CLINICAL AND MOLECULAR STUDIES OF LIPOSARCOMA

Göteborg 2007
To my dear family;
my husband Carl-Peter and my children Peter and Carolina
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ABSTRACT

Clinical and Molecular Studies of Liposarcoma

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Aims: (1) To analyse clinicopathological characteristics, treatment and outcome of liposarcoma, and to determine whether, and how, the Scandinavian Sarcoma Group (SSG) treatment guidelines were followed; (2) to analyse tumour volume and morphology response after radiotherapy in myxoid/round cell liposarcoma (MLS/RCLS); (3) to examine the role of the MLS-specific fusion gene FUS-DDIT3 in development of liposarcomas; and (4) to analyse expression patterns of cell cycle regulating proteins in MLS.

Methods: (1) A total of 319 liposarcomas reported between 1986–1998 to the SSG Register were reviewed. Altogether 237 patients without metastasis were analyzed for local recurrences in relation to surgical margins and radiotherapy, metastasis and survival. (2) Thirty-three primary or metastatic MLSs/RCLSs were treated with radiotherapy. Tumour size was measured by MRI or CT. Histopathology was performed of both non-irradiated and irradiated lesions. (3) The fibrosarcoma cell line HT1080 was transfected with the recombinant vectors pFUS-DDIT3-EGFP, pDDIT3-EGFP and pFUSa-EGFP. The tranfectants and the HT1080 cell line were injected into SCID mice, followed by histopathology. The transfected and non-transfected cells were cultured with adipogenic induction medium and microarray-based expression comparison of the different cell lines was performed. (4) Cell cycle controlling factors were analysed by immunohistochemistry and Western blotting in non-irradiated and irradiated MLSs/RCLSs.

Results: (1) Altogether 78% were primarily operated at a sarcoma centre, 45% with wide margins. Only 58% of high-grade (Grades III-IV) lesions with non-wide surgery had postoperative radiotherapy. The risk of local recurrence in this group was 47%, if not irradiated. The estimated 10-year local recurrence-free and metastasis-free survival in the low-grade (Grades I-II) group was 87% and 95% respectively, while in the high-grade group it was 75% and 61%, respectively. Independent adverse prognostic factors for local recurrence were surgery outside a sarcoma centre and dedifferentiated liposarcoma. For metastases, they were old age, large tumour size, high grade and histological type MLS/RCLS. (2) Irradiated MLS/RCLS showed median tumour volume reduction of 52% in 23 tumours. The morphology showed...
paucicellularity, hyalinization and lipoma-like appearance. There were no obvious differences in volume reduction or morphologic response in MLSs/RCLSs in comparison with MLSs. (3) Cells expressing *FUS-DDIT3* and *DDIT3* grew in SCID mice as liposarcomas and the capillary network was similar to that found in MLSs/RCLSs. Cells transfected with *DDIT3* responded in vitro to adipogenic factors by accumulation of fat, and microarray-based comparison showed that the *DDIT3* and *FUS-DDIT3* transfected variants shifted toward an MLS/RCLS-like expression pattern. (4) High expression of cyclin D1 and E, their kinases and kinase-inhibitors P16, P27 and P57 was observed, together with low Ki67 and normal cyclin A.

**Conclusion:** Liposarcoma should be treated at specialized centres and postoperative radiotherapy is indicated for high-grade lesions, at least after non-wide surgery. Low-grade MLSs have high radio-responsiveness, and radiotherapy is indicated after non-wide surgery or in a preoperative setting. The fusion oncogene *FUS-DDIT3* and *DDIT3* may induce a liposarcoma phenotype, with *DDIT3* being the tumour type-determining part of the fusion oncogene. Deregulation of G1-controlling proteins is common and indicates that MLS cells accumulate in late G1 phase.

**Key words:** Liposarcoma, radiotherapy, prognostic factors, transfection, *FUS-DDIT3*, adipogenesis, microarray expression analysis, cell cycle, immunohistochemistry
LIST OF PUBLICATIONS

This thesis is based upon the following papers, which are referred to in the text by their Roman numerals:

**Paper I**

Katarina Engström, Peter Bergh, Pelle Gustafson, Ragnar Hultborn, Helena Johansson, Rickard Lofvenberg, Kirsten Sundby Hall, Clement Trovik, Ola Wahlström, Henrik C.F. Bauer.


**Paper II**

Katarina Engström, Peter Bergh, Claes-Göran Cederlund, Ragnar Hultborn, Helena Willen, Pierre Åman, Lars-Gunnar Kindblom, Jeanne M. Meis-Kindblom.


**Paper III**

Katarina Engström, Helena Willén, Christina Kåbjörn-Gustafsson, Carola Andersson, Marita Olsson, Melker Göransson, Sofia Järnum, Anita Olofsson, Elisabeth Warnhammar, Pierre Åman.

The myxoid/round cell liposarcoma (MLS/RCLS) fusion oncogene *FUS-DDIT3* and the normal *DDIT3* induce a liposarcoma phenotype in transfected human fibrosarcoma cells. *Am J Pathol 2006, 168:*5.

