specifically expressed in hematopoietic cells. This PTP has been shown to be an essential regulator of T- and B-cell antigen receptor signalling (http://www.genecards.org).

The purpose of this study was to clarify the prognostic significance of lymphocyte infiltration in non-gastrointestinal

stromal tumor (GIST) STSs. To achieve this, we analyzed the expression of CD3+, CD4+, CD8+, CD20+ and CD45+ lymphocytes in 249 patients with non-GIST STS in relation to other clinicopathological variables.

Materials and Methods

Patients and Clinical Samples

The National Cancer Data Inspection Board and The Regional Committee for Research Ethics approved the study. The Regional Committee approved that written consent from the patients for their information to be stored in the hospital database and used for research was not needed. This because most of the material was more than 10 years old, and most of the patients being dead. The material was collected from our approved biobank for paraffin embedded material and slides. Data were analyzed anonymously.

Primary tumor tissues from patients diagnosed with STS at the University Hospital of North Norway (UNN) from 1973 to 2006 and the Hospitals of Arkhangelsk region, Russia, were used in this retrospective study. 496 potentially suitable patient records were identified from the hospital database but only 249 of these were eligible for this study because they had complete medical records and adequate paraffin-embedded tissues blocks. This report includes follow-up data for 167 Norwegian patients and 82 Russian patients up to September 2009. The median follow-up was 38 (range 0–392) months. Complete demographic and clinical data were collected retrospectively. Formalin-fixed and paraffin-embedded tumor specimens were obtained from the archives of the Departments of Pathology at UNN and Archangelsk. The tumors were graded according to the French Fèdèration Nationales des Centres de Lutte Contre le Cancer (FNCLCC), [WHO Tumors of Soft Tissue and bone, 2002]. Wide resection margins were defined as wide local resection with free microscopic margins or amputation of the affected limb or organ. Non-wide resection margins were defined as either marginal or intralesional resection margins, or no surgery.

Microarray construction

The histology of all soft tissue sarcoma cases were reviewed by two pathologists (AV and SWS). Tissue microarrays (TMAs) were constructed for high-throughput molecular pathology research[12]. The most representative areas of viable tumor cells

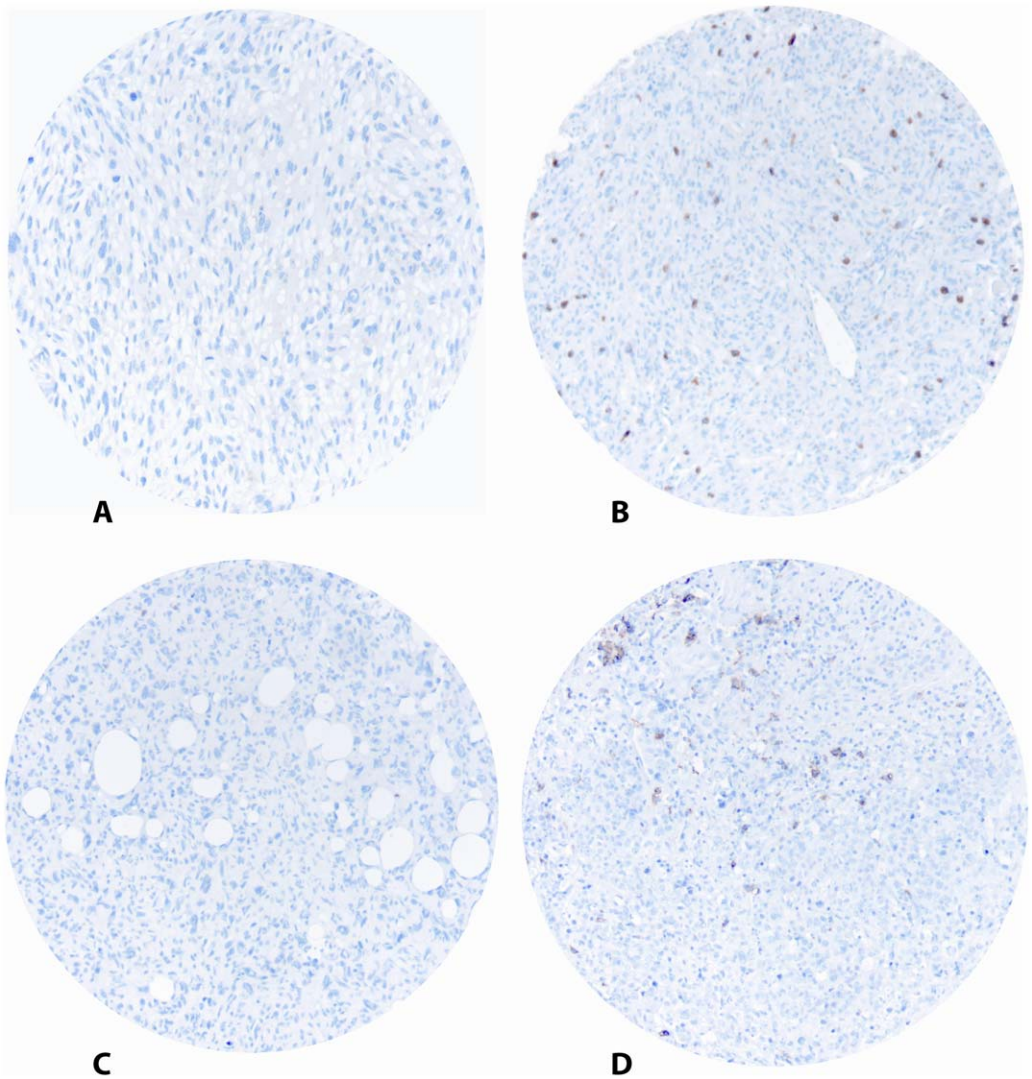

Figure 1. IHC microscopic pictures of TMA of soft tissue sarcoma representing different scores for CD4+ and CD20+ lymphocytes. (A) CD4 low score; (B) CD4 high score; (C) CD20 low score; (D) CD20 high score. Original magnification X 400.
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Table 1. Prognostic clinicopathologic variables as predictors for disease-specific survival soft tissue sarcomas (univariate analysis, log rank test), N = 249.

Characteristic	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Age					
≤20 years	20	8	15	40	0.126
21–60 years	113	45	68	52	
>60 years	116	47	30	40	
Gender					
Male	110	44	41	46	0.390
Female	139	56	45	45	
Nationality					
Norwegian	167	67	63	51	0.011
Russian	82	33	22	34	
Histology					
Undifferentiated pleomorphic sarcoma	68	27	29	40	0.102
Leiomyosarcoma	67	27	45	46	
Liposarcoma	34	14	NR	67	
MF/MFT	20	8	43	50	
Angiosarcoma	13	5	10	31	
Rhabdomyosarcoma	16	6	17	38	
MPNST	11	4	49	45	
Synovial sarcoma	16	6	31	29	
Other STS	4	2	NR	75	
Tumor localization					
Extremities	89	36	100	53	0.348
Trunk	47	29	32	44	
Retroperitoneum	37	25	25	38	
Head/Neck	18	7	15	41	
Visceral	58	23	30	42	
Tumor size					
≤5 cm	74	30	127	57	0.027
5–10 cm	91	37	44	45	
>10 cm	81	32	28	37	
Missing	3	1			
Malignancy grade FNCLCC					
1	61	25	NR	74	<0.001
2	98	39	41	45	
3	90	36	16	26	
Tumor depth					
Superficial	17	7	NR	93	<0.001
Deep	232	93	36	42	
Metastasis at time of diagnosis					
No	206	83	76	53	<0.001
Yes	43	17	10	10	
Surgery					
Yes	228	92	59	50	<0.001
No	21	8	5	0	
Surgical margins					
Wide	108	43	NR	62	<0.001

Table 1. Cont.

Characteristic	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Non-wide	141	57	19	33	
Chemotherapy					
No	191	77	52	47	0.424
Yes	58	23	29	40	
Radiotherapy					
No	176	71	48	46	0.590
Yes	73	29	38	43	

Abbreviations: MF/MFT, malignant fibroblastic/myofibroblastic tumors; MPNST, malignant peripheral nerve sheath tumor; STS, soft tissue sarcomas; NR, not reached; NOS, non specified.

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were carefully selected and marked on the hematoxylin and eosin (HE) slides for the corresponding donor blocks and sampled for the tissue microarray collector blocks. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments).

Studies suggest that punching multiple 0.6 mm cores from different regions captures the heterogeneity of the tumors more accurately than single 2 to 4 mm core[13]. Hence, we chose using two 0.6-mm cores of viable neoplastic tissue that were selected to be as representative as possible (different areas), after reviewing all original sections of the tumor and taking the heterogeneity in consideration. To include all core samples, 12 tissue array blocks were constructed. Multiple 4-µm sections were cut with a Micron microtome (HM355S) and stained by specific antibodies for immunohistochemistry (IHC).

Immunohistochemistry (IHC)

The applied antibodies were subjected to in-house validation by the manufacturer for IHC analysis on paraffin-embedded material. Ventana Benchmark, XT automated slide stainer (Ventana Medical System, France) was used for IHC. Sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the specimens in 0.01 M citrate buffer at pH 6.0 and exposing them to two repeated microwave heatings of 10 minutes each at 450W. The DAKO Envision+ System-HRP (DAB) kit was used as endogen peroxidase blocking. As negative staining controls, the primary antibodies were replaced with the primary antibody diluents. Primary mouse monoclonal antibodies were incubated for 16 minutes (CD20, clone L26 Ventana), 20 minutes (CD4, clone 1F6 Novocastra, dilution 1:5) and 32 minutes (CD8, clone 1A5 Ventana) at room temperature. The Ventana antibodies were pre-diluted from the manufacturer. Biotinylated goat anti-mouse IgG and mouse anti-rabbit IgM were used as secondary antibodies. The DAB was used to visualize the antigens. This was followed by application of liquid diaminobenzidine and substrate-chromogen, yielding a brown reaction product at the site of the target antigen. Finally, slides were counterstained with hematoxylin to visualize the nuclei. For each antibody, including negative controls, all TMA staining was performed in a single experiment.

Scoring of IHC

The ARIOL imaging system (Genetix, San Jose, CA) was used to scan the slides for antibody staining of the TMAs. The specimens were scanned at a low resolution (1.25×) and high resolution (20×) using an Olympus BX 61 microscope with an automated platform (Prior). The slides were loaded in the automated slide loader (Applied Imaging SL 50). Representative and viable tissue sections were scored manually on a computer screen semi-quantitatively for cytoplasmic staining. Tumors were scored as 0 (no cells), 1 (1–5 cells), 2 (6–19 cells) or 3 (20+ cells) (Figure 1). All samples were made anonymous and independently scored by two pathologists (AV and SWS). Where there was disagreement, the slides were re-examined and a consensus was reached by the observers. When assessing a variable for a given score, the scores of the other variables and the outcome were hidden from the observers.

Statistical Methods

All statistical analyses were done using the statistical package SPSS (Chicago, IL), version 16. The immunohistochemistry scores from each observer were compared for interobserver reliability by use of a two-way random effect model with absolute agreement definition. The intraclass correlation coefficient (reliability coefficient) was obtained from these results.

The Chi-square test and Fishers Exact test were used to examine the association between molecular marker expression and various clinicopathological parameters. Univariate analyses were done using the Kaplan-Meier method, and statistical significance between survival curves was assessed by the log rank test. Disease-specific survival (DSS) was determined from the date of histological-confirmed STS diagnosis.

Multivariate analysis was carried out using the Cox proportional hazards model to assess the specific impact of each pre-treatment variable on survival in the presence of other variables. Only variables of significant value from the univariate analysis were entered into the Cox regression analysis. Probability for stepwise entry and removal was set at 0.05 and 0.10, respectively. The significance level used was $p<0.05$.

Results

Clinicopathological Variables

Demographic, clinical, and histopathological variables are shown in Table 1. Patient age range was 0–91 years (mean 55 years), and 44% of the patients were males. The non-GIST STS comprised 68 undifferentiated pleomorphic sarcoma, 67 leiomyosarcoma, 34 liposarcoma, 20 malignant fibroblastic/myofibroblastic tumors, 16 rhabdomyosarcoma, 16 synovial sarcoma, 13 angiosarcoma, 11 malignant peripheral nerve sheath tumors (MPNST) and 4 other STS. There were 61 low grade STS (24%) and 188 high grade (FNCLCC grade 2 and 3) STS (76%).

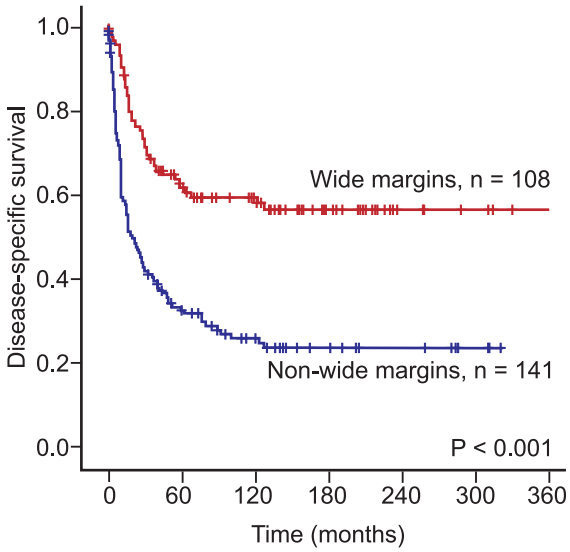

Figure 2. Disease-specific survival curves for patients with wide resection margins compared to patients with non-wide resection margins.
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The treatment option of choice was surgery (n = 228): 118 patients received surgery only; 55 patients received surgery and radiotherapy; 40 patients received surgery and chemotherapy; 13 patients received surgery, radiotherapy and chemotherapy; 2 patients received chemotherapy only; 3 patients received chemotherapy and radiotherapy; 2 patients received radiotherapy only; and16 patients received no therapy. The 5-year survival with non-wide resection margins was 33% and with wide resection margins it was 62%.

Inter-observer variability

There was good reproducibility between the two investigating pathologists. Their scoring agreement was tested for CD8 and CD20. The IHC scores from each observer were compared using a two-way random effect model with absolute agreement definition. The intra-class correlation coefficients (reliability coefficients, r) obtained from these results were 0.90 for CD8 ($P<0.001$) and 0.90 for CD20 ($P<0.001$).

Univariate analyses

Nationality, tumor size, malignancy grade, tumor depth, metastasis at time of diagnosis, surgery and surgical margins were all significant indicators for disease-specific survival (DSS) in univariate analyses (Table 1, Figure 2). Most of the patients with non-GIST STS who did not survive their disease, died within the first 10 years (120 months). After 10 years almost 60% (n = 108) of the patients with wide resection margins were alive, but only 20% (n = 141) of patients with non-wide resection margins or no surgery ($P<0.001$), (Figure 2).

Furthermore, increasing numbers of CD4+ ($P = 0.008$) and CD20+ lymphocytes in tumor ($P = 0.006$) correlated significantly

Table 2. Intratumoral lymphocyte infiltration and their prediction for disease-specific survival in patients with soft tissue sarcomas (univariate analysis; log-rank test), N = 249.

	Non-wide resections margins, n = 141					Wide resection margins, n = 108				
Marker expression	**Patients (n)**	**Patients (%)**	**Median survival (months)**	**5-Year survival (%)**	**P**	**Patients (n)**	**Patients (%)**	**Median survival (months)**	**5-Year survival (%)**	**P**
CD 3										
Low	95	67	26	39	0.028	81	75	NR	64	0.983
High	26	18	15	28		19	18	NR	61	
Missing	20	14				8	7			
CD 4										
Low	112	79	23	35	0.474	85	79	NR	57	0.008
High	18	13	12	33		16	15	NR	94	
Missing	11	8				7	6			
CD 8										
Low	98	70	22	37	0.349	85	79	NR	61	0.150
High	21	15	26	29		19	18	NR	79	
Missing	22	16				4	4			
CD 20										
Low	103	73	26	34	0.447	83	77	NR	55	0.006
High	20	14	11	35		22	20	NR	86	
Missing	18	13				3	3			
CD 45										
Low	113	80	21	33	0.745	87	81	NR	61	0.530
High	19	13	25	37		18	17	NR	67	
Missing	9	6				3	3			

Abbreviations: NR, not reached.
doi:10.1371/journal.pone.0014611.t002

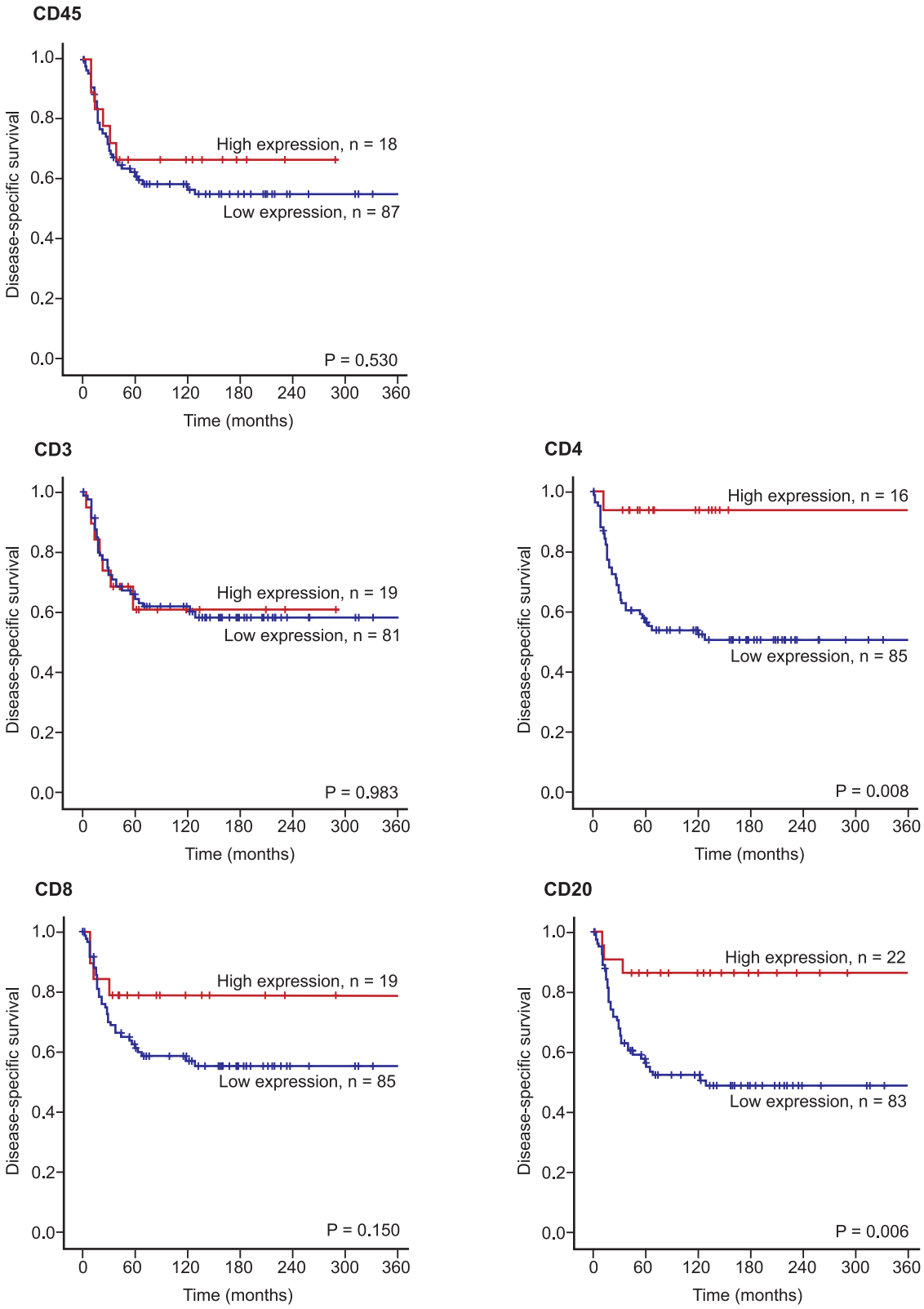

Figure 3. Disease-specific survival curves for CD3+, CD4+, CD8+, CD20+ and CD45+ lymphocytes in STS with wide resection margins.
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with an improved DSS in patients with wide resection margins (n = 108), (Table 2 and Figure 3). No such relationship was apparent for CD3+, CD8+ and CD45+ lymphocytes. In patients with non-wide resection margins (n = 141) increasing numbers of CD3+ lymphocytes correlated significantly (P = 0.028) with shorter DSS, (Table 2).

Improved survival was seen in patients younger than 60 years (P = 0.005), in tumors of histological grade 1 and 2 (P = 0.011), in tumors less than 5 cm (P = 0.018) and in patients who received chemotherapy (P = 0.024). This was shown through subgroup analysis of patients with high CD20+ lymphocytes in tumor and wide resection margins, Table 3. The same statistical trend was seen for gender, nationality, histology, tumor localization and in patients with or without radiotherapy, but these were not statistically significant (data not shown). There were no significant differences in the expression of the different immunomarkers in the different tumor groups (data not shown).

Multivariate analyses

Significant demographic, clinicopathological, and lymphocyte infiltrate variables from the univariate analyses were entered into the multivariate Cox regression analysis. An independent positive prognostic factor for improved DSS in patients with wide resection margin was a high number of CD20+ lymphocytes in the tumor (HR 5.5, CI 95% 1.62–18.61, P = 0.006).

Independent negative prognostic variables were Russian nationality (P = 0.020), high malignancy grade (P = 0.016) and metastasis at time of diagnosis (P = 0.001, Table 4). In patients with non-wide resection margins (n = 141) increasing numbers of CD3+ lymphocytes was an independent negative prognostic factor for DSS, (HR 2.2, CI 95% 1.25–3.89, P = 0.007), (Table 4).

Discussion

In this large scale study, we evaluated whether there is an association between the prevalence of CD3+, CD4+, CD8+, CD20+ and CD45+ lymphocytes in tumors and survival prognosis in 249 non-GIST STS patients. Interestingly, high intensities of CD20+ cells in tumors were an independent positive prognostic factor in patients with wide resection margins.

To our knowledge, this is the first report on CD20 expression in non-GIST STS and the first evidence of its possible clinical

Table 3. Results of subgroup analysis of patients with CD20+ lymphocytes in tumor and wide resection margins, n = 108.

Subgroup	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Age					
<60, CD20 Low	49	79	-	56	0.005
<60, CD20 High	11	18	-*	100	
Missing	2	3			
>60, CD20 Low	34	74	NR	54	0.347
>60, CD20 High	11	24	NR	73	
Missing	1	2			
Histological grade					
1 or 2, CD20 Low	52	76	-	67	0.011
1 or 2, CD20 High	14	21	-	100	
Missing	2	3			
3, CD20 Low	31	78	28	34	0.155
3, CD20 High	8	20	NR	63	
Missing	1	3			
Tumor size					
<5 cm, CD20 Low	28	70	-	63	0.018
<5 cm, CD20 High	10	25	-	100	
Missing	2	5			
>5 cm, CD20 Low	53	78	63	52	0.169
>5 cm, CD20 High	12	18	NR	75	
Missing	3	4			
Chemotherapy					
Yes, CD20 Low	24	80	-	82	0.024
Yes, CD20 High	5	17	-	100	
Missing	1	3			
No, CD20 Low	59	76	NR	59	0.080
No, CD20 High	17	22	NR	46	
Missing	2	3			

* Median survival is not computed because all cases are censored.
Abbreviations: NR, not reached.
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Table 4. Results of Cox regression analysis summarizing some significant independent prognostic factors in patients with soft tissue sarcomas, N = 249.

	Non-wide resections margins, n = 141			Wide resection margins, n = 108		
Factor	**Hazard Ratio**	**95% CI**	**P**	**Hazard Ratio**	**95% CI**	**P**
Nationality						
Norwegian	1.000			1.000		
Russian	1.635	0.978–2.731	0.061	2.246	1.135–4.444	0.020
Tumor size			0.428*			0.874*
≤5 cm	1.000			1.000		
5–10 cm	0.826	0.463–1.605		1.217	0.547–2.708	
>10 cm	1.232	0.690–2.199		1.045	0.389–2.806	
Malignancy grade FNCLCC			0.024*			0.016*
1	1.000			1.000		
2	1.237	0.611–2.506		3.464	1.081–11.096	0.036
3	2.214	1.108–4.425		5.046	1.656–15.376	0.004
Metastasis at time of diagnosis						
No	1.000			1.000		
Yes	3.651	2.081–6.409	<0.001	3.872	1.696–8.836	0.001
CD3						
Low	1.000			NIA		
High	2.202	1.245–3.893	0.007			
CD4						
Low	NIA			4.126	0.551–30.895	0.168
High				1.000		
CD20						
Low	NIA			5.503	1.627–18.606	0.006
High				1.000		

* Overall significance as a prognostic factor.
Abbreviations: NIA, not included in analysis.
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relevance in non-GIST STS patients with wide resection margins. This may suggest that CD20+ cells in the tumor are mediating a strong anti-tumor immune response in STS, but this effect is not strong enough to improve survival in patients without wide resection margins.

Activation of the adaptive immune system may suppress malignant cells, whereas activation of various types of innate immune cells may promote tumor growth[14]. The adaptive immunity, orchestrated by antigen-specific T and B-lymphocytes, inhibits tumor growth through both direct killing by cytotoxic T-lymphocytes, and a combination of cytokine and antibody mediated tumor cell lysis[14]. Cancer infiltration by tumor reactive T-lymphocytes is required for efficient tumor eradication[15]. However, cancer cells can escape the immune system in several ways including suppression of cytotoxic T-cells, by regulatory T-cells and by accumulation of myeloid suppressor cells[15–17].

Tumor-infiltration CD3+ cells are reported to be strongly associated with favorable prognosis in epithelial tumors in several studies[18–22]. The CD3+ cell is an independent positive predictor of response to neoadjuvant chemotherapy in breast cancer[23]. Low numbers of CD3+ lymphocytes predicted shorter disease-free survival in colon cancer[24] and cervical cancers[25]. However, T-cell parameters including CD3 values showed no correlation with survival in cases of metastatic ovarian carcinoma[26]. Accordingly, we did not find any such association in our mesenchymal material in patients with wide resection margins, but CD3 was a negative prognostic factor in patients with non-wide resection margins.

The role of CD4+ T and B lymphocytes is controversial in many cancers including STS; CD4+ cells in the absence of the CD8+ cytotoxic T cells are critical and sufficient for NKT cell-dependent rejection of experimental tumours[27]. In lung cancer the prognostic impact of CD4 is controversial[28,29], but in our material CD4+ cells were a positive prognostic factor in univariate analyses.

CD8+ cells in malignant tumors have been associated with a better survival in many types of cancer including: non-small cell lung carcinoma; carcinomas of the endometrium, bile duct, colon, oesophagus, urothelium; and uveal melanoma and follicular lymphoma[28,30–37]. However, the role of CD8+ lymphocytes in STS is controversial and most of the studies contain relatively few cases. There was a positive correlation between a high density of CD4+ and CD8+ lymphocytes in stroma and improved disease-specific survival in non-small cell lung cancer[28]. In our material CD8 was not a statistically significant factor (P = 0.15).

CD20+ cells are associated with a better survival in lung cancer, cervical cancer, prostate cancer and ovarian cancer[25,28,38–40]. CD20+ B-cells in metastatic lymph nodes are associated with favourable outcome in patients with oro- and hypopharyngeal

carcinoma[41]. On the other hand, B-cell infiltration detected by flowcytometry with CD19 were correlated with unfavourable outcome in metastatic ovarian carcinoma[26]. In our material high density of CD20+ lymphocytes was an independent positive prognostic indicator.

In cervical cancer no significant impact of CD45+ cells were seen [25], neither was it in non-GIST STS in this study.

The optimal chance for curing localized STS is based on wide resection surgery. Given that of the majority of STS patients succumb to this disease within 5 years, there is an apparent need for better systemic therapy including novel molecularly targeted therapies[42]. In our study, there was a 33% 5-year survival in the group with non-wide resection margins and 62% of those with wide resection margins.

Among STS patients who have had wide resection margins, it will be essential to identify those who will relapse and succumb this disease as these patients may benefit from adjuvant therapy, including immunotherapy. Until now adjuvant chemotherapy has been controversial due to inadequate selection criteria.

The human immune system contains specialized cells that are able to eliminate cancer cells[26], and tumor-infiltrating B-cells are able to produce tumor-specific antibodies[43]. Through external stimulation of the immune response, these cells may have the potential to aid the immune system in destroying single tumor cells and micro-metastases after surgery. This topic is investigated in the ongoing international osteosarcoma protocol EURAMOS where those who respond well to chemotherapy are randomized to receive interferon or no interferon, in an attempt to improve the immune response (http://www.ctu.mrc.ac.uk/euramos/).

In conclusion, high density of CD20+ lymphocytes in STS with wide resection margins is an independent positive prognostic indicator for these patients. Further research to define if CD20+ cells can modify tumors in a way that reduces disease progression and metastatic potential is needed.

Author Contributions

Conceived and designed the experiments: SWS TK AV TD RMB LTB. Performed the experiments: SWS TK AV. Analyzed the data: SWS TK AV TD RMB LTB. Contributed reagents/materials/analysis tools: SWS TK AV TD ES KAS LTB. Wrote the paper: SWS TD ES KAS RMB LTB.

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BMC Clinical Pathology

RESEARCH ARTICLE Open Access

Prognostic impact of CD57, CD68, M-CSF, CSF-1R, Ki67 and TGF-beta in soft tissue sarcomas

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Abstract

Background: Prognostic markers in curable STS may have the potential to guide therapy after surgical resection. The purpose of this study was to clarify the prognostic impact of the presence of cells and growth factors belonging to the innate immune system in soft tissue sarcomas (STS). The significance of macrophages (CD68), their growth factor macrophage colony-stimulating factor (M-CSF), its receptor colony-stimulating factor-1 receptor (CSF-1R), natural killer cells (CD57) and the general immunomodulating molecule (TGF-beta) are all controversial in STS. Herein, these markers are evaluated and compared to the cell proliferation marker Ki67.

Methods: Tissue microarrays from 249 patients with non-gastrointestinal (non-GIST) STS were constructed from duplicate cores of viable and representative neoplastic tumor areas and duplicate cores of peritumoral capsule. Immunohistochemistry was used to evaluate the expression of CD68, M-CSF, CSF-1R, CD57, TGF-beta and Ki67 in tumor and peritumoral capsule.

Results: In univariate analyses increased expression of M-CSF ($P = 0.034$), Ki67 ($P < 0.001$) and TGF-beta ($P = 0.003$) in tumor correlated with shorter disease-specific survival (DSS). Increased expression of CD68 in tumor correlated significantly with malignancy grade ($P = 0.016$), but not DSS ($P = 0.270$). Increased expression of Ki67 in peritumoral capsule tended to correlate with a shorter DSS ($P = 0.057$). In multivariate analyses, co-expression of M-CSF and TGF-beta ($P = 0.022$) in tumor and high expression of Ki67 ($P = 0.019$) in peritumoral capsule were independent negative prognostic factors for DSS.

Conclusions: Increased co-expression of M-CSF and TGF-beta in tumor in patients with STS, and increased expression of Ki67 in peritumoral capsule were independent negative prognostic factors for DSS.

Keywords: Soft tissue sarcomas, STS, Malignancy grade, DSS, Macrophages, NK cells, CD57, Ki67, TGF-beta, TMA

Background

Soft tissue sarcomas (STS) are heterogeneous malignancies originating from the mesenchymal lineage. There are more than 50 different histological entities and they comprise less than 1% of adult malignancies [1]. The STS are among the most aggressive cancer types with a lethality of 40–50% due to metastasis or local relapse [2]. There are several prognostic factors which determine tumor progression, and ultimately the patient's outcome, including positive resection margins, presence of local recurrence, histological entity and tumor grade, size, location and depth [3-9].

Many studies have been designed to investigate the prognostic factors of STS by using immunohistochemical methods [10], and most of the published data have focused on the expression of markers for cell kinetics and regulatory proteins of the cell cycle.

Immunotherapy and vaccines with the capability to activate the host immune system may have a role as second-line therapy, and characterization of the in situ cellular and molecular immunology form the basis for such therapy [11]. Hence, clinical data on the prognostic significance of different immunological cells are warranted.

The innate immune system consists mainly of granulocytes, macrophages, natural killer (NK) cells, dendritic cells (DCs) and their corresponding growth factors and receptors [12]. They mediate major histocompatibility

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complex unrestricted cytotoxicity and are essential in the immediate limitation and elimination of foreign challenges to the host, including defense against cancer, but lack the ability of 'memory' when re-exposed to the same antigen[12,13]. The NK cell has a well-established role in tumor rejection in a variety of cancers[14-16], and the mechanism by which these cells discriminate tumor from normal cells has provided new insights into tumor immunosurveillance and has suggested new strategies in the treatment of human cancer [17,18].

Ki67 expression increases with increasing malignancy grade in many cancer types of different lineages [19-23]. In Ewing's sarcoma, high Ki67 expression was an independent prognostic factor for progression free survival and overall survival independent of treatment type [24].

We have previously reported the prognostic significance of the humoral immune system by lymphocyte infiltration in tumor [25] and peritumoral capsule [26] of STS. We have also reported the significance of the innate immune system by the correlation of expression of macrophages (CD68), their growth factor macrophage colony-stimulating factor (M-CSF), its receptor colony-stimulating factor-1 receptor (CSF-1R) and histological grade in STS [27]. It was important to validate these findings in a different material, explore the relationship to expression of Ki67, disease-specific survival (DSS) and include other markers as CD57 and TGF-beta. The purpose of this study was to examine the prognostic role of the innate immune system in STS by assessing the expression of CD68, M-CSF, CSF-1R, CD57, TGF-beta and Ki67.

Methods

Patients and clinical samples

The National Cancer Data Inspection Board and The Regional Committee for Research Ethics approved the study. The material was collected from our approved biobank for paraffin embedded material and slides. Data were analyzed anonymously.

Primary tumor tissue from untreated patients diagnosed with STS at the University Hospital of North Norway (UNN) from 1973 to 2006 and the Hospitals of Arkhangelsk region, Russia, from 1996 to 2006 was used in this retrospective study. 496 potentially suitable patient records were identified from the hospital database, but only 249 of these were eligible based on complete medical records and adequate paraffin-embedded tissue blocks. In 80 of these cases it was also possible to obtain tissue from the peritumoral capsule for TMA [26]. This report includes follow-up data for 167 Norwegian and 82 Russian patients until September 2009. The median follow-up was 38 (range 0–392) months.

The histology of all soft tissue sarcoma cases was reviewed according to modern classification (WHO, 2002) by two dedicated pathologists (AV and SWS). For the Russian material, new slides were made from all paraffin blocks. For the Norwegian material, new slides were made when necessary. All biopsies were immunostained with cytokeratin (CK), c-kit (CD117), Actin, smooth muscle actin (SMA), vimentin (VIM) and CD34. Some slides were also stained with S100 if necessary to rule out differential diagnoses. Further molecular methods were, in general, not considered necessary for differential diagnostics, but in some cases PCR or FISH were performed. About 10% of the initial diagnoses were revised due to altered classification and the appearance of new entities such as GIST. All carcinosarcomas, endometrial sarcomas, carcinomas and lymphomas were excluded.

Microarray construction

Tissue microarrays (TMAs) were constructed for high-throughput molecular pathology research[28-30]. The slides were evaluated by two pathologists (AV and SWS) using light microscope to identify the tumor and the peritumoral capsule. The most representative areas of the tumor and peritumoral capsule were carefully selected and marked on the hematoxylin and eosin (HE) slides for the corresponding donor blocks and sampled for the tissue microarray collector blocks[26]. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments).

Studies suggest that punching multiple 0.6 mm cores from different regions captures the heterogeneity of the capsule more accurately than single 2 to 4 mm cores [30]. Hence, we obtained two 0.6-mm cores of tumor and two cores from peritumoral capsule (four cores from each patient). These were secured from different representative areas of the tissue block and selected to be as representative as possible. To include all core samples, 12 tissue array blocks were constructed. Multiple 4-μm sections were cut with a Micron microtome (HM355S) and specific antibodies were stained for immunohistochemistry (IHC).