**Paper IV**


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATM</td>
<td>ataxia-telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ataxia-telangiectasia mutated and rad3-related</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic leucine zipper region</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CHK</td>
<td>checkpoint kinase 2</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>DDIT3</td>
<td>DNA-damage-inducible transcript 3 (CHOP, GADD153)</td>
</tr>
<tr>
<td>DDLS</td>
<td>dedifferentiated liposarcoma</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EWS</td>
<td>Ewing sarcoma</td>
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<tr>
<td>FNA</td>
<td>fine needle aspiration</td>
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<tr>
<td>FUS</td>
<td>fusion (TLS)</td>
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<tr>
<td>GADD153</td>
<td>growth arrest and damage-inducible153</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-Isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>MLS</td>
<td>myxoid liposarcoma</td>
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<tr>
<td>MM</td>
<td>maintenance medium</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<tr>
<td>PDGFR</td>
<td>platelet derived growth factor receptor</td>
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<tr>
<td>PLS</td>
<td>pleomorphic liposarcoma</td>
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<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>pRB</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>RCLS</td>
<td>round cell liposarcoma</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
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<td>RXR</td>
<td>retinoid X receptor</td>
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<tr>
<td>SCID</td>
<td>severe combined immune deficient</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>SSG</td>
<td>Scandinavian Sarcoma Group</td>
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<tr>
<td>STS</td>
<td>soft tissue sarcoma</td>
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<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
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<td>TLS</td>
<td>translocated in liposarcoma (FUS)</td>
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<tr>
<td>UVB</td>
<td>ultraviolet B</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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<td>WDLS</td>
<td>well-differentiated liposarcoma</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>YB-1</td>
<td>Y-box-binding protein-1</td>
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INTRODUCTION

Soft tissue sarcoma (STS) represents a heterogeneous group of diseases accounting for less than 1% of all malignant tumours in Sweden (1). Liposarcomas are among the most common malignant mesenchymal tumours, comprising 10-18% of all STSs (2-4). Various histological typing has been used since Virchow first described liposarcoma 150 years ago (5). In 1935 Ewing (6) broadened the scope of histological criteria by which liposarcoma was recognized and others such as Stout (7), Enterline (8), and Enzinger and Winslow (9) developed the nomenclature and suggested several subgroups. The World Health Organization (WHO) have published three editions on classification of soft tissue tumours, the first in 1969. The current nomenclature is based on a proposal by the WHO Committee for the Classification of Soft Tissue Tumours in 2002 (10) (Table 1).

Table 1. Liposarcoma subtypes, according to the World Health Organization (WHO) classification of 2002.

<table>
<thead>
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<th>Subtype</th>
<th>Description</th>
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<tr>
<td><em>Atypical lipomatous tumour/Well-differentiated liposarcoma</em> (WDLS)</td>
<td>Adipocytic (lipoma-like), sclerosing, inflammatory and spindle cell</td>
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<td><em>Dedifferentiated liposarcoma</em> (DDLS)</td>
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<td><em>Myxoid liposarcoma</em> (MLS)</td>
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<tr>
<td><em>Pleomorphic liposarcoma</em> (PLS)</td>
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<tr>
<td><em>Mixed-type liposarcoma</em></td>
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Epidemiology

One study, based on the Swedish Cancer Registry including all sites, reported an annual incidence of 2.5 per million (11) while a population-based study in the Southern Swedish Health Care Region reported an annual incidence of 1.2 per million for liposarcoma of the extremity and trunk wall (12). Liposarcoma is a tumour primarily found in adults, with a peak incidence between 40 and 60 years of age. Children between 10 and 15 years of age are rarely also reported (13-15). In most larger series
the reported mean age is 52-56 years (16, 17). Individuals with retroperitoneal liposarcoma are 5-10 years older than are individuals with tumours in the extremities, probably owing to later detection. By contrast, patients with myxoid liposarcoma of the extremity and trunk are approximately 10 years younger than those with other histological subtypes (9, 18, 19).

Men are slightly more often affected than women (55%-61%) (16). The major sites of liposarcoma are extremities, particularly the thigh and retroperitoneum (20), but liposarcoma may be found throughout the body. The majority of liposarcomas occur in deep, non-fat soft tissue rather than subcutaneous tissues (16) in contrast to lipomas, which supports the theory that lipomas do not undergo transformation to become liposarcomas (20).

**Pathogenesis**

Because of the rarity of STS most studies deal with the whole group of STSs. The causes of STS are only poorly understood. The pathogenesis is based on various inherited and environmental factors and also, on pre-existing conditions (1, 21, 22). Among environmental factors exposure to ionizing irradiation including Thorotrust, alkylating agents, arsenical pesticides and medications, vinyl chloride, immunosuppressive drugs, human immunodeficiency virus (HIV), herpes virus type 8 and anabolic steroids are described as risk factors. Other chemicals such as phenoxyherbicides, dioxin and chlorophenol are possible risk factors (23, 24).

Inherited conditions associated with STSs are Li-Fraumeni syndrome, neurofibromatosis type1, Gardner’s syndrome, retinoblastoma, Werner’s syndrome and nevoid basal cell carcinoma syndrome (Gorlin’s syndrome). Pre-existing medical conditions such as long-standing lymphoedema (Stewart-Treves syndrome) can also cause STS (1). Several cases with trauma preceding development of STS have been reported in the literature but there is no proof of a causal relationship (2).

**Diagnosis**

**Clinical presentation**

The clinical manifestation is dependent on the localization of the tumour. The presentation of liposarcoma on the extremities and superficial trunk
is often a deep-seated mass, frequently of large size, painless or with mild or moderate pain and functional disturbances. Retroperitoneal liposarcomas tend to be diagnosed later, with more serious bowel symptoms such as gradual abdominal enlargement, pain, anorexia, vomiting and intestinal or urinary obstruction.

**Radiology**

Radiology can be performed either with computed tomography (CT) or with magnetic resonance imaging (MRI) but MRI is preferred because of its high intrinsic contrast resolution and multiplanar imaging possibilities. The different LS subtypes have different magnetic resonance (MR) appearance and diagnostic pitfalls.

The well-differentiated liposarcomas (WDLSs) of the extremities can be difficult to distinguish from lipomas, but the most important features that suggest malignancy are male sex, high age, large size, presence of thickened septa and nodular and/or globular areas of non-adipose tissue within the fatty lesion composing 25% or more of the volume (25, 26).