Immunohistochemistry (IHC)

Sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the specimens in 0.01 M citrate buffer at pH 6.0 and exposing them to two repeated microwave heatings of 10 min at 450 W. The slides were then transferred to the Ventana Benchmark, XT automated slide stainer (Ventana Medical System, Illkirch, France). Tissue sections were incubated with primary mouse monoclonal antibodies recognizing Ki67, CD68 and CD57 (Ventana Medical System), as well as rabbit polyclonal M-CSF, CSF-1R (clone H-300; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TGF-beta (clone sc-146; Santa Cruz). The dilution was 1:5 for M-CSF, 1:25 for CSF-1R and 1:50 for TGF-beta. All Ventana antibodies were

prediluted by the manufacturer. The incubation periods were 16 min for Ki67, CD57 and CD68, and 28 min for TGF-beta, M-CSF and CSF-1R. As secondary antibodies, biotinylated goat antimouse IgG and mouse antirabbit IgM, both 200 lg ml, were used. The Dako EnVision + System-Horseradish Peroxidase [diaminobenzidine (DAB)] kit (Dako, Glostrup, Denmark) was used to block endogenous peroxidase. This was followed by application of liquid diaminobenzidine as substrate-chromogen, yielding a brown reaction product at the site of the target antigen (iView DAB® procedure). Finally, slides were counterstained with hematoxylin to visualize the nuclei. For each antibody, including controls, all TMA staining were performed in a single experiment. As negative staining controls, the primary antibodies were replaced with the primary antibody diluents. In the TMA we also used cores from carcinomas and normal tissue as positive and negative controls.

Scoring of IHC

The ARIOL imaging system (Genetix, San Jose, CA) was used to scan the slides for antibody staining of the TMAs [26]. The number of CD57 positive cells (including NK cells) and CD68 positive cells (including macrophages) in tumors were scored as 0 (no cells), 1 (1–5 cells), 2 (6–19) or 3 (20+ cells) per 0.6 mm core. Examples are shown in Figure 1. Regarding M-CSF, CSF-1R, Ki67 and TGF-beta, expression was scored as: 0, negative; 1, weak; 2, intermediate; and 3, strong. The mean score from the duplicate cores from tumor or capsule, respectively, was used. Marker expression was dichotomised (low vs. high), and high expression defined as mean score ≥ 0.30 for CD68, ≥ 0.75 for TGF-beta, ≥ 2.00 for Ki67 and ≥ 0.01 for CD57, M-CSF and CSF-1R. All samples were anonymized and independently scored by two pathologists (AV and SWS). When disagreement, the slides were re-examined and consensus was reached by the observers. When assessing a variable for a given score, the scores of the other variables and the outcome were hidden from the observers.

Statistical methods

All statistical analyses were done using the statistical package SPSS (Chicago, IL), version 18. The immunohistochemistry scores from each observer were compared for interobserver reliability by use of a two-way random effect model with absolute agreement definition. The intraclass correlation coefficient (reliability coefficient) was obtained from these results.

The Chi-square test and Fishers Exact test were used to examine the association between molecular marker expression and various clinicopathological variables. Univariate analyses were performed using the Kaplan-Meier method, and statistical significance between survival curves was assessed by the log rank test. Disease-specific

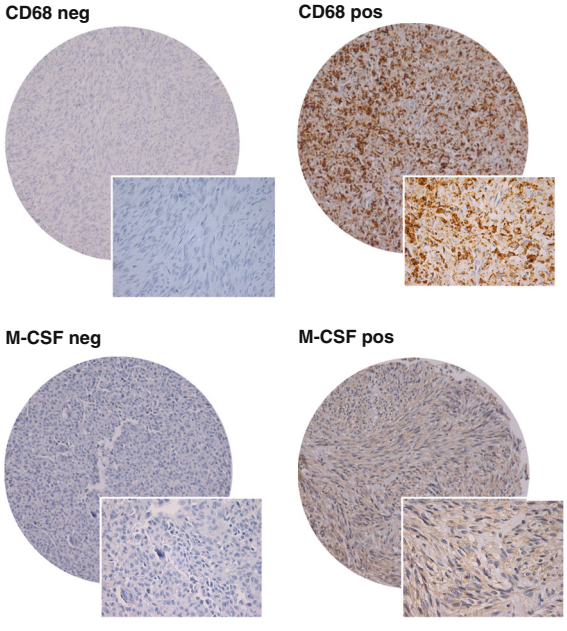

Figure 1 IHC microscopic pictures of TMA of soft tissue sarcoma representing different expression of CD68 and M-CSF. (A) CD68 low expression; (B) CD68 high expression; (C) M-CSF low expression; (D) M-CSF high expression. Original magnification X 100 and 400.

Table 1 Prognostic clinicopathological variables as predictors for disease-specific survival soft tissue sarcomas (univariate analysis, log rank test), N = 249

Characteristic	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Age					
≤ 20 years	20	8	15	40	0.126
21-60 years	113	45	68	52	
> 60 years	116	47	30	40	
Gender					
Male	110	44	41	46	0.390
Female	139	56	45	45	
Nationality					
Norwegian	167	67	63	51	0.011
Russian	82	33	22	34	
Histology					
Undifferentiated pleomorphic sarcoma	68	27	29	40	0.102
Leiomyosarcoma	67	27	45	46	
Liposarcoma	34	14	NR	67	
MF/MFT	20	8	43	50	
Angiosarcoma	13	5	10	31	
Rhabdomyosarcoma	16	6	17	38	
MPNST	11	4	49	45	
Synovial sarcoma	16	6	31	29	
Other STS	4	2	NR	75	
Tumor localization					
Extremities	89	36	100	53	0.348
Trunk	47	29	32	44	
Retroperitoneum	37	25	25	38	
Head/Neck	18	7	15	41	
Visceral	58	23	30	42	
Tumor size					
< 5 cm	74	30	127	57	0.027
5-10 cm	91	37	44	45	
> 10 cm	81	32	28	37	
Missing	3	1			
Malignancy grade FNCLCC					
1	61	25	NR	74	<0.001
2	98	39	41	45	
3	90	36	16	26	
Tumor depth					
Superficial	17	7	NR	93	<0.001
Deep	232	93	36	42	
Metastasis at time of diagnosis					
No	206	83	76	53	<0.001
Yes	43	17	10	10	

Table 1 Prognostic clinicopathological variables as predictors for disease-specific survival soft tissue sarcomas (univariate analysis, log rank test), N = 249 *(Continued)*

Surgery					
Yes	228	92	59	50	<0.001
No	21	8	5	0	
Surgical margins					
Wide	108	43	NR	62	<0.001
Non-wide	141	57	19	33	
Chemotherapy					
No	191	77	52	47	0.424
Yes	58	23	29	40	
Radiotherapy					
No	176	71	48	46	0.590
Yes	73	29	38	43	

Abbreviations: MF/MFT, malignant fibroblastic/myofibroblastic tumors; MPNST, malignant peripheral nerve sheath tumor; STS, soft tissue sarcomas; NR, not reached; NOS, non specified.

survival (DSS) was determined from the date of confirmed STS diagnosis.

The multivariate analysis was carried out using the Cox proportional hazards model to assess the independent impact of each pre-treatment variable on survival in the presence of other variables. Only significant variables from the univariate analyses were entered into the Cox regression analysis. Probability for stepwise entry and removal was set at 0.05 and 0.10, respectively. The significance level used was $p < 0.05$.

Results

Clinicopathological variables

Demographic, clinical, and histopathological variables are shown in Table 1. Patient age range was 0–91 years (mean 55 years), and 44% of the patients were males. The non-GIST STS comprised 68 undifferentiated pleomorphic sarcoma, 67 leiomyosarcoma, 34 liposarcoma, 20 malignant fibroblastic/myofibroblastic tumors, 16 rhabdomyosarcoma, 16 synovial sarcoma, 13 angiosarcoma, 11 malignant peripheral nerve sheath tumors (MPNST) and 4 other STS. There were 61 low grade STS (24%) and 188 high grade (FNCLCC grade 2 and 3) STS (76%).

The treatment option of choice was surgery (n = 228), seven patients received chemotherapy and/or radiotherapy only, and 14 patients received no therapy. A total of 120 patients received surgery only, 55 surgery and radiotherapy; 40 surgery and chemotherapy and 13 surgery, radiotherapy and chemotherapy. Two patients received chemotherapy only, three both chemotherapy and radiotherapy, and two radiotherapy only. The 5-year survival with non-wide resection margins was 33% and with wide resection margins 62%.

Inter-observer variability

There was good reproducibility between the two investigating pathologists. The scoring agreement was tested for M-CSF and CD68 expression in tumor. The IHC scores from each observer were compared using a two-way random effect model with absolute agreement definition. The intraclass correlation coefficients (reliability coefficients, r) obtained from these results were 0.85 for M-CSF ($P < 0.001$) and 0.90 for CD68 ($P < 0.001$).

Univariate analyses

Nationality, tumor size, malignancy grade, tumor depth, metastasis at time of diagnosis, surgery and surgical margins were all significant indicators for disease-specific survival (DSS) in univariate analyses (Table 1).

Besides, increased expression of M-CSF ($P = 0.034$), Ki67 ($P < 0.001$) and TGF-beta ($p = 0.003$) in tumor correlated significantly with a shorter DSS, (Table 2 and Figure 2). Co-expression of M-CSF and TGF-beta ($p = 0.004$) also correlated with shorter DSS. No such relationship was observed for CD57, CD68, and CSR-1R.

A shorter DSS with increased expression of M-CSF was seen in females ($P = 0.025$), Norwegian patients ($P = 0.015$) and in patients with tumors larger than 5 cm ($P = 0.018$, data not shown).

Increased expression of Ki67 in the peritumoral capsule correlated with a shorter DSS ($N = 80$, $P < 0.001$). Increased expression of CD68 in the peritumoral capsule tended to correlate with a shorter DSS, though not statistically significant ($N = 80$, $P = 0.057$), Table 3. No prognostic impact was observed for CD57, M-CSF, CSR-1R, TGF-beta or co-expression of M-CSF and TGF-beta. There was a correlation of expression of Ki67 in tumor ($N = 249$, $P = 0.001$) and metastasis at the time of the

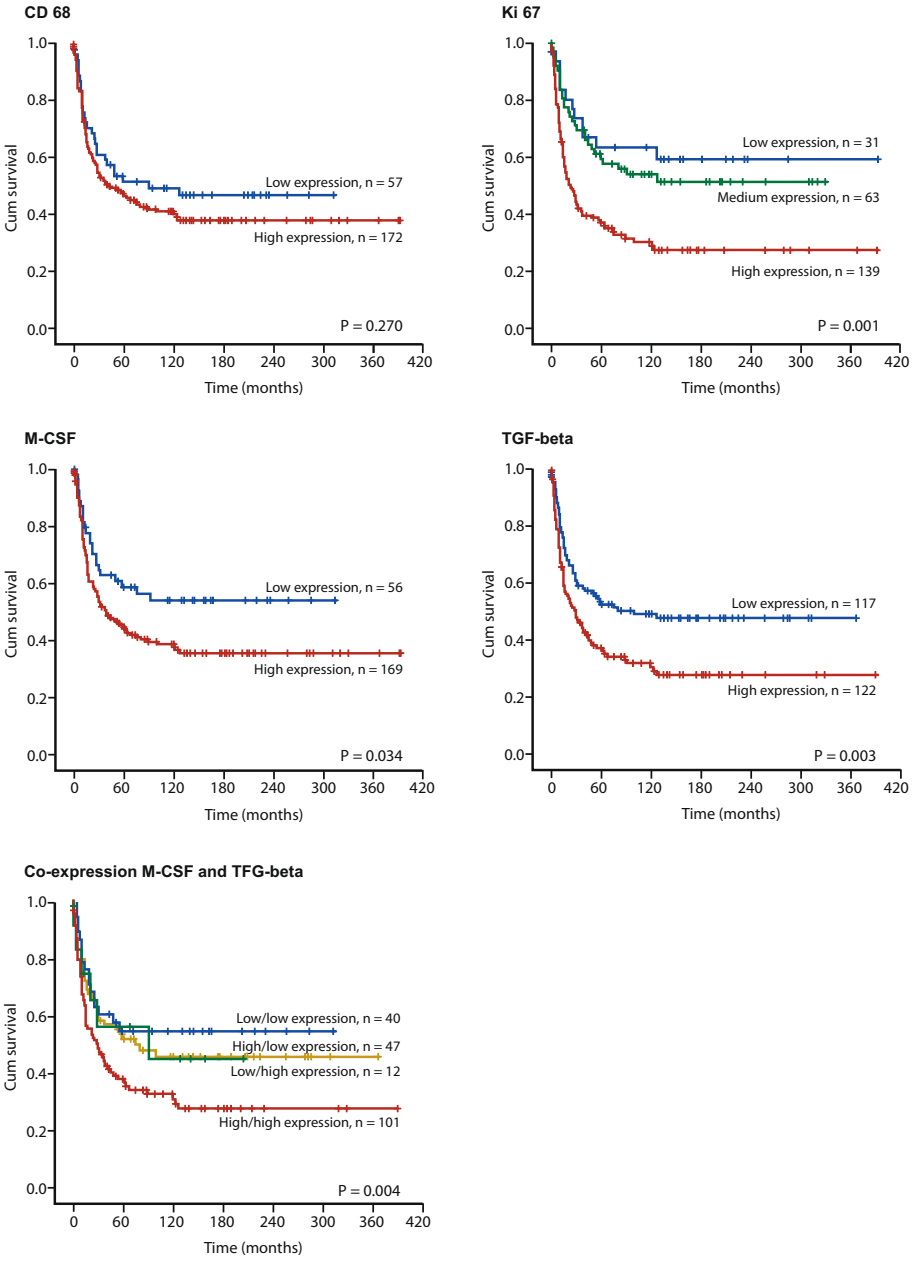

Figure 2 Disease-specific survival curves for high and low expression of CD68, Ki67, M-CSF, TGF-beta and co-expression M-CSF and TGF-beta in tumor in patients with STS (N = 249).

diagnosis, but not no correlation of expression of Ki67 in peritumoral capsule (N = 80, P = 0.395) and metastasis at the time of the diagnosis (data not shown).

In co-variation analyses between malignancy grade and expression of the different markers in tumor, Ki67, CD68, M-CSF and TGF-beta showed statistical significance (data not shown). Increased expression of CD68 in tumor correlated with malignancy grade (P = 0.016) and expression of Ki67 (P < 0.001). Increased expression of M-CSF in tumor correlated with malignancy grade (P = 0.010) and expression of Ki67 (P = 0.002). Increased expression of TGF-beta in tumor correlated with malignancy grade (P = 0.029) and expression of Ki67 (P = 0.005), table 4 and 5. There was a co-variation between expression of M-CSF

Table 2 Expression of markers in tumor and their prediction for disease-specific survival in patients with soft tissue sarcomas (univariate analysis; log-rank test), N = 249

Marker expression	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
CD 57					
Low	93	37	54	49	0.617
High	135	54	49	48	
Missing	21	8			
CD 68					
Low	57	23	91	52	0.270
High	172	69	45	47	
Missing	20	8			
M-CSF					
Low	56	22	NR	59	0.034
High	169	68	38	44	
Missing	24	10			
CSF-1R					
Low	38	15	41	44	0.832
High	191	77	38	46	
Missing	20	8			
Ki67					
Low	31	12	NR	63	<0.001
Medium	63	25	NR	59	
High	139	56	24	37	
Missing	16	6			
TGF-beta					
Low	117	47	99	53	0.003
High	122	49	29	37	
Missing	10	4			
M-CSF and TGF-beta					
Low	119	48	91	53	0.004
High	101	41	29	38	
Missing	29	12			

Abbreviations: NR, not reached.

and TGF-beta in tumor ($P < 0.001$, data not shown). In crosstabulation the expected count in the low M-CSF, high TGF-beta group was 26.7 patients (data not shown), but the observed count was 12 patients (Figure 2).

Multivariate analyses

Significant demographic, clinicopathological, and expression variables from the univariate analyses were entered into the multivariate Cox regression analysis. In the multivariate analysis, the co-expression of M-CSF and TGF-beta in the tumor was an independent prognostic factor for DSS. Other independent negative prognostic variables were malignancy grade ($P < 0.001$), metastasis at time of diagnosis ($P < 0.001$) and non-wide resection margins ($P = 0.001$, Table 6).

In patients with tissue from peritumoral capsule, independent negative prognostic variables were non-wide resection margins ($P = 0.031$) and high expression of Ki67 ($P = 0.019$, Table 6)

Discussion

In this study we evaluated whether there is an association between the expression of CD57, CD68, M-CSF, CSF-1R, Ki67 and TGF-beta in tumors or peritumoral

Table 3 Expression of markers in peritumoral capsule and their prediction for disease-specific survival in patients with soft tissue sarcomas (univariate analysis; log-rank test), N = 80

Marker expression	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
CD 57					
Low	50	63	38	47	0.797
High	29	36	123	55	
Missing	1	1			
CD 68					
Low	34	43	NR	61	0.057
High	45	56	31	43	
Missing	1	1			
M-CSF					
Low	36	45	75	54	0.608
High	39	49	36	46	
Missing	5	6			
CSF-1R					
Low	36	45	52	49	0.587
High	37	46	57	47	
Missing	7	9			
Ki67					
Low	32	40	NR	74	<0.001
High	37	46	29	35	
Missing	11	14			
TGF-beta					
Low	43	54	52	50	0.906
High	28	35	31	50	
Missing	9	11			
M-CSF and TGF-beta					
Low	24	30	80	57	0.626
High	42	53	31	45	
Missing	14	18			

Abbreviations: NR, not reached.

Table 4 Results of expression of CD68 and M-CSF in tumor versus malignancy grade in patients with soft tissue sarcomas, N = 249

Expression	Malignancy grade (%)			
	Grade 1	Grade 2	Grade 3	Total
CD68, Low	23 (40)	16 (28)	18 (32)	57 (100)
CD68, High	37 (22)	73 (42)	62 (36)	172 (100)
Total	60 (26)	89 (39)	80 (35)	229 (100)
Missing	20		Chi-Square	8.319
			P-value	0.016
	Grade 1	Grade 2	Grade 3	Total
M-CSF, Low	23 (41)	18 (32)	15 (27)	56 (100)
M-CSF, High	35 (21)	67 (40)	67 (40)	169 (100)
Total	58 (26)	85 (38)	82 (37)	225 (100)
Missing	24		Chi-Square	9.300
			P-value	0.010
	Grade 1	Grade 2	Grade 3	Total
TGF-beta, Low	15 (13)	66 (56)	36 (31)	117 (100)
TGF-beta, High	6 (5)	71 (58)	45 (37)	122 (100)
Total	21 (9)	137 (57)	81 (34)	239 (100)
Missing	10		Chi-Square	7.091
			P-value	0.029

capsule and survival in 249 non-GIST STS patients. Increased co-expression of M-CSF and TGF-beta in the tumor and increased expression of Ki67 in the peritumoral capsule were independent negative prognostic factors for DSS in patients with STS. High expression of M-CSF in tumor was correlated with high malignancy grade, increased Ki67 and short DSS. To our knowledge, this is the first report on co-expression of M-CSF and TGF-beta in STS and the first evidence of its possible clinical relevance in STS patients.