Dedifferentiated liposarcomas (DDLSs) occur within a WDLS. The radiological features suggesting dedifferentiation are areas of focal, nodular non-lipomatous region greater than 1 cm in size within a WDLS (26).

Myxoid liposarcomas (MLSs) often have a characteristic appearance with a large, well-defined and multi-lobulated lesion, localized intra- and/or intermuscularly. The high water content of the lesion gives a typical appearance on MRI. Adipose tissue in the lesion, that constitutes less than 10% of the lesion and is often seen in septa or as small nodules in the lesion, is an important diagnostic feature (26).

Pleomorphic liposarcomas (PLSs) and round cell liposarcomas (RCLSs) have a non-specific soft tissue tumour appearance on MRI, with prominent heterogeneity and, often, areas of necrosis and haemorrhage. Small amounts of fat in the lesion suggest the diagnosis (26).

**Biopsy options**

In the pre-operative diagnosis of soft tissue tumours, different biopsy techniques are used depending on the site, size and depth of the lesion. Many sarcoma centres use core-needle biopsy or longitudinally oriented incisional biopsy for extremity masses. Fine needle aspiration (FNA) cytology is preferred at sarcoma centres in the Scandinavian countries.
The advantage of using FNA is the small risk of seeding sarcoma cells to the surrounding tissue and allowing a reduced surgical margin, leading to better preservation of function. Fine needle aspiration is also useful for diagnosing intra-abdominal, retroperitoneal and mediastinal masses. The disadvantage of using FNA is that the cellular samples are small and it can be difficult to ascertain the tumour grade and histological type. For surgical planning, it is, however, most important to decide whether the lesion represents a soft tissue tumour and whether the tumour is benign or malignant (27). A 90% diagnostic accuracy of differentiating a benign lesion from a liposarcoma has been reported with FNA cytology (27-30). An experienced cytopathologist is essential for this method to be successful, as is well-functioning collaboration between the radiologist, orthopaedic surgeon and cytopathologist.

**Reverse transcription-polymerase chain reaction**

Reverse transcription-polymerase chain reaction (RT-PCR) is used to detect the *FUS-DDIT3* fusion gene transcripts, pathognomonic for MLS. This method is most suitable for fresh or frozen samples and helps to differentiate between MLSs and other myxoid variants of STSs, such as myxoid malignant fibrous histiocytoma (MFH), myxofibrosarcoma, predominantly myxoid WDLS of the retroperitoneum, and intramuscular myxoma. Even round cell variants of MLS without signs of lipogenesis can be diagnosed with RT-PCR (31).

**Treatment**

**Surgery**

Surgery is the most important treatment modality. Wide local excision with clear margins is important for local tumour control. The Scandinavian Sarcoma Group (SSG) follow the definition of surgical margins introduced by Enneking in 1980 (32). Until 2006 the margins were reported as:

1. “Intralesional margin” when the dissection passed within the lesion and microscopic or macroscopic tumour was left at the margin of the resection.
2. “Marginal margin” when the lesion was removed in one piece but the dissection was through the pseudocapsule or reactive tissue surrounding the lesion.
3. “Wide margin” when the tumour was removed en bloc completely surrounded by a cuff of normal tissue but the dissection was within the involved compartment.
(4) “Myectomy” meant that the muscle in which the tumour was located was removed without opening its fascia.
(5) “Compartmental resection” was performed when the tumour-involved compartment, beyond the fascial septa of the involved compartment, was removed en bloc.

Since 2006 there has been a modification in the classifications of margins as follows:
(1) “Positive margin” means that a gross tumour or microscopic tumour is left at the margin, which is reported.
(2) “Negative margin” means that there is no microscopic tumour at the margin. The extent of the margin is reported.

More than 20 mm of normal tissue around the tumour, or fascia completely surrounding the tumour is classified as “wide margin”.

**Radiotherapy**

Radiotherapy in STS is an established method for elimination of microscopic tumour cf Strander H (33). Radiotherapy as an adjunct to limited surgical excision was shown to achieve somewhat lower local control than radical resection alone but with greatly improved functional and cosmetic results and no differences in overall survival (34-37). In 1998 Yang et al. (38) assessed, in a prospective randomized trial, that adjuvant postoperative radiation therapy in combination with conservative surgery improves local control for extremity STSs of both low and high grade in patients with microscopic negative, marginal or minimal microscopic positive surgical margins. No difference was found in overall survival. It is interesting to note that in the low-grade group 40% of lesions were of the radio-sensitive MLS type.

Until 2005 the SSG recommended postoperative radiotherapy to a dosage of 50 Gy if the surgery was marginal and to 60–70 Gy if the surgery was intralesional and a resection was not possible. According to new recommendations, even deep-seated, high-grade (histopathological grade III-IV) STSs with wide surgery should be treated with 50 Gy.

Most STSs that are unresectable do not respond to radiation therapy with sufficient tumour volume regression, making them resectable. The few studies on this subject report a 5-year local control rate of approximately 30–45% for unresected STSs treated with radiotherapy alone, median 61- 64 Gy (39-41). Local tumour control was related to tumour size at radiation, and radiation dose. Kepka et al. (39) report that patients who
received doses of less <63 Gy had a 5-year local control rate of 22% compared with 60% for patients who received doses of ≥63 Gy or more. Local control at 5 years was 51%, 45% and 9%, respectively, for tumours <5 cm, 5-10 cm and >10 cm. However, there are some reports on high radiation responsiveness in MLSs, both as case reports in unresected tumours and in a pre-operative setting (11, 42-46).

Chemotherapy
Chemotherapy is used partly in a palliative setting and partly as adjuvant therapy for high-grade STSs in different protocols. Doxorubicin and ifosfamide are the most commonly used chemotherapeutics in the treatment of STSs. Several studies have shown a benefit of doxorubicin or ifosfamide-based chemotherapy treatment in terms of reduced local recurrence rate and distant metastases, but no lasting survival benefit (47-51). The SSG will start an adjuvant protocol for patients with high-grade STSs, identified as high-risk patients for metastases, if the following criteria are fulfilled: vascular invasion alone or together with the risk factors tumour size > 8 cm, necrosis or infiltrative growth. If vascular invasion is not present, at least two of the above risk factors should be identified.

Prognostic factors
Different adverse prognostic factors for local recurrence and tumour-related death have been identified for the whole group of STSs and specifically for LS.
High malignancy grade and positive microscopic margins have been identified as obvious adverse prognostic factors for local recurrence of liposarcoma (20, 52).
Tumour related factors, such as site (worse for retroperitoneal liposarcoma), size and depth, malignancy grade, presence of tumour necrosis, histological subtype and presentation with local recurrence, as well as treatment-related factors, such as non-wide resection, have been identified as unfavourable prognostic factors for tumour-related death (11, 12, 16, 19, 52-56). Prognostic factors vascular invasion and infiltrative growth pattern, recognized as risk factors for metastases in STSs (57), were not found to be independent predictors for clinical outcome in a study on liposarcomas by Gustafson P (12).
Histological subtypes

The different histological subtypes of liposarcoma all have different appearance and clinical behaviour (58). The histopathological low-grade group constitutes of WDLS (grade I) and MLS with <5% round cells (grade II) and the high-grade group of MLS with >5% round cells (grade III or IV) and DDLS and PLS (grade IV).

Well-differentiated liposarcomas

Well-differentiated liposarcomas represent 40-45% of all liposacomas, with a peak incidence between 50 and 60 years (59). The most common sites are limbs and the retroperitoneum. Four different histological subtypes are recognized, which is of limited practical importance. Sclerosing and inflammatory types are most often seen in the retroperitoneum.

Well-differentiated liposarcomas may recur locally but do not metastasize unless they undergo dedifferentiation, which is most common in the retroperitoneum (60, 61). The term “atypical lipomatous tumour” as an alternative to “WDLS” was proposed by Evans in 1979 (62, 63), and use of this heterogeneous terminology has been both debated and differentially applied at the different sarcoma centres. According to the update based on the new WHO classification of STSs (2002), the preferred term for lesions arising at surgically amenable locations should be “atypical lipomatous tumour” but for the lesions arising in the retroperitoneum and mediastinum, the term “WDLS” should be used because of their association with significant mortality (64).

Typical WDLS most often presents as large, deep-seated lesions of the thigh followed by lesions in the retroperitoneum. Tumours in the extremities and superficial trunk have disease-related mortality near 0% but in the retroperitoneum they have a high propensity of local recurrences and the disease-related mortality is high, either as a result of uncontrolled local disease or because of dedifferentiation and metastasis (61, 65). The risk of dedifferentiation of the lesions in extremities is <2% but in the retroperitoneum it is >20% (10).

The typical adipocytic (lipoma-like) WDLS has a characteristic morphology with relatively mature fat cells varying in size, enlarged atypical nuclei in varying number, low cellularity, few mitotic figures and minimal fibrous or myxoid zones. Mono- or multi-vacuolated lipoblasts may be found. Karyotypic analysis of WDLS/atypical
lipomatous tumour has shown the presence of extra ring and/or giant marker chromosomes derived from 12q (13-15) (64). Amplification of MDM2 and CDK4 from this region is frequently seen in WDLS but TP53 is very rarely mutated in WDLS cf Sandberg AA (66).

**Dedifferentiated liposarcoma**

Dedifferentiated liposarcoma is a high-grade tumour that occurs most commonly in the retroperitoneum. The dedifferentiation is most probably time-dependent and the majority of the tumours present as de novo lesions (10). Local recurrence rate after surgery of 41–52%, distant metastasis rate 15–17%, and a disease-related mortality rate of 28–30% have been reported. The tumours located in the retroperitoneum have significantly worse survival than tumours located at other sites (65, 67).

The most common histological features in DDLSs are transition areas from WDLS to non-pigmented sarcoma, which in most cases resemble high-grade fibrosarcoma or MFH. A minority of lesions contains only areas of low-grade dedifferentiation resembling fibromatosis or well-differentiated fibrosarcoma. Independently of the grade of dedifferentiation, the behaviour of DDLSs is that of a high-grade malignancy (68).

At a chromosomal level, DDLS frequently displays the same chromosomal abnormality associated with WDLS, i.e. presence of a supernumerary ring or giant chromosome derived from the 12q (13-15) region (67). TP53 mutations, determined by molecular methods, are rare but amplification levels of MDM2 and CDK4 are more frequently higher in DDLS than in WDLS cf Sandberg AA (66).

**Myxoid liposarcoma**

Myxoid liposarcoma represents about 40% of all liposarcomas (58). Myxoid liposarcomas and RCLSs were previously classified as two distinct subtypes but already 1979 Evans suggests that RCLSs represent a morphologic continuum of MLS (62). According to the most recent WHO classification of soft tissue tumours, RCLS is now included in the category of MLSs (10). MLS is a distinctive subgroup of liposarcoma with unique histological and cytogenetic features (69, 70). More than 95% of MLS cases carry the FUS-DDIT3 (also known as “TLS-CHOP”), and a few per cent carry the EWS-DDIT3 fusion oncogene. The molecular variability of the different fusion types has no significant impact on histological grade or clinical outcome (19). For further information see the chapter “Molecular biology of myxoid liposarcoma”.
Myxoid liposarcoma has a strong predilection for the thigh and occurs a decade earlier than other liposarcoma subtypes, but is very rarely reported in children. Myxoid liposarcoma presents with a continuum of cellularity that ranges from low or moderately cellular MLS to highly cellular round cell morphology, which correlates with the clinical behaviour. While classical MLS has a good prognosis with a low metastatic rate, the round cell type represents a high-grade malignancy (11, 16, 18, 53, 71, 72). Presence of round cell differentiation >5%, presence of necrosis and over-expression of P53 is associated with poorer disease-specific survival (19).