STS have varying biological characteristics regardless of histological entities. Its prognosis is poor, but also difficult to predict. This aggressive behavior reflects, at least in part, the capacity of the tumor to evade host immune surveillance. Evasion strategies can protect cancer cells from immune responses by a variety of mechanisms including self-tolerance, sequestration of tissue from surveillance, antigen shedding, lymphocyte killing, secretion of immunosuppressive cytokines, lack of MHC II expression, lack of co-stimulatory molecules and local secretion of prostaglandins.

CD57 positive cells have been implicated in the resistance against malignant and virally-infected cells. Presence of these cells was observed to be an independent prognostic marker for a better DSS in squamous cell carcinoma [31] and adenocarcinoma [32] of the lung, as well as in other cancers such as colonic and gastric carcinomas [14,15]. In NSCLS, high density of stromal CD57 positive cells was an independent, positive prognostic factor for DSS, whereas high density of CD57 positive cells within neoplastic cell areas was not [33]. In our material there was no such correlation in tumor or peritumoral capsule. The location of infiltrating lymphocytes may be important. There are major differences between 1) inflammatory cells within cancer cell nests in carcinomas (epithelial CD57 positive cells); 2) inflammatory cells present in the stroma of epithelial tumors (stromal CD57 positive cells), 3) inflammatory cells present along the invasive margins (peritumoral CD57 positive cells); and, 4) inflammatory cells in the peritumoral capsule of stromal tumors such as STS.

In addition to NK-cells, expression of CD57 is also found on T-lineage lymphocytes, where it is currently considered a marker-replicative senescence ("clonal exhaustion"), i.e., a high susceptibility to activation-induced cell death and the inability to undergo new cell-division cycles despite preserved ability to secrete cytokines upon encounter with their cognate antigen [34]. Even on NK cells it does not constitute a one-marker-labels-all solution: CD57 defines a functionally distinct population of mature NK cells in the human CD56dim CD16+ NK-cell subset [35].

Studies have demonstrated a close association between M-CSF and tumor progression in lung cancer cell lines [36]. In a NSCLC cohort studied by Kaminska et al. [37], high pretreatment serum levels of M-CSF were an independent predictor of poor survival in these patients.

Table 5 Results of expression of CD68 and M-CSF in tumor versus expression of Ki67 in patients with soft tissue sarcomas, N = 249

Expression	Ki67 (%)			
	Low	Medium	High	Total
CD68, Low	15 (27)	19 (35)	21 (38)	55 (100)
CD68, High	15 (9)	42 (25)	110 (66)	167 (100)
Total	30 (14)	61 (28)	131 (59)	222 (100)
Missing	27		Chi-Square	16.947
			P-value	<0.001
	Low	Medium	High	Total
M-CSF, Low	14 (26)	16 (30)	23 (43)	53 (100)
M-CSF, High	15 (9)	41 (25)	108 (66)	164 (100)
Total	29 (13)	57 (26)	131 (60)	217 (100)
Missing	32		Chi-Square	12.695
			P-value	0.002
	Low	Medium	High	Total
TGF-beta, Low	21 (18)	34 (30)	59 (52)	114 (100)
TGF-beta, High	7 (6)	28 (25)	79 (69)	114 (100)
Total	28 (12)	62 (27)	138 (31)	228 (100)
Missing	21		Chi-Square	10.749
			P-value	0.005

Table 6 Results of Cox regression analysis summarizing significant independent prognostic factors in patients with soft tissue sarcomas

	Tumor, N=249			Capsule, N=80		
Factor	**Hazard Ratio**	**95% CI**	**P**	**Hazard Ratio**	**95% CI**	**P**
Nationality						
Norwegian	1.000			1.000		
Russian	0.948	0.603-1.490	0.816	0.588	0.263-1.312	0.194
Tumor size						
< 5 cm	1.000		0.540*	1.000		0.342*
5-10 cm	1.103	0.687-1.770	0.685	0.888	0.376-2.099	
> 10 cm	1.310	0.797-2.153	0.287	1.671	0.660-4.233	
Malignancy grade FNCLCC						
1	1.000		0.001*	1.000		0.051*
2	1.997	1.129-3.531	0.017	1.402	0.383-5.137	0.610
3	2.874	1.617-5.107	<0.001	2.954	0.837-10.432	0.092
Metastasis at time of diagnosis						
No	1.000			1.000		
Yes	2.842	1.855-4.354	<0.001	2.101	0.901-4.898	0.086
Resection margins						
Wide	1.000			1.000		
Non-wide	2.523	1.706-3.730	<0.001	2.245	1.077-4-680	0.031
Ki67						
Low	1.000		0.432*	1.000		
Medium	1.059	0.528-2.163	0.876	-	-	-
High	1.365	0.710-2.625	0.351	2.553	1.167-5.584	0.019
M-CSF						
Low	1.000			NIA		
High	0.815	0.463-1.435	0.478			
TGF-beta						
Low	1.000			NIA		
High	0.682	0.247-1.881	0.460			
M-CSF and TGF-beta						
Low	1.000			NIA		
High	1.532	1.062-2.208	0.022			

* Overall significance as a prognostic factor. NIA = Not included in analysis.

However, Al-Shibli et al. [33] did not find any correlation between expression of M-CSF in NSCLC and DSS. CSF-1 protected osteoclasts from suppressive effects of transforming growth factor beta (TGF-beta) in a mouse mammary tumor cell line [38]. Kirma et al. studied M-CSF and TGF-beta in cervical cancer and found that CSF-1R (c-fms proto-oncogene product) activation may play a role in cervical carcinogenesis [39]. Richardsen et al. [27] showed that high M-CSF expression was correlated with a high malignancy grade in STS. In our study, high M-CSF expression in tumor correlated with a high malignancy grade, increased Ki67 and DSS in univariate analyses. But the expression of M-CSF in peritumoral capsule showed no correlation with DSS.

TGF-beta is a multifunctional cytokine known to induce G1 arrest in order to end proliferation, induce differentiation, or promote apoptosis in normal cells, thus being a natural tumor-suppressive agent. Though in tumorigenesis this mediator initiates EMT through activation of Smad and non-Smad signalling pathways[40]. Such pro-neoplastic action becomes possible through either blockade of the TGF-beta pathway with receptor-inactivating mutations, or

selective inactivation of the tumor-inhibiting arm of this pathway[41]. High TGF-beta expression was an independent negative prognostic factor for disease specific survival in STS[42]. In the multivariate analysis, co-expression of M-CSF and TGF-beta were an even stronger negative prognostic factor in this study. We found a co-variation of expression of M-CSF and TGF-beta in tumor. TFG-beta might regulate the expression of M-CSF. Grayfer et al. reported on the regulation of pro-inflammatory functions of goldfish macrophages and induction of gene expression by recombinant goldfish CSF-1 (rgCSF-1). At 72 h post treatment rgCSF-1 increased the expression of TGF-beta [43]. The combined expression of immunostimulatory granulocyte macrophage colony stimulating factor (GM-CSF) and anti-tumor suppressor TGF-β2 antisense (AS) transgenes can break tolerance and stimulate immune responses to cancer-associated antigens which make it possible to design bifunctional therapeutic anti-cancer vaccines[44].

Increased expression of Ki67 and M-CSF in tumor are negative prognostic indicators for patients with STS, but this is not independent of malignancy grade. In the univariate analysis presented TGF-beta seems to be the dominating factor, while low or high M-CSF expression in combination with low TGF-beta expression does not seem to influence prognosis significantly. Both expression of TGF-beta and M-CSF have co-variation with malignancy grade and expression of Ki67. In the multivariate analysis the co-expression of M-CSF and TGF-beta was a stronger prognosticator for DSS than each of the markers alone. Expression of Ki67 in tumor was not an independent prognosticator. As mitotic activity is one of the criteria determining the malignancy grade, expression of Ki67 is closely correlated to mitotic activity, hence also malignancy grade [45]. Archad et al. found that malignancy grade is a more important prognostic factor in glial neoplasms than Ki67 [19]. So Ki67 may not provide additional information if the tumor malignancy grade is classified correctly. The tumor stroma is important for cancer progression [46]. There is no evaluation of tumor stroma in the grading systems of STS. But Ki67 expression in the peritumoral capsule may have prognostic impact in addition to malignancy grading of the tumor. Further research is needed to determine whether an increased expression of Ki67 may be the result of on increased migration of fast-proliferating cells in the peritumoral capsule or an enhanced proliferation effect of tumor-released cytokines on the stromal cells.

Conclusion

In summary, increased co-expression of M-CSF and TGF-beta in tumor and increased Ki67 expression in the peritumoral capsule of STS patients were independent negative prognostic factors for DSS. This data may provide additional information to guide therapy after surgical resection.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SWS, TK, AV, TD, RMB and LTB participated in the design of the study. TK and AV collected clinical information. SWS and AV reviewed all the histological diagnosis, histological grading, selected and marked the slides for TMA construction. SWS, TK and AV performed the experiments. SWS, TK, AV, TD, RMB and LTB performed the statistical analysis. SWS, TK, AV, TD, ES, KAS and LTB contributed reagents/materials/analysis tools. SWS, TD, ES, KAS, RMB and LTB drafted the manuscript. All authors read and approved the final manuscript.

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Prognostic Impact of Jab1, p16, p21, p62, Ki67 and Skp2 in Soft Tissue Sarcomas

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Abstract

Purpose: The purpose of this study is to clarify the prognostic significance of expression of Jab1, p16, p21, p62, Ki67 and Skp2 in soft tissue sarcomas (STS). Optimised treatment of STS requires better identification of high risk patients who will benefit from adjuvant therapy. The prognostic significance of Jab1, p16, p21, p62, Ki67 and Skp2 in STS has not been sufficiently investigated.

Experimental Design: Tissue microarrays from 193 STS patients were constructed from duplicate cores of viable and representative neoplastic tumor areas. Immunohistochemistry was used to evaluate the expression of Jab1, p16, p21, p62, Ki67 and Skp2.

Results: In univariate analyses, high tumor expression of Ki67 ($P = 0.007$) and Skp2 ($P = 0.050$) correlated with shorter disease-specific survival (DSS). In subgroup analysis, a correlation between Skp2 and DSS was seen in patients with malignancy grade 1 or 2 ($P = 0.027$), tumor size >5 cm ($P = 0.018$), no radiotherapy given ($P = 0.029$) and no chemotherapy given ($P = 0.017$). No such relationship was apparent for Jab1, p16, p21 and p62; but p62 showed a positive correlation to malignancy grade ($P = 0.019$). Ki67 was strongly positively correlated to malignancy grade ($P = 0.001$). In multivariate analyses, Skp2 was an independent negative prognostic factor for DSS in women ($P = 0.009$) and in patients without administered chemotherapy or radiotherapy ($P = 0.026$).

Conclusions: Increased expression of Skp2 in patients with soft tissue sarcomas is an independent negative prognostic factor for disease-specific survival in women and in patients not administered chemotherapy or radiotherapy. Besides, further studies are warranted to explore if adjuvant chemotherapy or radiotherapy improve the poor prognosis of STS with high Skp2 expression.

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Introduction

Soft tissue sarcomas (STS) are a heterogeneous and highly malignant group of tumors originating from mesenchymal lineage. Local recurrence is common (20%) and metastases occur in one third of patients [1]. Prognostic markers in potentially curable STS should guide therapy after surgical resection. Neoadjuvant therapy is increasingly used and may improve prognosis in high-risk cases [2], but requires prognostic factors that can be evaluated preoperatively. Currently used prognostic factors mainly include clinicopathological variables such as tumor type, size, depth, malignancy grade, necrosis, vascular invasion, and growth pattern, which are combined into different prognostic systems [3–9].

The loss of cell cycle control is a critical step in the development of neoplasia. The cell cycle is a series of carefully coordinated and regulated steps that govern cellular proliferation. Cyclin-dependent kinases (CDK) phosphorylate the retinoblastoma (Rb) protein, a classic tumor suppressor and key component of the G1/S checkpoint. This allows DNA replication to proceed. Inhibitors of CDK, such as $p16^{INK4A}$, p21, and p27 act as brakes on progression through the cell cycle.

The human Jun activation domain binding protein 1 (Jab1) was originally identified as a coactivator of the gene regulatory AP-1 proteins (Jun/Fos protooncogenes) involved in the control of cell proliferation [10]. Jab1 directly binds to p27 and induces nuclear export and subsequent degradation [11]. Some studies indicated that Jab1 can interact specifically with the protein form of the CDK inhibitor 27 and shuttle p27 from the nucleus to the cytoplasm. And further to decrease the cellular amount of p27 by accelerating p27 degradation via the ubiquitin-proteasome system [12,13]. Other reports have shown that overexpression of Jab1 and low expression of p27 is associated with more advanced tumor stage and poor prognosis in several human cancers [11,14–16].

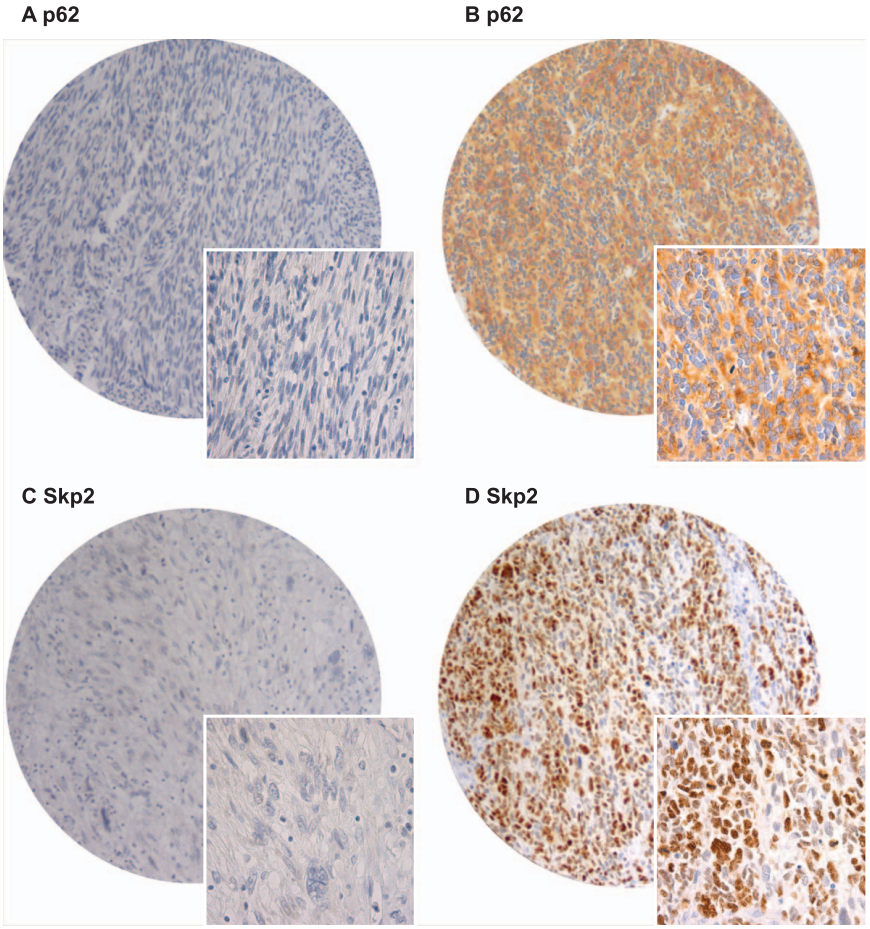

Figure 1. Immunohistochemistry microscopic pictures of tissue micro array of soft tissue sarcoma representing different expression of p62 and Skp2. (A) p62 low score; (B) p62 high score; (C) Skp2 low score; (D) Skp2 high score. Original magnification × 400.
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The CDK inhibitor $p16^{INK4a}$ (p16) protein belongs to the INK4 family of CDK inhibitors [17]. CDK inhibitors are negative regulators of the process of pRb hyperphosphorylation. The INK4 family of CDK inhibitors binds to CDK4/6 and the D family of cyclins to prevent formation of the cyclin–CDK complex required to phosphorylate pRb [17]. p16 has been identified as a tumor suppressor [18]. The gene encoding p16 is deleted in a high percentage of malignant cell lines and tissues [19–21]. p16 is important in cell senescence, and some studies have identified a role for p16 in cell proliferation and angiogenesis [22,23]. A model of murine rhabdomyosarcoma has been produced through a subsequent genetic manipulations, among others p16 deletion [24,25]. Still, the role of cell cycle regulators in the genesis of mesenchymal neoplasia is less well studied and the role of the p16 protein in STS has not been sufficiently investigated.

p21 (Waf1) is a cell cycle regulator, implicated in a variety of pathways [26]. The product of the CDKN1A gene (p21) binds to and inhibits the activity of CDK2/4 complexes, and thus functions as a regulator of cell cycle progression at the G1 checkpoint. Ki67 is involved in the synthesis of ribosomes and appears to be a necessary requirement for cell proliferation [27].

The intricate signalling network that determines whether cells grow, undergo senesce or die, achieves a remarkable degree of specificity with a relatively small number of signalling molecules [28]. Studies employing knockout, transgenic, and knockin mice have shown that p62 plays critical roles in a number of cellular functions, including bone remodelling, obesity, and cancer [29–31].

S-phase kinase-associated protein 2 (Skp2) is a member of mammalian F-box proteins, which displays S-phase-promoting function through ubiquitin-mediated proteolysis of the CDK inhibitor p27. Skp2 has been shown to regulate cellular proliferation by targeting several cell cycle-regulated proteins for ubiquitination and degradation. Skp2 has also been demonstrated to display an oncogenic function since its overexpression has been observed in many human cancers [32].

The purpose of this study was to clarify the prognostic significance of Jab1, p16, p21, p62, Ki67 and Skp2 expression in non-gastrointestinal stromal tumor (non-GIST) STS. To achieve this, we analyzed the expression of these markers in 193 patients with non-GIST STS in relation to demographic and other clinicopathological variables.

Materials and Methods

Patients and Clinical Samples

The National Cancer Data Inspection Board and The Regional Committee for Research Ethics (REK nord) approved the study. The material was collected from our approved biobank for paraffin embedded material and slides. The Regional Committee

Table 1. Prognostic clinicopathological variables as predictors for disease-specific survival of soft tissue sarcomas (univariate analysis, log rank test), N = 193.