Most STSs metastasize preferentially to the lungs but high-grade MLS has also a predilection for other locations, such as subcutaneous tissue, bone, and retroperitoneum.

The morphology of a pure MLS is characterized by hypocellular spindle cell proliferation in a myxoid background, lipoblasts around vessels or at the periphery of the lesion and a typical plexiform capillary bed. Lipoblasts are neoplastic cells that, to some extent, recapitulate the differentiation cascade of normal fat (20). Presence of hypercellular areas with undifferentiated round cell morphology has previously been classified as mixed myxoid/round cell variant (MLS/RCLS) when ranging in extent between 5-80% and as pure RCLS when more than 80% of areas had round cell morphology. In the most recent WHO classification >5% round cell areas are defined as high grade MLS.

**Pleomorphic liposarcoma**

Pleomorphic liposarcoma is the rarest type involving about 10% of liposarcomas (16, 20). It is a high-grade tumour that may mimic MFH or even carcinoma or melanoma.

The diagnosis depends on the identification of, at least focally, lipogenic differentiation and/or lipoblasts that are pleomorphic and multi-vacuolated with hyperchromatic and scalloped nuclei (73, 74). The tumour suppressor gene *TP53* in PLS is often mutated together with complex chromosomal imbalances, mostly gains but also, though less frequently, losses (75).

This subtype has a high propensity to recur locally and metastasize, and a 5 year local recurrence-free and metastasis-free survival of 50–58% and 48–58%, respectively, has been reported (73, 74).
**Mixed-type liposarcoma**

Mixed-type liposarcoma is a very rare entity. Mentzel et al. (76) report 4% mixed-type liposarcoma in a group of 569 liposarcomas. The tumour is, according to the WHO classification, characterized by a combination of MLS and WDLs, or MLS and PLS. The tumour occurs most frequently in retroperitoneal or intra-abdominal locations, predominantly in elderly patients (10).

**Molecular mechanism of radiation-induced cell cycle arrest**

Radiation therapy may induce differentiation. This has been reported in different mammalian cell lines such as an erytroleukemic cell line (through induction of hemoglobin and increase in cell size and protein content) and human rhabdomyosarcoma RMZ-RC2 clone cells (through induction of myogenic differentiation) (77). The mechanism behind this phenomenon is probably a prolongation of the G1 phase by arrest of the cell cycle at the G1/S transition.

**The G1/S transition in normal cell**

The cell cycle of normal cell consists of mitosis and interphase, divided into G1, S and G2, during which the cell grows, replicates its deoxyribonucleic acid (DNA) and prepares for the division. Cells that do not proliferate exit G1 phase and enter a quiescent stage, G0 until different growth factor signals promote the cell to re-enter the cell cycle. A complex system of cell cycle checkpoints and regulatory proteins, called cyclins, together with enzymes, called cyclin-dependent kinases (CDKs), controls the progression through the different phases of the cell cycle (78). Phosphorylation and dephosphorylation of different amino acids of the CDK proteins play both an activating and an inhibiting role in the regulation of the cell cycle. The mandatory proteins that regulate the cell cycle are called cyclin D, cyclin E, cyclin A and cyclin B. These proteins are required as subunits for the catalytic activity of CDKs. Cyclin D interacts with CDK4 and CDK6, cyclin E with CDK2, cyclin A with CDK2 and CDK1 and cyclin B with CDK1. Promotion of the G1/S transition is mediated by CDC25A by dephosphorylation and thereby activation of CDK2 (79).

The **MDM2** gene is a proto-oncogene (promotes the cell cycle) that encodes a nuclear phosphoprotein MDM2. The transcription of the **MDM2** is activated by P53 tumour suppressor protein and the encoded phosphoprotein MDM2 itself binds to and inhibits the **TP53** gene
tranactivation domain. MDM2 also promotes degradation of P53 by ubiquitination mechanism. MDM2 further degrades pRB and thereby supports the transcription activity of E2F family and cell cycle progress, (Figure 1).

Proliferating cell nuclear antigen (PCNA) interacts with multiple partners involved in DNA repair, DNA synthesis and cell cycle regulation. It also binds to different cyclin-CDKs complexes and helps them to reach their targets (80).

Different tumour suppressor genes mediate the inhibition of cell cycle progression. They encode proteins such as retinoblastoma protein, and two different CDK- inhibitors families: the INK 4 family includes P16, P15, P18, P19, and the Cip/Kip family includes P21, P27, P57.

Retinoblastoma protein (pRB), a tumour suppressor protein, associates with the transcription factor E2F in hypophosphorylated form and prevents the E2F transcription activity of genes that mediate progression through S phase (e.g. cyclin E), G2/M transition, DNA replication and repair. The cyclin D/CDK 4/6 complex phosphorylates pRB and this leads to its dissociation from E2F, which in turn leads to cyclin E expression and further phosphorylation of pRB by cyclin E/CDK2.

**Figure 1.** Cell cycle of normal cell and G1 arrest induced by deoxyribonucleic acid (DNA) damage
Radiation-induced cell cycle arrest at the G1/S transition

Cells respond to radiation-induced DNA damage by induction of arrest in the G1, S and G2 phases of the cell cycle to allow repair (see Figure 1). The arrest is mediated by the related proteins ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia mutated and rad3-related ATR), which are activated by double-strand and single-strand DNA breaks, respectively.

1. ATM is the main protein of the early arrest response induced by ionizing radiation. In the initial phase ATM phosphorylate the human checkpoint kinase 2 (CHK2), which in turn phosphorylate CDC25A.
2. Phosphorylation of CDC25A leads to its degradation and thereby inactivation of cyclin E/CDK2.
3. The initial phase is followed by a prolonged G1 arrest through stabilization of the P53 tumour supressor protein.
4. ATM kinase activity will itself and by activating the CHK2 phosphorylate the P53 protein, stabilizing and prolonging its half-life.
5. ATM also disrupts P53-MDM2 binding by phosphorylation of serines 15/20 on P53, which leads to inhibition of the MDM2-induced P53 degradation.
6. P53 induces P21 expression, which in turn inactivates cyclin E/CDK2 and down-regulates PCNA.
7. Inactivation of cyclin E/CDK2 leads to inhibition of pRB phosphorylation. This results in continued binding of E2F transcription factors.
8. Hypophosphorylated pRB also represses the transcription of E2F-regulated genes by recruiting repressor molecules to their promoters, as well as repressing cyclin A, cyclin E, CDK2 and PCNA (81).

The P53-P21-pRB pathway leads to cell cycle arrest at the G1/S transition (82-84).

Molecular mechanism of adipogenesis

The knowledge of the molecular mechanism of adipogenesis is essential for the molecular understanding of myxoid liposarcoma. Multipotent mesenchymal stem cells will, on receiving certain signals, be destined towards the adipocytic lineage and adipoblasts will
develop. Overlapping molecular phenomena drive the adipoblasts to differentiate to pre-adipocytes and finally to mature adipocytes. Different hormones, cytokines, nutrients and signalling molecules control the activity of different transcription factors that are responsible for the complex genetic fat conversion, as illustrated in Figure 2.

**Figure 2.** Mechanism in pre-adipocyte adipogenesis and effect of deoxyribonucleic acid (DNA) damage

Factors that promote adipogenesis include insulin, glucose, glucocorticoides, prostaglandins, fatty acids and amino acids. Factors that inhibit adipogenesis are, among others, tumour necrosis factor alpha (TNF-α), interleukins (IL)-1, -6 and -11 and interferon gamma (IFN-γ) (85) (Figure 2).
Many different pro- and anti-adipogenic transcription factors are important for adipogenesis. Pro-adipogenic transcription factors involved in terminal differentiation by activation of adipocyte-specific genes include the different isoforms of CCAAT/enhancer binding protein (C/EBP) and the isoform peroxisome proliferator-activated receptor (PPAR)-γ2. One of the anti-adipogenic transcription factors is DNA-damage-inducible transcript 3 (DDIT3).

The different isoforms of C/EBP (β, δ, α) belong to a family of bZIP class of leucine zipper transcription factors. The bZIP region is characterized by juxtaposition of a region rich in basic amino acids to a region containing a repeat of hydrophobic amino acids, often leucines. The two regions form an α-helical conformation. This region dimerizes with an analogous region of a second bZIP protein forming a leucine zipper dimer. The basic region of the paired subunits interacts with a DNA binding site forming a “scissors” grip around the DNA substrate.

The different isoforms of C/EBP form stable homodimers. The DDIT3 gene, (also known as “C/EBP-homologous protein (CHOP)” and “growth arrest and DNA damage inducible (GADD 153)”) encodes for a nuclear protein, which is a member of C/EBP family. The DDIT3 protein is incapable of homodimer formation but forms stable heterodimers with the isoforms of C/EBP α and β. This family of regulatory molecules activates genes involved in metabolism, cytokine cascade and terminal differentiation.

The C/EBP are abundant proteins present in many different cell types while DDIT3 is expressed at very low levels under normal conditions. The DDIT3 gene is induced in G0 cells after growth cessation and DDIT3 proteins are found in different cell types in connection with terminal differentiation, e.g. erythroid and keratocytes differentiation. The DDIT3 gene is also induced after exposing the cell to metabolic stress or DNA damage, e.g. treatment with alkylating agents, ultraviolet (UV) light, nutritional deprivation, oxidative stress and stress that disturb endoplasmic reticulum function. It is believed that the DDIT3 protein has a role in induction of cell death under conditions associated with endoplasmic reticulum stress (86).

Complex, hierarchical expression of the different C/EBP proteins and DDIT3 were identified in different experiments with 3T3-L1-mouse fibroblast induced to adipocytic differentiation (87, 88). C/EBP-β and -δ are induced early in the terminal differentiation programme and in turn activate PPAR-γ2. The PPAR isoforms α, δ and γ belong to a nuclear hormone receptor family. The PPAR-γ protein forms a dimer with
retinoid X receptor (RXR). This complex regulates transcription of adipocyte-specific genes upon binding of ligands for either receptor. Peroxisome proliferator-activated receptor-γ plays an important role in glucose homeostasis and insulin sensitivity. C/EBP-α expression rises after induction of PPAR-γ2 and these two transcription factors co-regulate each other’s expression. The DDIT3 protein was defined in different experiments with 3T3-L1-mouse fibroblast as an inhibitor of gene transcription in adipocytic differentiation by forming heterodimer with C/EBP-α and modulating the activity of C/EBP-α DNA-binding sites of various genes, important for maintenance of fat cell phenotype. C/EBP-α and DDIT3 have been reported to decrease when dedifferentiation of adipocytes is induced by cytokines (88). Nutrition deprivation in the adipocyte system reportedly induces the DDIT3 mRNA expression and the differentiation slows down (89).

Exit from the cell cycle is a prerequisite for terminal differentiation in adipogenesis and both PPAR-γ2 and C/EBP-α act as inhibitors of the proliferation:

1. PPAR-γ2 inhibits the E2F from exerting its transcriptional activity on S-phase genes and up-regulates P21 (90, 91).
2. C/EBP-α interacts with cell cycle protein E2F, binds CDK2 and CDK4 and up-regulates P21 expression, which prevents the CDK-mediated phosphorylation of pRB, thus leading the cell to exit from the cell cycle (85, 92).