Characteristic	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Age					
≤20 years	17	9	190	47	0.064
21–60 years	85	44	235	63	
>60 years	91	47	111	51	
Gender					
Male	81	42	235	60	0.087
Female	112	58	180	53	
Nationality					
Norwegian	131	68	228	62	0.005
Russian	62	32	81	44	
Histology					
Pleomorphic sarcoma	57	30	52	45	0.031
Leiomyosarcoma	47	24	89	64	
Liposarcoma	32	17	NR	71	
MF/MFT	16	8	123	56	
Angiosarcoma	8	4	10	38	
Rhabdomyosarcoma	9	5	NR	67	
MPNST	9	5	NR	56	
Synovial sarcoma	12	6	31	30	
Other STS	3	2	NR	–	
Tumor localization					
Extremities	78	40	201	56	0.922
Trunk	37	19	214	53	
Retroperitoneum	27	14	135	51	
Head/Neck	13	7	191	58	
Visceral	38	20	202	62	
Tumor size					
<5 cm	57	30	257	69	0.026
5–10 cm	73	38	183	54	
>10 cm	61	32	127	48	
Missing	2	1			
Malignancy grade FNCLCC					
1	61	28	316	81	<0.001
2	98	39	173	55	
3	90	33	103	36	
Surgical margins					
Wide	97	50	254	66	<0.001
Non-wide	96	50	128	46	
Chemotherapy					
No	156	81	207	57	0.669
Yes	37	19	180	51	
Radiotherapy					
No	132	68	216	58	0.190
Yes	61	32	152	52	

Abbreviations: MF/MFT, malignant fibroblastic/myofibroblastic tumors; MPNST, malignant peripheral nerve sheath tumor; STS, soft tissue sarcomas; NR, not reached; NOS, non specified.

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approved that written consent from the patients for their information to be stored in the hospital database and used for research was not needed because most of the material was more than 10 years old, and most of the patients being dead. The ethics committee specifically waived the need for consent. Data were analyzed anonymously.

Primary tumor tissues from patients diagnosed with STS at the University Hospital of North Norway (UNN) from 1973 to 2006 and the Hospitals of Arkhangelsk region, Russia, were used in this retrospective study. In total, 496 potentially suitable patient records were identified from the hospitals databases. Of these, 247 patients were excluded due to missing clinical data (n = 86) or inadequate material for histological examination (n = 161). In addition 33 were excluded because of metastasis at the time of the diagnosis, 13 were excluded because they had no surgery, and 10 patients had both metastasis and no surgery. Eligible for this study were 193 patients. This report includes data for 131 Norwegian patients and 62 Russian patients followed until September 2009. The median follow-up was 38 (range 0–392) months. Complete demographic and clinical data were collected retrospectively. Formalin-fixed and paraffin-embedded tumor specimens were obtained from the archives of the Departments of Pathology at UNN and Archangelsk. The tumors were graded according to the French Fèdèration Nationales des Centres de Lutte Contre le Cancer (FNCLCC), [WHO Tumors of Soft Tissue and bone, 2002]. Wide resection margins were defined as wide local resection with free microscopic margins or amputation of the affected limb or organ. Non-wide resection margins were defined as either marginal or intralesional resection margins.

Tissue Microarray Construction

The histology of all soft tissue sarcoma cases were reviewed by two pathologists (AV and SWS). Tissue microarrays (TMAs) were constructed for high-throughput molecular pathology research [33]. The most representative areas of viable tumor cells were carefully selected and marked on the hematoxylin and eosin (HE) slides for the corresponding donor blocks and sampled for the tissue microarray collector blocks. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments).

Studies suggest that punching multiple 0.6 mm cores from different regions captures the heterogeneity of the tumors more accurately than single 2 to 4 mm core [34]. Hence, we chose using two 0.6-mm cores of viable neoplastic tissue that were selected to be as representative as possible (different areas), after reviewing all original sections of the tumor and taking the heterogeneity in consideration. To include all core samples, 12 tissue array blocks were constructed. Multiple 4-μm sections were cut with a Micron microtome (HM355S) and stained by specific antibodies for immunohistochemistry (IHC).

Immunohistochemistry (IHC)

All staining were performed in the Ventana Benchmark XT automated slide stainer (Ventana Medical System, Illkirch, France). Before staining the sections were incubated at 60 degrees Celsius over night. Tissue sections were incubated with primary mouse monoclonal antibodies recognizing Jab1 (Zymed, catalog number 18–7386, 1:50), Skp2 (Zymed, catalog number 18–0307, 1:10), p62 (BD Biosciences, catalog number 610832, 1:50), Ki67 (Ventana, catalog number 790–4286, ready to use) and p21 (Dako, catalog number M7202, 1:25), p16 (MTM lab, Germany, catalog number 9511, ready to use). We used a Ventana antibody diluent (catalog number 251-018). The incubation periods were 28 minutes for Jab1, 28 minutes for p16, 32 minutes for p62 and Ki67, 40 minutes for p21 and Skp2. This was followed by application of liquid diaminobenzidine as substrate-chromogen, yielding a brown reaction product at the site of the target antigen (Ventana iView DAB Detection Kit, catalog number 760–091). iVIEW DAB Detection Kit is an indirect biotin streptavidin system for detecting mouse and rabbit primary antibodies. The DAB chromogen produces a dark brown precipitate that is readily visualized by light microscopy. All reagents are provided pre-diluted by the manufacturer for use in Ventana Benchmark XT. Finally, slides were counterstained with hematoxylin to visualize the nuclei. For each antibody, including negative controls, all TMA staining were performed in a single experiment. In the TMA we also used cores from carcinomas and normal tissue as positive and negative controls.

Scoring of IHC

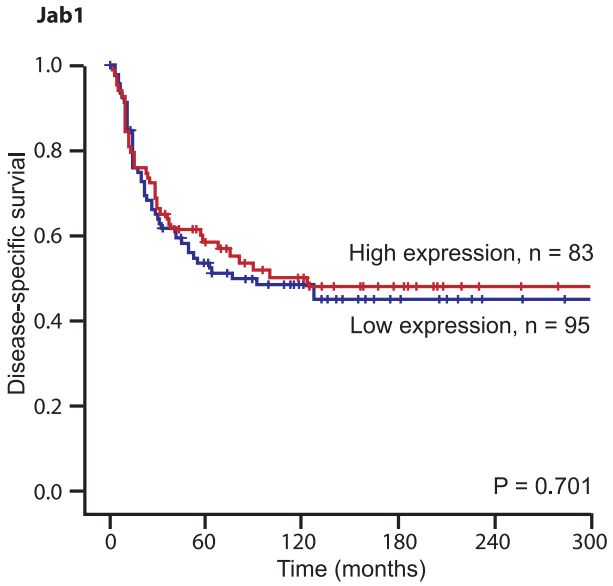
The ARIOL imaging system (Genetix, San Jose, CA) was used to scan the slides for antibody staining of the TMAs. The specimens were scanned at a low resolution (1.25×) and high resolution (20×) using an Olympus BX 61 microscope with an automated platform (Prior). The slides were loaded in the automated slide loader (Applied Imaging SL 50). In our experience it was difficult for the ARIOL imaging system to distinguish between tumor and stroma in soft tissue sarcomas. Representative and viable tissue sections were therefore scored manually on a computer screen semi-quantitatively for nuclear and/or cytoplasmic staining, Figure 1. The expression of Jab1, p16, p21, p62, Ki67 and Skp2 was scored as: 0, negative; 1, weak; 2, intermediate; 3, strong. The score for each patient was based on the mean scoring of cores from one or several biopsies. To achieve maximal reproducibility in all cases, every staining was dichotomised (low and high expression). Instead of using the overall mean score as cutoff, the cutoffs were chosen at levels securing statistically sufficient numbers in each group and appearing most biologically plausible. Hence, in this study the cutoff values varied among the different markers. High expression was defined as mean score >0 for p21 and Skp2, ≥ 0.33 for p62, ≥ 0.75 for p16 and ≥ 2.00 for Jab1 and Ki67. All samples were anonymized and independently scored by two pathologists (AV and SWS). In case of disagreement, the slides were re-examined and a consensus was reached by the observers. When assessing a variable for a given score, the scores of the other variables and the outcome were hidden from the observers.

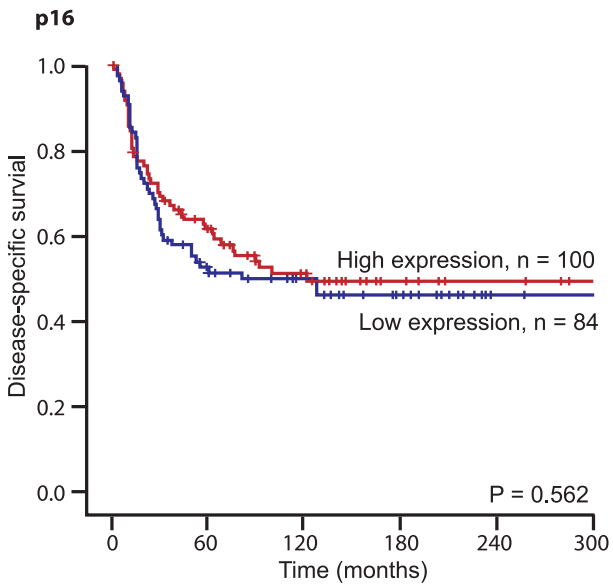
Statistical Methods

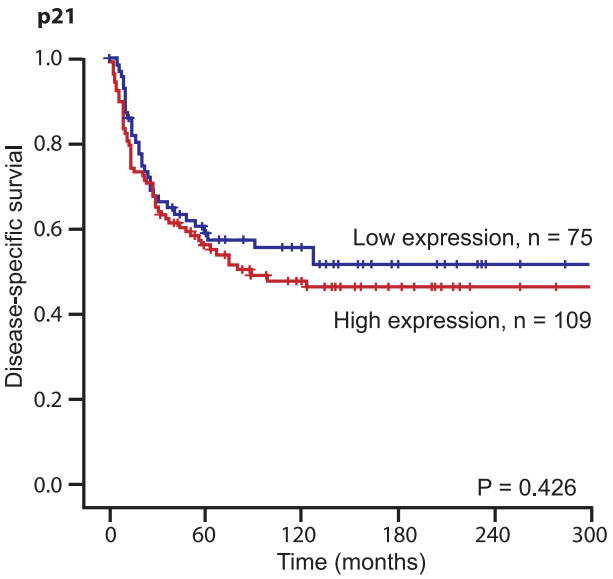
All statistical analyses were done using the statistical package SPSS (Chicago, IL), version 18. The IHC scores from each observer were compared for interobserver reliability by use of a two-way random effect model with absolute agreement definition. The intraclass correlation coefficient (reliability coefficient) was obtained from these results.

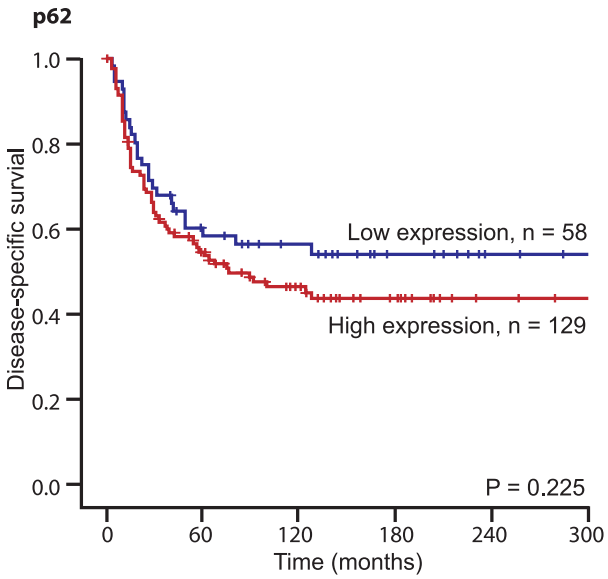
The Chi-square test and Fishers Exact test were used to examine the association between molecular marker expression and various clinicopathological parameters. Univariate analyses were done using the Kaplan-Meier method, and statistical significance between survival curves was assessed by the log rank test. Disease-specific survival (DSS) was determined from the date of histological-confirmed STS diagnosis. Correlation of marker expression was done using the Pearson correlation (2 tailed) at the 0.05 and the 0.01 level.

Multivariate analysis was carried out using the Cox proportional hazards model to assess the specific impact of each pre-treatment variable on survival in the presence of other variables. Only variables of significant value from the univariate analysis were entered into the Cox regression analysis. Probability for stepwise


Jab1
Disease-specific survial
1.0
0.8
0.6
0.4
0.2
0.0
High expression, n = 83
Low expression, n = 95
P = 0.701
0
60
120
180
240
300
Time (months)


p16
Disease-specific survial
1.0
0.8
0.6
0.4
0.2
0.0
High expression, n = 100
Low expression, n = 84
P = 0.562
0
60
120
180
240
300
Time (months)


p21
Disease-specific survial
1.0
0.8
0.6
0.4
0.2
0.0
Low expression, n = 75
High expression, n = 109
P = 0.426
0
60
120
180
240
300
Time (months)


p62
Disease-specific survial
1.0
0.8
0.6
0.4
0.2
0.0
Low expression, n = 58
High expression, n = 129
P = 0.225
0
60
120
180
240
300
Time (months)

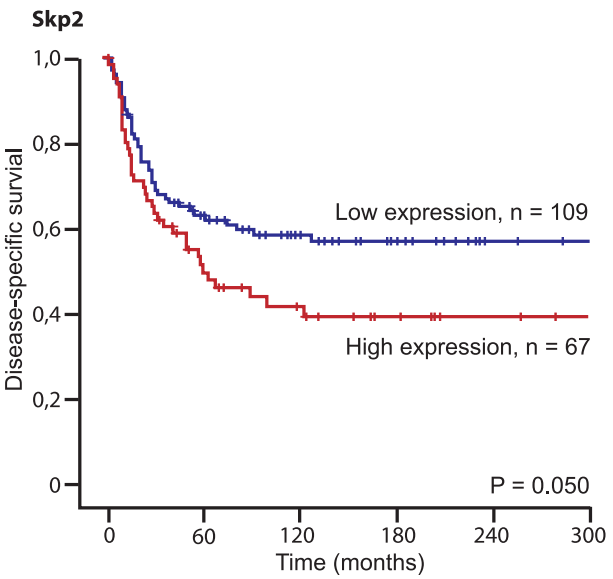

Skp2
Disease-specific survial
1,0
0,8
0,6
0,4
0,2
0
Low expression, n = 109
High expression, n = 67
P = 0.050
0
60
120
180
240
300
Time (months)

Figure 2. Disease-specific survival curves for high and low expression of different markers in patients with soft tissue sarcomas (N = 193).
doi:10.1371/journal.pone.0047068.g002

entry and removal was set at 0.05 and 0.10, respectively. The significance level used was $P<0.05$.

Results

Clinicopathological Variables

Demographic, clinical, and histopathological variables are shown in Table 1. Patient age ranged from 0–89 years (mean 55 years), and 42% of patients were male. The treatment option of choice was surgery (N = 193): 104 patients received surgery only; 52 patients received surgery and radiotherapy; 28 patients received surgery and chemotherapy; 9 patients received surgery, radiotherapy and chemotherapy. The 5-year survival for patients with wide and non-wide resection margins was 66% and 46% respectively, Table 1.

Inter-observer Variability

There was good scoring reproducibility between the two investigating pathologists. Their scoring agreement was tested for p62 and Skp2. The IHC scores from each observer were compared using a two-way random effect model with absolute agreement definition. The intra-class correlation coefficients (reliability coefficients, r) obtained from these results were 0.82 for p62 ($P<0.001$) and 0.94 for Skp2 ($P<0.001$).

Univariate Analyses

Nationality, histology, tumor size, malignancy grade and surgical margins were all significant indicators for disease-specific survival (DSS) in univariate analyses, Table 1.

In univariate analyses, increased expression of Ki67 ($P = 0.007$) and Skp2 ($P = 0.050$) correlated significantly with a shorter DSS, Table 2 and Figure 2. No such relationship was apparent for Jab1, p16, p21 and p62, but expression of p62 was positively correlated to malignancy grade ($P = 0.019$), Table 3. Ki67 was strongly positively correlated to malignancy grade ($P = 0.001$), Table 3.

High expression of the different markers was significantly correlated. There was weak ($r = 0.20–0.29$), moderate ($r = 0.30–0.39$) and strong ($r = 0.40–0.69$) positive correlations between the various examined markers. There was strong correlation between p16 and p62, strong correlation between Ki67 and p21/Skp2,

Table 2. Expression of markers and their prediction for disease-specific survival in patients with soft tissue sarcomas (univariate analysis; log-rank test), N = 193.

Marker expression	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Jab1					
Low	95	49	76	54	0.701
High	83	43	123	59	
Missing	15	8			
p16					
Low	84	13	80	51	0.562
High	100	52	123	62	
Missing	9	5			
p21					
Low	75	39	NR	59	0.426
High	109	56	89	56	
Missing	9	5			
p62					
Low	58	30	NR	59	0.255
High	129	67	76	55	
Missing	6	3			
Ki67					
Low	28	15	NR	68	0.007
Medium	56	29	NR	66	
High	99	51	57	48	
Missing	10	5			
Skp2					
Low	109	56	NR	63	0.050
High	67	45	59	50	
Missing	17	9			

Abbreviations: NR, not reached.
doi:10.1371/journal.pone.0047068.t002

Table 3. Results of expression of p21, p62, Ki67 and Skp2 versus malignancy grade in patients with soft tissue sarcomas, N = 193.

Expression	Malignancy grade (%)						
	Grade 1	Grade 2	Grade 3	Total	Missing	Chi-Square	P-value
p21, Low	25 (33)	26 (35)	24 (32)	75	9	1.420	0.492
p21, High	28 (26)	45 (41)	36 (33)	109			
Total	53 (29)	71 (39)	60 (33)	184			
p62, Low	24 (41)	17 (29)	17 (29)	58	6	7.893	0.019
p62, High	28 (22)	55 (43)	46 (36)	129			
Total	52 (25)	72 (38)	63 (38)	187			
Ki67, Low	13 (46)	8 (29)	7 (25)	28	10	18.525	0.001
Ki67, Medium	22 (39)	22 (39)	12 (21)	56			
Ki67, High	15 (15)	42 (42)	42 (42)	99			
Total	50 (27)	72 (39)	61 (33)	183			
Skp2, Low	36 (33)	41 (38)	32 (29)	109	17	1.797	0.407
Skp2, High	16 (24)	27 (40)	24 (36)	67			
Total	52 (30)	68 (39)	56 (32)	176			

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moderate correlation between Jab1 and p21/p62/Skp2, moderate correlation between p21 and p62/Skp2 and moderate correlation between Ki67 and Jab1/p16/p62, Table 4.

In subgroup analyses (Table 5), increased Skp2 expression was associated with a shorter DSS in Norwegian patients (P = 0.021), those with malignancy grade 1 and 2 tumors (p = 0.027), tumors larger than 5 cm (P = 0.018), without radiotherapy (P = 0.029) and without chemotherapy (P = 0.017), Figure 3. There were no significant differences in the expression of the different immunomarkers in the different histological tumor groups (data not shown).

Multivariate Analyses

Demographic, clinicopathological, and expression variables from the univariate analyses were entered into the multivariate Cox regression analysis (Table 6). In the multivariate analysis, age (P = 0.012), malignancy grade (P<0.001) and wide resection margins (P = 0.001) were independent prognostic factors for DSS. In addition, Skp2 had an independent prognostic impact in women (P = 0.009) and in patients not treated with chemotherapy or radiation (P = 0.026).

Table 4. Correlation of marker expression in patients with soft tissue sarcomas (Pearson correlation), N = 193.

Marker expression	Jab1	p16	p21	P62	Skp2	Ki67
Jab1	–	0.243**	0.372**	0.315**	0.305**	0.398**
p16	0.243*	–	0.208**	0.418**	0.275**	0.332**
p21	0.372**	0.208**	–	0.360**	0.312**	0.418**
p62	0.315**	0.418**	0.360**	–	0.195*	0.368**
Skp2	0.305**	0.275**	0.312**	0.195*	–	0.456**
Ki67	0.398**	0.332**	0.418**	0.368**	0.456**	–

*Correlation is significant at the 0.05 level (2 tailed).
**Correlation is significant at the 0.01 level (2 tailed).
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Discussion

In this large scale study, we evaluated whether there is an association between tumor cell expression of Jab1, p16, p21, p62, Ki67 and Skp2 and survival in 193 non-GIST STS patients. Increased expression of Skp2 in patients with STS was an independent negative prognostic factor for DSS in women and in patients not administered chemotherapy or radiotherapy. To our knowledge, this is the first report where Skp2 is compared with Jab1, p16, p21 and p62 in STS and the first evidence of its possible clinical relevance in STS patients regarding potential benefits for adjuvant treatment with chemotherapy or radiation in the subgroup of patients with high expression of Skp2.

STS has different biological characteristics regardless of its histological phenotype. Its prognosis is in general poor, but also difficult to predict. In potentially curable STS prognostic markers should, ideally, guide further therapy following surgical resection. In our material, high expression of the different cell cycle control markers were significantly correlated. This is, however, expected since there is overlap in their mechanisms of action. Tsuchida et al. [35] suggested that Jab1 may play an important role in determining the differentiation stage of rhabdomyosarcoma cells by modulating the activity of CDK inhibitor p27. However, in our material, Jab1 showed no correlation with malignancy grade and had no prognostic impact on DSS in STS.

Epigenetic silencing of p16 might be critical early initiating events in the tumorigenesis of Ewing sarcoma family tumors [36]. p16 has been shown as a sensitive and specific marker for distinguishing atypical lipomatous tumor-well-differentiated liposarcoma and dedifferentiated liposarcoma from benign adipocytic neoplasms [37]. There is overexpression of p16 in uterine leiomyosarcoma compared to benign leiomyoma and normal myometrium [38]. p16 and pRb immunohistochemical expression increases with increasing tumour grade in mammary phyllodes tumours [39]. In a series of 38 pediatric osteosarcomas there was an inverse correlation between loss of pRB and p16 expression. Absence of p16 expression significantly correlated with decreased survival in univariate analysis [40]. Immunohistochemically decreased expressions of p16 was associated with poor prognosis in malignant peripheral nerve sheath tumor [41]. In a series of 21

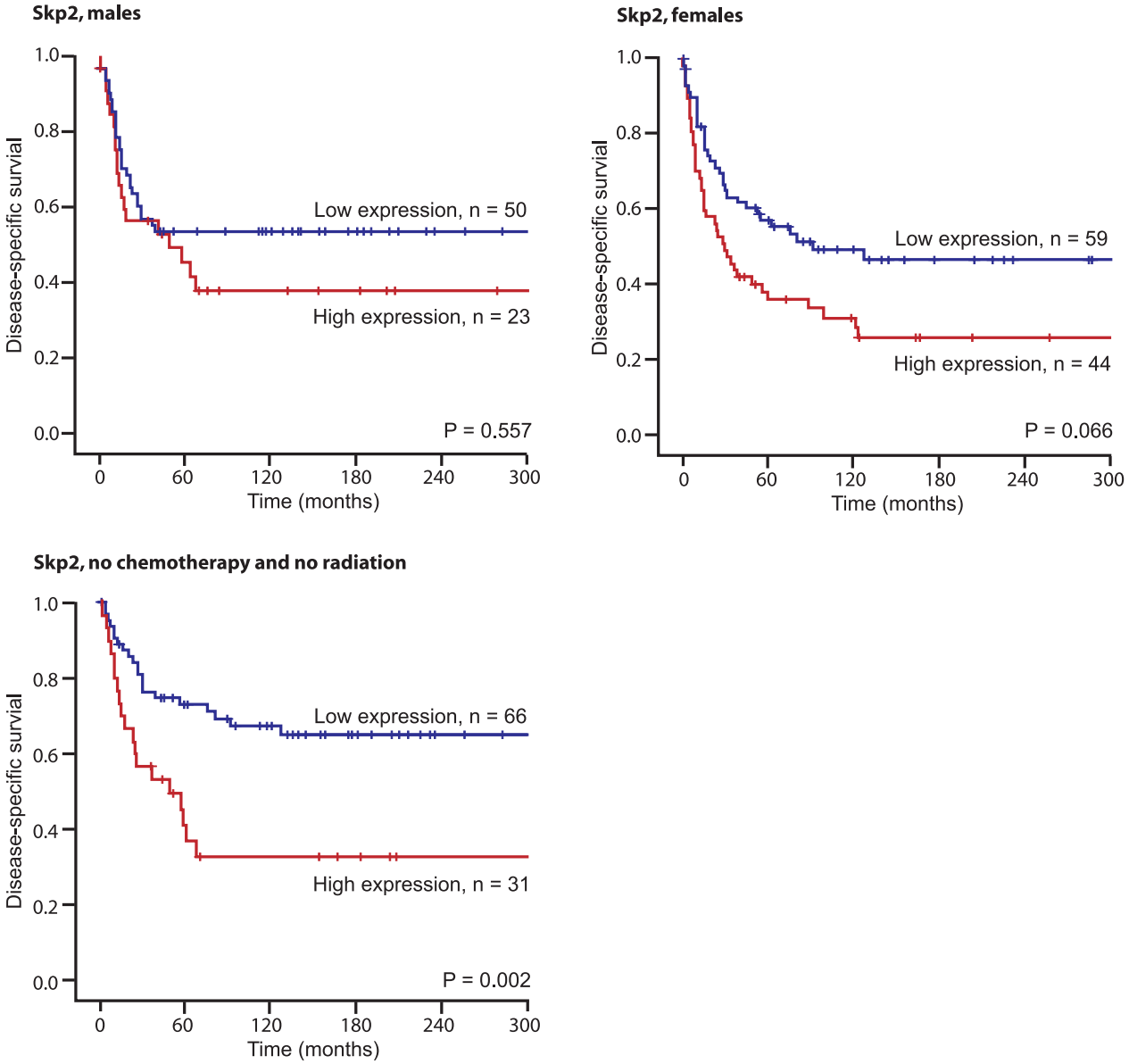

Figure 3. Disease-specific survival curves for high and low expression of Skp2 in males (N = 81), females (N = 112) and in patients not treated with chemotherapy or radiation (N = 104).
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uterine leiomyosarcomas, no statistically significant correlation between p16 expression and clinical stage, age, vascular space involvement, and recurrent disease could be found. Additionally, the overall survival did not significantly differ between p16-positive and p16-negative cases [42]. In a series of 84 uterine leiomyosarcomas, p16 did not show any significant correlation with survival [43]. Shim et al. [44] found no significant difference between the survival rate according to the p16 expression in 66 soft tissue sarcomas. In our material there was no correlation of p16 and malignancy grade or DSS.

Using *in vivo* RNA interference, Young et al. implicated the p53 target gene p21 as a critical mediator in sarcomagenesis [45]. The expression of p21 was closely associated with tumor malignancy grade, and therefore considered used as prognostic markers in a series of 152 STS [46]. López-Guerrero et al observed that the expression of p21 ($P<0.015$) was higher in disseminated than localized disease in patients with Ewing's sarcoma tumors, but p21 did not influence progression free or overall survival [47]. In a series of 36 patients with leiomyosarcoma, p21 was not correlated with time to recurrence or overall survival [48]. Similarly, in our material p21 was not correlated to malignancy grade or DSS. This can be due to other bypass molecules involved in p53 suppression functions.

There are few publications regarding p62 and STS. Rolland et al. demonstrated that p62 expression in breast cancer is associated with tumor progression, but not DSS [49]. In a series of 109 non-small cell lung cancers, p62 were an independent factors predicting worse lung cancer-specific survival [50]. Kitamura et al. demonstrated cytosolic overexpression of p62 in prostate adenocarcinoma and high-grade PIN, suggesting that p62 might be a novel marker for prostatic malignancy [51]. However, in a series of 59 colorectal carcinomas, p62 had no prognostic value [52]. In our material, p62 correlated with malignancy grade, but not DSS.

Table 5. Results of subgroup analysis of patients with expression of Skp2, N = 193.

Subgroup	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Age					
0–60 years, Skp2 Low	52	53	NR	71	0.074
0–60 years, Skp2 High	38	38	67	56	
Missing	9	9			
>60 years, Skp2 Low	57	61	80	57	0.188
>60 years, Skp2 High	29	31	36	42	
Missing	8	9			
Gender					
Male, Skp2 Low	50	62	NR	63	0.577
Male, Skp2 High	23	28	67	61	
Missing	8	10			
Female, Skp2 Low	59	53	NR	63	0.066
Female, Skp2 High	44	39	49	44	
Missing	9	8			
Nationality					
Norwegian, Skp2 Low	67	51	NR	71	0.021
Norwegian, Skp2 High	57	44	89	54	
Missing	7	5			
Russian, Skp2 Low	42	68	91	51	0.177
Russian, Skp2 High	10	16	41	16	
Missing	10	16			
Malignancy grade					
1 or 2, Skp2 Low	77	59	NR	74	0.027
1 or 2, Skp2 High	43	33	89	56	
Missing	10	8			
3, Skp2 Low	32	51	26	37	0.970
3, Skp2 High	24	38	31	39	
Missing	7	11			
Tumor size					
<5 cm, Skp2 Low	37	63	NR	64	0.610
<5 cm, Skp2 High	17	29	NR	75	
Missing	5	8			
>5 cm, Skp2 Low	71	53	NR	63	0.018
>5 cm, Skp2 High	50	37	49	41	
Missing	13	10			
Radiotherapy					
No, Skp2 Low	77	58	NR	68	0.029
No, Skp2 High	45	34	58	46	
Missing	10	8			
Yes, Skp2 Low	32	52	62	53	0.744
Yes, Skp2 High	22	36	100	58	
Missing	7	11			
Chemotherapy					
No, Skp2 Low	94	60	NR	66	0.017
No, Skp2 High	49	31	58	45	
Missing	13	8			
Yes, Skp2 Low	15	41	45	47	0.743
Yes, Skp2 High	18	49	89	61	
Missing	4	11			

Abbreviations: NR, not reached.

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Table 6. Results of Cox regression analysis summarizing prognostic factors in patients with soft tissue sarcomas.

	All patients, N = 193			No chemotherapy, no radiation, N = 104			Women, N = 112		
Factor	**Hazard Ratio**	**95% CI**	**P**	**Hazard Ratio**	**95% CI**	**P**	**Hazard Ratio**	**95% CI**	**P**
Age									
0–60 years	1.00			1.00			1.00		
>60 years	1.79	1.14–2.81	0.012	3.64	1.64–8.09	0.001	1.60	0.89–2.90	0.118
Nationality									
Norwegian	1.00			1.00			1.00		
Russian	1.49	0.89–2.48	0.129	1.67	0.72–3.86	0.232	2.33	1.17–4.66	0.016
Tumor size									
<5 cm	1.00		0.260*	1.00		0.963*	1.00		0.017*
5–10 cm	1.34	0.75–2.38	0.325	0.89	0.40–1.98	0.783	1.69	0.80–3.60	0.166
>10 cm	1.65	0.91–3.02	0.101	0.97	0.43–2.20	0.936	3.15	1.41–7.03	0.005
Malignancy grade FNCLCC									
1	1.00		<0.001*	1.00		0.004*	1.00		0.002*
2	2.70	1.37–5.32	0.004	2.36	1.04–5.34	0.040	4.79	1.93–11.88	0.001
3	4.86	2.45–9.65	<0.001	4.66	1.87–11.61	0.001	4.86	1.91–12.34	0.001
Resection margins									
Wide	1.00			1.00			1.00		
Non-wide	2.19	1.38–3.48	0.001	3.32	1.65–6.69	0.001	1.03	0.56–1.92	0.923
Ki67									
Low	1.00		0.392*	1.00		0.272*	1.00		0.214*
Medium	0.98	0.42–2.27	0.961	0.97	0.25–3.74	0.965	0.48	0.17–1.32	0.154
High	1.44	0.62–3.36	0.393	1.88	0.50–7.01	0.349	0.85	0.30–2.36	0.750
Skp2									
Low	1.00			1.00			1.00		
High	1.35	0–86−2.11	0.194	2.05	1.09–3.86	0.026	2.32	1.23–4.36	0.009

*Overall significance as a prognostic factor.

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High expression of Skp2 was reported to correlate with reduced overall survival in patients with myxofibrosacroma [53,54]. Di Vizio et al. [55] found that Skp2 expression correlated with poor prognosis in gastrointestinal stromal tumors (GIST). Oliveira found that Skp2 expression is associated with cell proliferation and a worse prognosis in 182 soft tissue sarcomas [56]. In our material, high expression of Skp2 was a negative prognostic factor for DSS. Interestingly, this correlation was statistically significant in women only (P = 0.009), not men (P = 0.577). This may be related to differences in expression of sexual hormone receptors (ER and PGR) in male and female STS patients [57,58]. An inverse correlation between Skp2 expression and the expression of ER and PGR has been reported by others investigating breast cancer [59] and other studies suggest that Skp2B may modulate the activity of the estrogen receptor [60,61]. It should be a priority in further studies to explore the relations of Skp2, gender and DSS.

Since the regulation of p27Kip1 degradation is mediated by its specific ubiquitin ligase subunits S-phase kinase protein (Skp)2 and cyclin-dependent kinase subunit (Cks)1, many have an inverse correlation regarding overexpression of Skp2 and decreased expression of p27Kip1, an analysis of p27Kip1 and other Skp2 target proteins such as p53 and Rb would be helpful for substantiating the Skp2 observations. It has already been shown that Skp2 deficiency can enhance sensitivity of leukemia cells to chemotherapy [62] and Skp2 is itself being increasingly considered a possible target for breast cancer and prostate cancer therapy [63,64]. Wang et al. found a significantly negative correlation between Skp2 expression and the survival of patients administered radiotherapy, indicating that overexpression of Skp2 was correlated with an increased radioresistance of esophageal squamous cell carcinoma. This is in contrast to our findings where chemotherapy and radiotherapy appear to reduce the negative survival impact in patients with Skp2 expressing STS tumors.

In conclusion, our data suggest that an increased Skp2 expression in women with STS was an independent indicator of a poor survival. Skp2 expression data may provide additional information to guide adjuvant therapy after surgical resection. Future studies are warranted to evaluate whether adjuvant chemotherapy or radiotherapy will improve the poor prognosis of Skp2 expressing soft tissue sarcoma patients.

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Author Contributions

Conceived and designed the experiments: SWS TK AV TD RMB LTB. Performed the experiments: SWS TK AV. Analyzed the data: SWS TK

AV TD RMB LTB. Contributed reagents/materials/analysis tools: SWS TK AV TD ES KAS LTB. Wrote the paper: SWS TK TD ES KAS RMB LTB.

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Prognostic impact of Skp2, ER and PGR in male and female patients with soft tissue sarcomas

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Abstract

Background: S-phase kinase-associated protein 2 (Skp2) is a member of mammalian F-box proteins. The purpose of this study is to clarify the prognostic significance of expression of Skp2 related to gender, estrogen receptor (ER) and progesterone receptor (PGR) in soft tissue sarcomas (STS). Skp2 has been demonstrated to display an oncogenic function since its overexpression has been observed in many human cancers. Optimized treatment of STS requires better identification of high-risk patients who will benefit from adjuvant therapy. The prognostic significance of Skp2 related to ER and PGR in STS has not been sufficiently investigated.

Methods: Tissue microarrays from 193 STS patients were constructed from duplicate cores of viable and representative neoplastic tumor areas. Immunohistochemistry was used to evaluate the expression of Skp2, ER and PGR.

Results: In univariate analyses, high tumor expression of Skp2 correlated ($p = 0.050$) with reduced disease-specific survival (DSS). In subgroup analyses expression of PGR in males ($p = 0.010$) and in patients older than 60 years ($p = 0.043$) were negative prognostic factors for DSS. Expression of ER in females was a positive prognostic factor for DSS ($p = 0.041$). In co-expression analyses in the whole cohort, low expression of Skp2 in combination with low expression of ER was positive for DSS ($p = 0.049$). In females high expression of Skp2 in combination with low expression of ER was a negative prognosticator ($p = 0.021$). In the multivariate analyses, age ($p = 0.012$), malignancy grade ($p < 0.001$), wide resection margins ($P = 0.010$), ER negative / PGR positive co-expression profile ($p = 0.002$) and ER positive / PGR negative co-expression profile ($p = 0.015$) were independent negative prognostic factors for DSS. In females expression of Skp2 ($p = 0.006$) was associated with shorter DSS.

Conclusions: We found diverse prognostic impacts of expression of Skp2, ER, PGR and DSS in male and female patients with STS. In men, but not women, ER positive / PGR negative co-expression profile was an independent negative prognostic factor for DSS. In women, but not men, high expression of Skp2 was associated with reduced DSS.