**Molecular biology of myxoid liposarcoma (MLS)**

**The FUS gene**

The FUS gene (also known as “TLS”) encodes a ribonucleic acid (RNA)-binding protein that is very similar to the protein encoded by EWSRI and TAF15. The normal biological function of these proteins is not well understood. There is evidence that FUS protein participates in RNA processing. The N-terminal domains of FUS, EWSR1 and TAF 15 associate partly with components of the RNA Polymeras II complex and partly possess a potent transcriptional activator region. The C-terminal domain of FUS binds to RNA and shuttles between the nucleus and the cytoplasm. This part of FUS also binds to Y-box binding protein-1 (YB-1=transcription and translation factor), which participates in RNA splicing. Ribonucleic acid Polymerase II has been shown to couple gene
transcription with RNA splicing in vivo (93) and FUS molecules may have a critical function in promoting this RNA processing (94). FUS-deficient mice exhibit increased radiation sensitivity, deficient DNA repair and male sterility (95).

**The FUS-DDIT3 gene**

The *DDIT3* gene on chromosome 12q13 fuses with the *FUS* gene on 16p11 or with *EWS* on 22q12 in MLS (19, 96, 97) in MLS. The translocation results in a fusion gene which codes for an oncogenic protein where the C-terminal, RNA-binding part in FUS or EWSR1 is replaced by the full length DDIT3 protein with its C-terminal part containing the DNA-binding domain. Reported molecular heterogeneity is dependent on different exon combinations from the *FUS* gene fused with the exon 2 of the *DDIT3* gene. A number of different variants have been reported, the most common combinations of which are Type I and II (Type I: *FUS* exon 7 binds to *DDIT3* exon 2; Type II: *FUS* exon 5 binds to *DDIT3* exon 2), with Type II being seen in the majority of cases (see Figure 3).

**Figure 3.** DDIT3, FUS and FUS-DDIT3 fusion oncoprotein.

N-term SYGQQS = a region containing the degenerated hexapeptide repeat (serine, glycine, glutamine and tyrosine-rich region), RGG = multiple repeats of tripeptide arginine-glycine-glycine (98, 99)

The N-terminal part of FUS is a strong autonomous transcription activator and controls DDIT3. The fusion oncoproteins act as abnormal transcription factors and are believed to induce abnormal expression of growth-controlling genes as part of their transforming activities (100, 101). It has been demonstrated in vitro by Adelmant et al. (102) that
FUS-DDIT3 inhibits adipocyte differentiation by blocking the C/EBPβ activity. In vitro analysis by Göransson et al. (103) demonstrated that the fusion gene up-regulates the IL-6 and IL-8 gene. Interleukin-6 belongs to the group of factors that inhibit adipogenesis.

Experiments with transgenic mice showed that mice expressing DDIT3 protein did not develop liposarcoma and the white adipose tissue had normal architecture but accumulated glycogen within adipocytes, unlike normal adipose tissue (104). Transgenic mice expressing FUS protein did not develop liposarcoma either and the white adipose tissue had normal histology (105). Transgenic mice expressing FUS-DDIT3 protein developed liposarcoma but only in adipose tissue.

Thiazolidinedione, an antidiabetic drug, is a ligand for PPAR-γ. Human liposarcomas were found to express PPAR-γ at a significant level and treatment with thiazolidenediones induced a change in morphology and gene expression consistent with terminal adipocyte differentiation both in cell lines and in humans (106, 107).

**Aberrant expression of cell cycle regulating proteins in myxoid liposarcoma**

In MLS the mutation of *TP53* is reported to involve anything from a few per cent up to 30% (108-111). MDM2 over-expression has been found to correlate significantly with high-grade morphology, and abnormalities involving the pRB-cyclin D pathway have been observed in more than 90% of cases (108). *MYC* has been found amplified in about 50% of MLSs and especially in the round cell component (109). Low expression of P27 (less than 75% of cell nuclei being immunostained) has been shown to correlate with decreased metastasis-free and overall survival in MLS (112). Significantly reduced expression of P16 and P14 has been reported in MLS with more than 5% of round cells (111).
**BACKGROUND AND AIMS**

**Paper I. Liposarcoma – outcome based on 237 patients from the Scandinavian Sarcoma Group Register**

The SSG Register for STSs started in March 1986. All the Nordic countries are members but only the sarcoma centres in Norway and Sweden report their patients to the register. Approximately 90% of all STS patients in Norway and Sweden are treated at a centre and therefore the SSG Register is considered population-based for these two countries (113). Within the SSG there has been consensus on surgical and radiation treatment policy.

The aim of this study was to investigate the occurrence of local recurrences and metastases, and analyse survival data in a large group of liposarcoma patients aimed to be treated according to the guidelines of the SSG as specified in protocol SSG VII: 1 and 2.

**Paper II. Irradiation of myxoid/round cell liposarcoma induces volume reduction and lipoma-like morphology**

Experience with radiation therapy in the treatment of the different liposarcoma subtypes is limited. A few reports on MLSs illustrate the tumours’ response to radiation therapy, with reduction in tumour size with radiation doses as low as 30 and even 10 Gy (42-45, 114).

The aim of this study was to analyse the effects of pre-operative radiation in MLSs/RCLSs with regard to morphology and changes in tumour volume.