Background

S-phase kinase-associated protein 2 (Skp2), a mammalian F-box protein, displays S-phase-promoting function, through ubiquitin-mediated proteolysis of the CDK inhibitor p27. Skp2 has been shown to regulate cellular proliferation by targeting several cell cycle-regulated proteins for ubiquitination and degradation. Skp2 has also been demonstrated to display an oncogenic function since its overexpression has been observed in many human cancers [1]. High expression of Skp2 was reported to correlate with reduced overall survival in patients with myxofibrosarcoma [2,3]. Di Vizio et al. [4] found that Skp2 expression correlates with poor prognosis in gastrointestinal stromal tumors (GIST). Oliveira found that Skp2 expression is associated with cell proliferation and a worse prognosis in 182 soft tissue sarcomas [5]. In a previous study we showed that high expression of Skp2 was a negative prognostic factor for DSS [6]. Interestingly, this correlation was statistically significant in females only, not in males. This may be related to differences in expression of sexual hormone receptors (ER and PGR) in male and female STS patients [7,8]. In previous studies, we have shown the prognostic value of female steroid hormone receptors in STSs, both alone and in coexpression with TGF-β, fascin and Akt isoforms [7-9]. Such prognostic impact is not surprising,

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since both ER and PGR regulate growth and cell differentiation upon ligand-dependent and ligand-independent activation and are in essence growth factors. However, the prognostic significance of Skp2 related to ER and PGR in STS has not been sufficiently investigated.

The purpose of this study is to clarify the prognostic significance of expression of Skp2 related to age, gender and female steroid hormone receptors (ER and PGR) in non-gastrointestinal stromal tumor (non-GIST) STS. To achieve this, we analyzed the expression of these markers in 193 patients with non-GIST STS in relation to demographic and other clinicopathological variables. Our major hypothesis is that a different prognostic significance of Skp2 in men and women exists and is related to diverse gender expressions of ER and PGR.

Methods

Primary tumor tissues from patients diagnosed with STS at the University Hospital of North Norway (UNN) from 1973 to 2006 and the Hospitals of Arkhangelsk region,

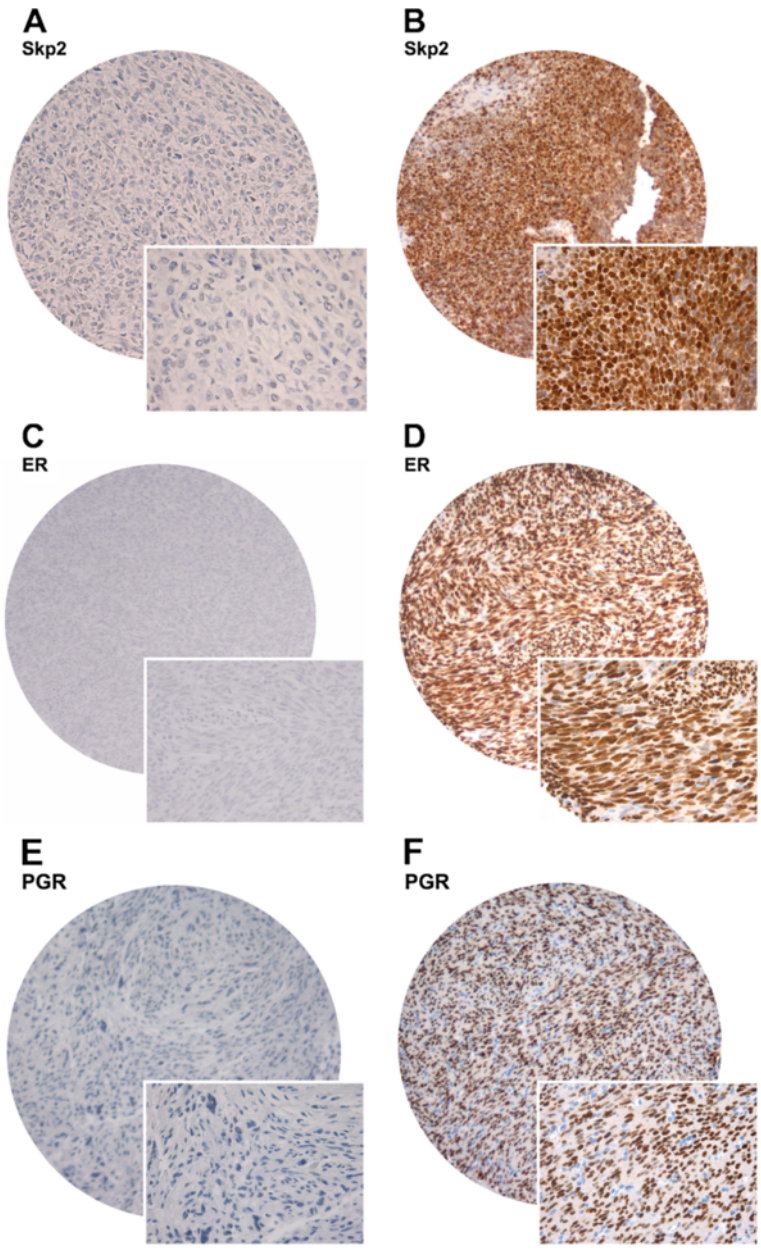

Figure 1 Pictures of cores. Immunohistochemistry microscopic pictures of tissue micro array of soft tissue sarcoma representing different expression of Skp2 and ER. (**A**) Skp2 low score; (**B**) Skp2 high score; (**C**) ER low score; (**D**) ER high score; (**E**) PGR low score; (**F**) PGR high score; Original magnification ×100 and ×400.

Table 1 Prognostic clinicopathological variables as predictors for disease-specific survival of soft tissue sarcomas (univariate analysis, log rank test), N = 193

Characteristic	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Age					
<20 years	17	9	190	47	0.064
20–59 years	85	44	235	63	
≥60 years	91	47	111	51	
Gender					
Male	81	42	235	60	0.087
Female	112	58	180	53	
Nationality					
Norwegian	131	68	228	62	0.005
Russian	62	32	81	44	
Histology					
Pleomorphic sarcoma	57	30	52	45	0.031
Leiomyosarcoma	47	24	89	64	
Liposarcoma	32	17	NR	71	
MF/MFT	16	8	123	56	
Angiosarcoma	8	4	10	38	
Rhabdomyosarcoma	9	5	NR	67	
MPNST	9	5	NR	56	
Synovial sarcoma	12	6	31	30	
Other STS	3	2	NR	-	
Tumor localization					
Extremities	78	40	201	56	0.922
Trunk	37	19	214	53	
Retroperitoneum	27	14	135	51	
Head/Neck	13	7	191	58	
Visceral	38	20	202	62	
Tumor size					
<5 cm	57	30	257	69	0.026
5–9 cm	73	38	183	54	
≥10 cm	61	32	127	48	
Missing	2	1			
Malignancy grade FNCLCC					
1	54	28	NR	81	<0.001
2	76	39	80	55	
3	63	33	28	36	
Surgical margins					
Wide	97	50	254	66	<0.001
Non-wide	96	50	128	46	
Chemotherapy					
No	156	81	207	57	0.669
Yes	37	19	180	51	
Radiotherapy					
No	132	68	216	58	0.190
Yes	61	32	152	52	

Abbreviations: MF/MFT, malignant fibroblastic/myofibroblastic tumors; *MPNST*, malignant peripheral nerve sheath tumor; *STS*, soft tissue sarcomas; *NR*, not reached; *NOS*, non specified.

Russia, were used in this retrospective study. In total, 496 potentially suitable patient records were identified from the hospitals' databases. Of these, 247 patients were excluded due to missing clinical data (n = 86) or inadequate material for histological examination (n = 161). In addition, 33 were excluded because of metastasis at the time of the diagnosis, 13 were excluded because they had no surgery, and 10 patients had both metastasis and no surgery, leaving a total of 193 patients eligible for this study. This report includes data for 131 Norwegian patients and 62 Russian patients followed until September 2009. The median follow-up was 38 (range 0–392) months. Complete demographic and clinical data were collected retrospectively. Formalin-fixed and paraffin-embedded tumor specimens were obtained from the archives of the Departments of Pathology at UNN and Arkhangelsk. The tumors were graded according to the French Fédération Nationales des Centres de Lutte Contre le Cancer (FNCLCC) system [WHO Tumors of Soft Tissue and bone, 2002]. Wide resection margins were defined as wide local resection with free microscopic margins or amputation of the affected limb or organ. Non-wide resection margins were defined as either marginal or intralesional resection margins.

Microarray construction

Two pathologists (AV and SWS) reviewed the histology of all soft tissue sarcoma cases. Tissue microarrays (TMAs) were constructed for high-throughput molecular pathology research [10]. The most representative areas of viable tumor cells were carefully selected and marked on the hematoxylin and eosin (HE) slides for the corresponding donor blocks and sampled for the tissue microarray collector blocks. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments).

Studies suggest that punching multiple 0.6 mm cores from different regions captures the heterogeneity of the tumors more accurately than a single 2 to 4 mm core [11]. We therefore chose to use two 0.6-mm cores of

Table 2 Percentage of high expression of ER, PGR and Skp2 in the different histological subtypes N = 193

Histology	N	ER (%)*	PGR (%)**	Skp2 (%)***
Pleomorphic sarcoma	57	40	26	37
Leiomyosarcoma	47	50	43	40
Liposarcoma	32	35	23	21
MF/MFT	16	27	29	36
Angiosarcoma	8	25	13	29
Rhabdomyosarcoma	9	50	56	67
MPNST	9	11	11	44
Synovial sarcoma	12	40	27	50
Other STS	3	67	33	67
Total	193	39	30	38

* Chi 8.516, p = 0.385.
** Chi 10.238, p = 0.249.
*** Chi 8.596, p = 0.377.
Abbreviations: MF/MFT, malignant fibroblastic/myofibroblastic tumors; *MPNST*, malignant peripheral nerve sheath tumor.
Chi-square test showed no differences in percentage of high expression of ER, PGR and Skp2 in the different histological subtypes.

viable neoplastic tissue. After reviewing all original sections of the tumor and taking heterogeneity into consideration, the two cores were selected to be as representative as possible (different areas). To include all core samples, 12 tissue array blocks were constructed. Multiple 4-μm sections were cut with a Micron microtome (HM355S) and stained with specific antibodies for immunohistochemistry (IHC).

Immunohistochemistry (IHC)

The applied antibodies were subjected to in-house validation by the manufacturer of IHC analysis on paraffin-embedded material. All staining was performed in the Ventana Benchmark XT automated slide stainer (Ventana Medical System, Illkirch, France). Before staining, the sections were incubated over night at 60 degrees Celsius. Tissue sections were incubated with primary mouse monoclonal antibodies recognizing Skp2 (Zymed, catalog number 18–0307, 1:10), ER (Ventana, catalog number 790–4324, ready to use) and PGR (Ventana, catalog number 790–4296). The incubation periods were 40 minutes for Skp2, 32 minutes for ER and 24 min for PGR. This was followed by application of liquid diaminobenzidine as substrate-chromogen, yielding a brown reaction product at the site of the target antigen (Ventana iView DAB Detection Kit, catalog number 760–091). iVIEW DAB Detection Kit is an indirect biotin streptavidin system for detecting mouse and rabbit primary antibodies. The DAB chromogen produces a dark brown precipitate that is readily visualized by light microscopy. All reagents are provided pre-diluted by the manufacturer for use in Ventana Benchmark XT. Finally, slides were counterstained with hematoxylin to visualize the nuclei. For each antibody, including negative controls, all TMA staining were performed in a single experiment. In the TMA we also used cores from carcinomas and normal tissue as positive and negative controls.

Scoring of IHC

The ARIOL imaging system (Genetix, San Jose, CA) was used to scan the slides for antibody staining of the TMAs. The specimens were scanned at a low resolution (1.25×) and a high resolution (20×) using an Olympus BX 61 microscope with an automated platform (Prior). The slides were loaded in the automated slide loader (Applied Imaging SL 50). Representative and viable tissue sections were scored manually on a computer screen semi-quantitatively for nuclear and/or cytoplasmic staining. The expression of Skp2, ER and PGR was scored as: 0, negative; 1, weak; 2, intermediate and 3, strong (Figure 1). The score for each patient was based on the mean scoring of cores from one or several biopsies. To achieve maximal reproducibility in all cases, every staining was dichotomized (negative and positive expression). Positive expression was defined as mean score > 0. All samples were anonymized and independently scored by two pathologists (AV and SWS). In case of disagreement, the slides were re-examined and the observers reached a consensus. When assessing a variable for a given score, the scores of the other variables and the outcome were hidden from the observers.

Statistical methods

All statistical analysis was performed using the statistical package SPSS (Chicago, IL), version 18. The IHC scores from each observer were compared for inter-observer reliability by use of a two-way random effects model with absolute agreement definition. The intra-class correlation coefficient (reliability coefficient) was obtained from these results.

Chi-square and Fisher exact tests were used to examine the association between molecular marker expression and various clinicopathological parameters. Univariate analyses were done using the Kaplan-Meier method, and statistical significance between survival curves was assessed by the log rank test. Disease-specific survival (DSS) was determined from the date of histologically confirmed STS diagnosis. Correlation of marker expression was done using the Pearson correlation (2-tailed) at the 0.05 and 0.01 levels.

Multivariate analysis was carried out using the Cox proportional hazards model to assess the specific impact of each pre-treatment variable on survival in the presence of other variables. Variables of significant value from the univariate analysis were entered into the Cox regression analysis. Probability for stepwise entry and removal was set at 0.05 and 0.10, respectively. The significance level used was $p < 0.05$.

Table 3 Expression of markers, gender and their prediction for disease-specific survival in patients with soft tissue sarcomas (univariate analysis; log-rank test), All = 193, Males = 81, Females = 112

Marker expression	Patients (n)	Patients (%)	Median survival (months)	5-year survival (%)	P
Skp2, all					
Low	109	56	NR	63	0.050
High	67	45	59	50	
Missing	17	9			
Skp2, men					
Low	50	62	NR	63	0.577
High	23	28	67	61	
Missing	8	10			
Skp2, women					
Low	59	53	NR	63	0.066
High	44	39	49	44	
Missing	9	8			
ER, all					
Low	112	58	123	57	0.725
High	72	67	91	57	
Missing	9	5			
ER, men					
Low	49	60	NR	69	0.089
High	29	36	58	49	
Missing	3	4			
ER, women					
Low	63	56	57	47	0.041
High	43	38	NR	62	
Missing	6	5			
PGR, all					
Low	132	68	NR	62	0.101
High	57	30	52	46	
Missing	4	2			
PGR, men					
Low	64	79	NR	69	0.010
High	15	19	41	33	
Missing	2	2			
PGR, women					
Low	68	61	80	55	0.832
High	42	38	74	51	
Missing	2	2			

Abbreviations: NR, not reached.

Consent

The National Cancer Data Inspection Board and The Regional Committee for Research Ethics (REK nord) approved the study. The material was collected from our approved biobank for paraffin embedded material and slides. The Regional Committee approved that written consent from the patients for their information to be stored in the hospital database and used for research was not needed because most of the material was more than 10 years old, and most of the patients being dead. The ethics committee specifically waived the need for consent. Data were analyzed anonymously.

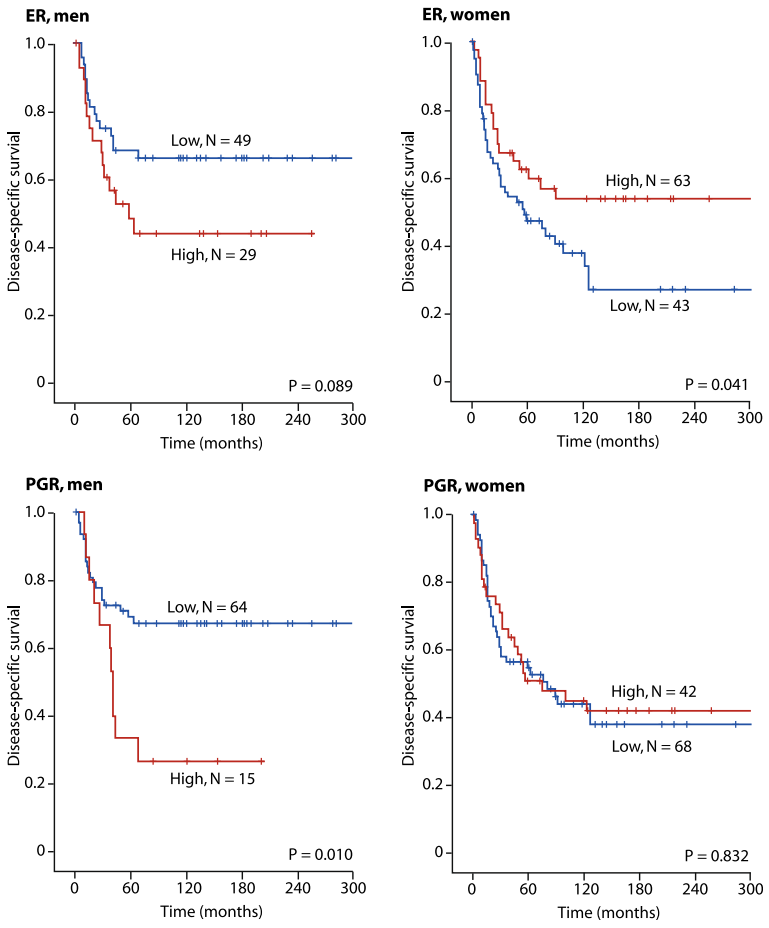

Figure 2 Survival plots ER and PGR. Disease-specific survival curves for high and low expression of ER and PGR in male (N = 81) and female (N = 112) patients with soft tissue sarcomas.

Results

Clinicopathological variables

Demographic, clinical, and histopathological variables are shown in Table 1. Patient age ranged from 0–89 years (mean 55 years), and 42% of patients (81/193) were male. Treatment for all patients included surgery: 104 patients received surgery only; 52 patients received surgery and radiotherapy; 28 patients received surgery and chemotherapy; 9 patients received surgery, radiotherapy and chemotherapy. The 5-year survival for patients with wide and non-wide resection margins was 66% and 46% respectively, Table 1.

Inter-observer variability

There was good scoring agreement between the two investigating pathologists. The IHC scores from each observer were compared using a two-way random effects model with absolute agreement definition. The intra-class correlation coefficients (reliability coefficients, r) obtained from these results were 0.94 for Skp2 ($p < 0.001$), 0.92 for ER ($p < 0.001$) and 0.96 for PGR ($p < 0.001$).

Univariate analyses

Nationality, histology, tumor size, malignancy grade and surgical margins were all significant indicators for disease-specific survival (DSS) in univariate analyses (Table 1). Table 2 shows the percentage of high expression of ER, PGR and Skp2 in the different histological subtypes. Chi-square test showed no differences in overall expression of ER, PGR and Skp2 with respect to the different histological subtypes.

In univariate analyses, increased expression of Skp2 ($p = 0.050$) correlated significantly with reduced DSS, (Table 3 and Figure 2). No such relationship was apparent for ER and PGR when males and females were combined in one group.

In subgroup analyses (Tables 3 and 4), increased PGR expression in men ($p = 0.010$) and in patients older than 60 years ($p = 0.043$) was associated with a reduced DSS. Increased ER expression in women was associated with longer DSS ($p = 0.041$). High expression of ER were associated with favorable survival in patients with rhabdomyosarcoma ($N = 9$, $p = 0.040$). High expression of ER was

Table 4 Expression of markers, age and their prediction for disease-specific survival in patients with soft tissue sarcomas (univariate analysis; log-rank test)

Marker expression	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Skp2, <60 years, N = 99					
Low	52	53	NR	71	0.074
High	38	38	67	56	
Missing	9	9			
Skp2, ≥60 years, N = 94					
Low	57	61	80	57	0.188
High	29	31	36	42	
Missing	8	9			
ER, <60 years, N = 99					
Low	55	56	127	59	0.197
High	40	40	NR	67	
Missing	4	4			
ER, ≥60 years, N = 94					
Low	57	61	80	55	0.293
High	32	34	52	44	
Missing	5	5			
PGR, <60 years, N = 99					
Low	63	64	NR	67	0.488
High	34	34	NR	55	
Missing	2	2			
PGR, ≥60 years, N = 94					
Low	69	73	91	57	0.043
High	23	24	39	32	
Missing	2	2			

Abbreviations: NR, not reached.

associated with poor survival in patients with synovial sarcoma (N = 12, p = 0.010). There were no significant differences in survival according to high or low expression of Skp2 in any of the histological subtypes (data not shown).

In patients with low expression of ER (N = 112), men had better 5-year survival (69%) compared to women (47%, p = 0.002), while there were no differences (p = 0.376) between men and women in patients with high expression of ER (N = 72). In patients with low expression of PGR (N = 132), men had better 5-year survival (69%) compared to women (55%, p = 0.013), while there were no differences (p = 0.271) between men and women in patients with high expression of PGR (N = 57). There were no differences in survival between men and women in univariate analyses of patients with low (N = 109, p = 0.529) or high (N = 67, p = 0.233) expression of Skp2 (data not shown).

In co-expression analyses (Table 5) Skp2 negative / ER negative profile was associated with longer DSS (p = 0.049). In women a Skp2 positive and ER negative profile was associated with reduced DSS (p = 0.021), Table 5 and Figure 3. In men a double negative ER/PGR profile was associated with longer DSS (p = 0.013) while in women a double positive ER/PGR was associated with longer DSS (p = 0.001). In patients younger than 60 years the combination ER negative and PGR positive was associated with shorter DSS. In the whole cohort of patients a triple positive expression of ER, PGR and Skp2 was associated with longer DSS (p = 0.005), Figure 3. Triple negative expression of ER, PGR and Skp2 was also associated with longer DSS, but not statistically significant (p = 0.068), Figure 3. ER negative / PGR positive co-expression was associated with shorter DSS regardless of Skp2 expression, Table 6.

Taking into consideration the possible distortion of results by gender-related sarcomas (i.e. leiomyosarcoma in uterus) we have attempted to exclude these sarcomas and recalculate all analyses. There were no significant differences in the results compared to those obtained without exclusion of gender-related sarcomas (data not shown).

Multivariate analyses

Significant demographic, clinicopathological and expression variables from the univariate analyses were entered into the

Table 5 Co-expression of Skp2/ER, Skp2/PGR and their prediction for disease-specific survival in patients with soft tissue sarcomas (univariate analysis; log-rank test), All = 193, Men = 81, Women = 112

Co-expression	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Skp2 / ER, all					
Low/low	66	34	NR	67	0.049
Low/high	39	20	91	59	
High/low	35	18	57	44	
High/high	30	16	NR	58	
Missing	23	12			
Skp2 / ER, men					
Low/low	33	41	NR	72	0.427
Low/high	16	20	37	50	
High/low	11	14	NR	72	
High/high	11	14	63	58	
Missing	10	12			
Skp2 / ER, women					
Low/low	33	29	127	61	0.021
Low/high	23	21	91	65	
High/low	24	21	31	32	
High/high	19	17	NR	58	
Missing	13	12			
Skp2 / PGR, all					
Low/low	80	41	NR	71	0.056
Low/high	25	13	54	46	
High/low	40	21	59	49	
High/high	27	14	67	51	
Missing	21	11			
Skp2 / PGR, men					
Low/low	41	51	NR	73	0.141
Low/high	7	9	26	29	
High/low	18	22	NR	61	
High/high	5	6	67	60	
Missing	10	12			
Skp2 / PGR, women					
Low/low	39	35	NR	68	0.234
Low/high	18	16	75	54	
High/low	22	20	29	39	
High/high	22	20	57	49	
Missing	11	10			

Abbreviations: NR, not reached.

multivariate Cox regression analysis (Table 7). In the multivariate analyses, age ($p = 0.012$), malignancy grade ($p < 0.001$), wide resection margins ($p = 0.010$), ER negative / PGR positive co-expression ($p = 0.002$) and ER positive / PGR negative co-expression ($p = 0.015$) were independent negative prognostic factors for DSS. In women, expression of Skp2 ($p = 0.006$) was associated with reduced DSS. In women, tumor size ($p = 0.020$) and nationality ($p = 0.014$) were independent prognostic factors for DSS, Table 7. In multivariate analyses co-expression of Skp2/ER or Skp2/PGR were not stronger prognosticators for DSS than single expression of Skp2, ER and PGR (data not shown).

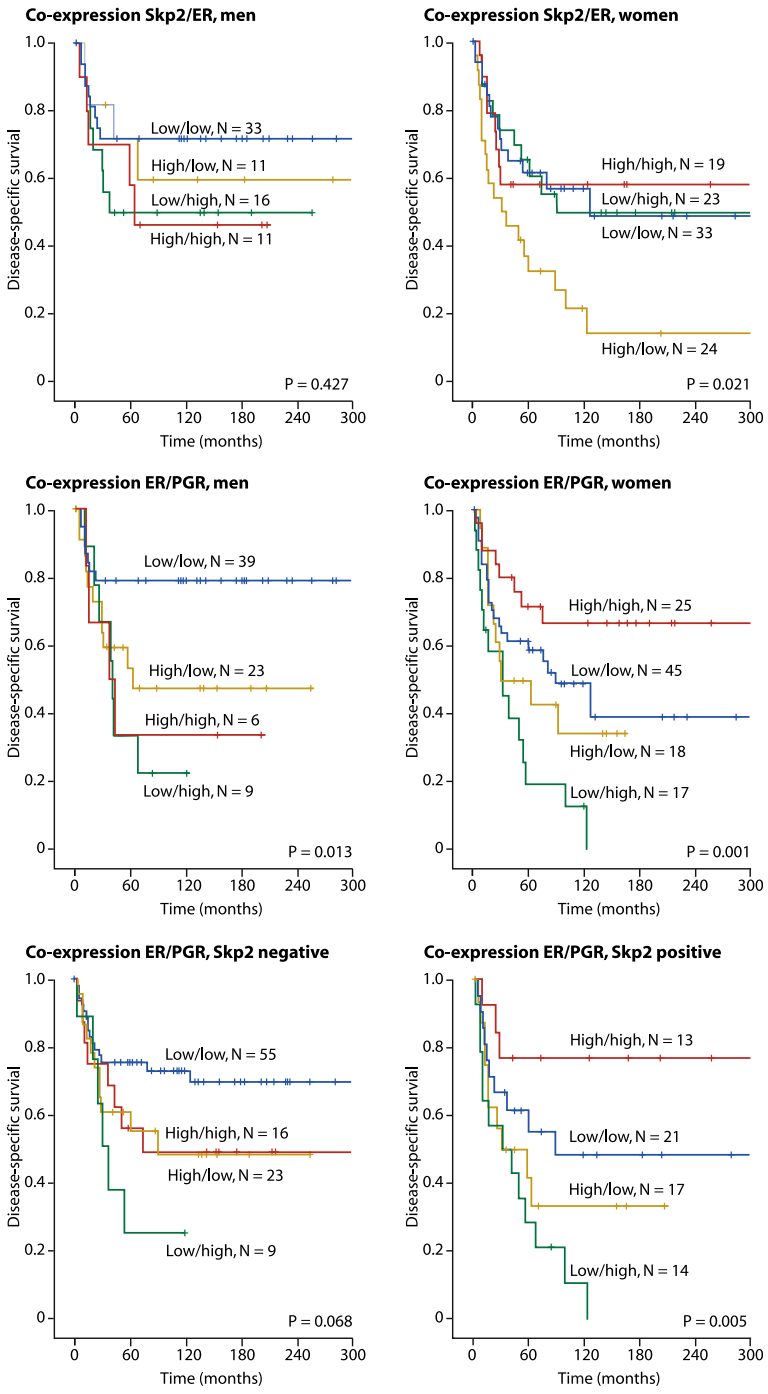

Figure 3 Survival plots co-expression. Disease-specific survival curves for co-expression of Skp2, ER or PGR in males (N = 81), females (N = 112) and co-expression of ER and PGR in Skp2 negative (N = 109) and Skp2 positive (N = 67) patients.

Discussion

In this large-scale study, we evaluated the prognostic significance of expression of Skp2 related to age, gender, ER and PGR in 193 STS patients. Our hypothesis was confirmed. We found diverse prognostic DSS impacts from gender related expression of Skp2, ER, PGR and DSS in STS. In men, but not women, an ER positive/PGR negative co-expression profile was an independent negative prognostic factor for DSS. In women, but not men, high expression of Skp2 was associated with reduced DSS. High expression of ER reduced the negative impact of Skp2 in women. While women with the Skp2 positive / ER positive phenotype had favorable survival, women with the Skp2 positive / ER negative phenotype had

Table 6 Co-expression of ER/PGR and their prediction for disease-specific survival in patients with soft tissue sarcomas (univariate analysis; log-rank test)

Co-expression	Patients (n)	Patients (%)	Median survival (months)	5-year survival (%)	P
ER / PGR, all, N = 193					
Low/low	84	44	NR	69	<0.001
Low/high	26	13	38	24	
High/low	41	21	62	52	
High/high	31	16	NR	64	
Missing	11	6			
ER / PGR, men, N = 81					
Low/low	39	48	NR	79	0.013
Low/high	9	11	41	33	
High/low	23	28	63	53	
High/high	6	7	37	33	
Missing	4	5			
ER / PGR, women, N = 121					
Low/low	45	40	89	59	0.001
Low/high	17	15	31	19	
High/low	18	16	29	50	
High/high	25	22	NR	72	
Missing	7	6			
ER / PGR, <60 years, N = 99					
Low/low	41	41	NR	72	0.001
Low/high	13	13	31	23	
High/low	19	19	NR	58	
High/high	21	21	NR	76	
Missing	5	5			
ER / PGR, ≥60 years, N = 94					
Low/low	43	46	NR	64	0.052
Low/high	13	14	39	26	
High/low	22	23	58	47	
High/high	10	11	37	40	
Missing	6	6			
ER / PGR, Skp2 low, N = 109					
Low/low	55	50	NR	76	0.068
Low/high	9	8	68	25	
High/low	23	21	91	61	
High/high	16	15	75	56	
Missing	6	6			
ER / PGR, Skp2 high, N = 67					
Low/low	21	31	89	55	0.005
Low/high	14	21	31	29	
High/low	17	25	29	42	
High/high	13	19	NR	77	
Missing	2	3			

Abbreviations: NR, not reached.

Table 7 Results of Cox regression analysis summarizing prognostic factors in patients with soft tissue sarcomas

	All patients, N = 193			Men, N = 81			Women, N = 112		
Factor	**Hazard ratio**	**95% CI**	**P**	**Hazard ratio**	**95% CI**	**P**	**Hazard ratio**	**95% CI**	**P**
Age									
0–59 years	1.00			1.00			1.00		
≥60 years	1.84	1.15-2.95	0.012	1.69	0.65-4.41	0.282	1.51	0.83-2.77	0.179
Nationality									
Norwegian	1.00			1.00			1.00		
Russian	1.49	0.88-2.52	0.143	1.39	0.41-4.66	0.598	2.51	1.20-5.21	0.014
Tumor size									
<5 cm	1.00		0.138*	1.00		0.668*	1.00		0.020*
5–9 cm	1.47	0.79-2.73	0.226	1.68	0.54-5.25	0.372	1.71	0.77-3.77	0.187
≥10 cm	1.91	1.01-3.60	0.047	1.32	0.40-4.39	0.652	3.14	1.38-7.15	0.006
Malignancy grade FNCLCC									
1	1.00		<0.001*	1.00		<0.001	1.00		0.004*
2	2.72	1.36-5.46	0.005	3.07	0.86-10.96	0.084	4.33	1.76-10.67	0.001
3	4.61	2.26-9.40	<0.001	15.47	4.36-54.97	<0.001	4.23	1.64-10.89	0.003
Resection margins									
Wide	1.00			1.00			1.00		
Non-wide	1.87	1.16-3.02	0.010	7.69	2.67-22.16	<0.001	0.81	0.42-1.54	0.512
Skp2									
Low	1.00			1.00			1.00		
High	1.48	0.87-2.52	0.151	0.46	0.19-1.12	0.088	2.52	1.31-4.85	0.006
ER / PGR									
Low/low	1.00		0.006*	1.00		0.004*	1.00		0.216*
Low/high	2.64	1.43-4.85	0.002	4.99	1.31-18.97	0.018	1.91	0.89-4.11	0.097
High/low	2.07	1.15-3.73	0.015	8.35	2.55-27.36	<0.001	1.76	0.79-3.93	0.170
High/high	1.16	0.57-2.38	0.682	4.50	0.96-21.13	0.056	0.92	0.36-2.35	0.868

* Overall significance as a prognostic factor. The difference between the individual p-value and total p-value in the multivariate analysis is relevant in cases where there are more than two categories for a given variable. Overall p-value is calculated based on a general assessment of all categories for the given variable, but the individual p-value only calculates the significance of a given category versus the reference category.

poor survival. To the best of our knowledge, this is the first prognostic evaluation of Skp2 related to the female hormone receptors ER and PGR in STS.

Expression of ER and PGR is a routinely investigated indicator of endocrine therapy success in breast cancer [12,13] and a modest, but significantly better overall survival of anti-estrogen receptor therapy has been documented [14]. ER and PGR are also reported to be positive prognosticators of uterine leiomyosarcomas [15]. However, extra-uterine sarcomas have barely been explored in this context. The distribution and prognostic value of expression of these steroid hormone receptors in STS are therefore of great scientific interest. In our study, in the univariate analyses, ER showed a significantly favorable influence on survival in female patients, but not in males. PGR was an unfavorable prognosticator for men, but not for women. In multivariate analysis ER positive / PGR negative co-expression is an independent negative prognostic factor for DSS in males, but not in females.

We have modified the Allred score for STS and used 1% positivity as cut-off value [7,16]. The strong and moderate (score 3 and 2, respectively) hormone receptor expression occurred mostly in sarcomas of uterus, pelvis and breast, while the weak (score 1) expression of both ER and PGR was surprisingly evenly distributed among location, gender and age. Generally, 39% of the tumors expressed ER and 30% expressed PGR in our material. Roughly half of the patients expressed at least one of these receptors. The findings are in partial agreement with findings of Chaudhuri et al. [17] who found ER to be positive in 24% of STS.

Huang et al. suggested that the therapeutic strategies designed to reduce Skp2 may play an important clinical role in treatment of breast cancer cells, especially ER/HER2 negative breast cancers [18]. Voduc et al. found

cyclin E and Skp2 to be prognostic for breast cancer-specific survival in univariate analyses. Double positive expression of cyclin E / Skp2 was associated with young age at diagnosis, grade 3 tumors, ER-negative status and HER2 negative status [19]. Zheng et al. found that higher levels of Skp2 were detected more frequently in ER-negative breast cancer tumors and tumors metastatic to the axillary lymph nodes [20]. Signoretti et al. also found that higher levels of Skp2 are present more frequently in ER-negative tumors than in ER-positive cases. The subset of Skp2 positive / ER negative breast carcinomas were also characterized by high tumor grade and HER2 negative [21]. In our material, the five year DSS in Skp2 positive / ER negative women with STS was 32% compared to 58% in Skp2 positive / ER positive women (P = 0.021).

In our previous work we have shown that ER and PGR expression possess variable prognostic significance depending on gender, both *per se* and in co-expression with TGF-β, fascin and Akt isoforms [7-9]. In the present study, the prognostic diversity of Skp2, ER and PGR in men and women was seen in the different co-expression profiles: female patients with Skp2 positive / ER negative profile had decreased survival rates. For men, the Skp2 negative / ER negative profile was the most favorable phenotype. PGR expression in men, but not women, was associated with a shorter DSS. ER expression in women, but not men, was associated with a longer DSS. The ER negative / PGR positive profile was a significantly unfavorable factor for the whole patient cohort both in univariate and multivariate analysis. Interestingly, such a profile occurred in only 2% of patients in one large-scale study based on 3000 breast cancer cases [22], while in our STS study this profile was seen in 13% of tumors.

The data collection introduced problems in identifying adequate numbers of similar patients with similar tumors and with the same treatment traditions. These are well known problems when conducting STS studies. Our findings are in large hypothesis generating, and to be more conclusive future STS studies must be based on large, multi-institutional and multinational studies with possibilities to establish adequately sized STS patient cohorts of homogenous tumor groups. However, all tumors investigated herein had mesenchymal derivation and belong to the same generic group.

Conclusions

In conclusion, there were different prognostic impacts of expression of Skp2, ER, PGR and DSS in male and female patients with STS. In men, but not in women, ER positive / PGR negative co-expression was an independent negative prognostic factor for DSS. In women, but not in men, expression of Skp2 was associated with reduced DSS.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SWS, TK, AV, TD, RMB and LTB participated in the design of the study. TK, ES and AV collected clinical information. SWS and AV reviewed all the histological diagnosis, histological grading, selected and marked the slides for TMA construction. SWS, TK and AV performed the experiments. SWS, TK, AV, TD, RMB and LTB performed the statistical analysis. SWS, TK, AV, TD, ES, KAS and LTB contributed reagents/materials/analysis tools. SWS, TD, ES, KAS, RMB and LTB drafted the manuscript. All authors read and approved the final manuscript.

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