**Paper III. The myxoid/round cell liposarcoma fusion oncogene FUS-DDIT3 and the normal DDIT3 induce a liposarcoma phenotype in transfected human fibrosarcoma cells**

It has been postulated that MLS/RCLS develops from pre-adipocytes carrying FUS-DDIT3, making them incapable of terminal differentiation (115-117). However, most liposarcomas do not occur in lipomatous tissue. Rather, they occur in or between the large muscles of the limb.
The aim of this study was to analyse how the oncogene FUS-DDIT3 affects the morphology of a poorly differentiated, human fibrosarcoma cell line, HT1080.

**Paper IV. Abnormal expression of cell cycle regulators in FUS-CHOP carrying liposarcomas**

The fusion oncoproteins act as abnormal transcription factors and are believed to induce abnormal expression of growth-controlling genes as part of their transforming activities.

The aim of this study was to search for recurrent abnormal expression patterns of cell cycle-regulating proteins and growth factor receptors.
MATERIAL AND METHODS

Paper I

The study was based on 319 patients with primary liposarcomas of the trunk wall and extremities diagnosed between 1986 and 1998 and reported to the SSG Register by sarcoma centres in Norway and Sweden. After a review by the SSG Pathology Board (118) the diagnosis of liposarcoma was retained in 242 patients. A further four cases with metastases and one without surgery with curative intention were excluded, leaving 237 patients for analysis.

Time for local recurrence, metastases, and death, or last follow-up of living patients was given from the date of diagnosis, i.e. the first surgical treatment. Overall and disease-specific survival, and metastasis-free and local recurrence-free survival was estimated using Kaplan-Meier survival curves. Uni- and multivariate analysis of adverse prognostic factors for metastasis and local recurrence was performed using the Poisson regression model. Analysis of prognostic factors for metastases was performed excluding the subtype WDLS which does not metastasize. The analysed factors were sex, age, tumour size, histopathological subtype, grade and depth, surgery at versus surgery outside a sarcoma centre, primary surgical margin, margin at resection, tumour location defined as trunk, upper and lower extremity, thigh and lower leg.

Paper II

The study was based on 15 patients, ten males and five females patients with 33 primary or metastatic MLS/RCLS tumours treated with radiation therapy in a pre-operative setting or as the sole treatment modality. All patients referred to the Department of Orthopaedics at the Sahlgrenska University Hospital, Göteborg, Sweden, during the period 1994–2004 were included consecutively. In 14/15 patients the pre-operative diagnosis was made by FNA cytology and/or core needle biopsy. One patient underwent surgical resection of abdominal tumours without a pre-operative diagnosis. The median age was 45 years (28–76 years) and the median follow-up time was 44 months (24–99 months).
The tumours were investigated before and in all but three cases also after radiation therapy with MRI or CT. The volume response to radiotherapy was calculated according to the formula

\[ \pi \times d_1 \times d_2 \times d_3 / 6 \]

expressing the outcome as the per cent volume change from the initial volume. Histopathology was performed in 62 surgically removed tumours, 27 after irradiation, one 16 months after irradiation and 34 without irradiation, the non-irradiated ones being from three patients with metastases. Reverse transcription-polymerase chain reaction was performed on 13 tumours from eleven patients to detect the fusion gene \textit{FUS-DDIT3}.

External radiotherapy was planned using CT-based, three-dimensional technique in all patients. The radiotherapy was administered by a linear accelerator with photon energies 4–15 MV in multiple fields, 1.75–2.0 Gy per fraction, five times weekly to 34–46 Gy. One inoperable lesion was irradiated to 60 Gy.

**Paper III**

**Transfection**

In this study, PCR fragments containing the full length coding regions of \textit{FUS-DDIT3} type II, \textit{DDIT3}, or the sequences encoding the first 180 amino acids of \textit{FUS} were cloned into a pEGFPN1, a plasmid expression vector (Clontech) containing a gene encoding enhanced green fluorescent protein (EGFP) and a geneticine resistance selection gene (G418). The resulting vectors were designated \textit{pFUS-DDIT3-EGFP}, \textit{pDDIT3-EGFP}, and \textit{pFUSa-EGFP} (Figure 4).

The fibrosarcoma cell line HT1080 was transfected with the recombinant vectors. After resistance selection the surviving cells were harvested after 10 days. Clones showing nuclear EGFP fluorescence were expanded in the presence of geneticine and recombinant protein expression was confirmed by Western blot analysis.
SV40=Semian virus 40, CMV=cytomegalovirus, EGFP=enhanced green fluorescent protein

**Adipogenes**

pFUS-DDIT3-EGFP, pDDIT3-EGFP transfected and wild-type HT1080 cell lines were cultured with adipogenic induction medium (Cambrex product PT3004 containing human recombinant insulin, dexamethasone, Indomethacine and 3-Isobutyl-1-methylxanthin (IBMX), followed by maintainance medium (MM), or only MM. Accumulation of fat was detected by staining the cells with Oil Red after fixation with 4% buffered formalin.

**Microarray**

Total RNA was extracted from cultured cells and used for cDNA synthesis with Cy3 and Cy5-labeled nucleotides. The cDNA was hybridized to microarrays comprising 23,707 genes. Hybridized microarrays were scanned in a microarray scanner and fluorescence intensities were extracted to numerical values.

Minimum median intensity was set to 1 to avoid data loss when the ratio between the samples and the reference cell line (wild-type HT1080) was calculated. Genes with the signal intensity of the transfected HT1080 variants
being at least three times smaller or larger than that of the HT1080 reference cell line were scored as up- or down-regulated. Genes with a normalized intensity <25 in the transfected cell lines were rejected from the lists of up-regulated genes. Similarly, genes with a normalized intensity <25 in the HT1080 reference was rejected from the lists of down-regulated genes. Ribonucleic acid from the MLS cell line 402-91, which carries the FUS-DDIT3 gene, was also tested with the HT1080 cell line as a reference (see Figure 5).

**Figure 5.** Microarray gene expression analysis, 23,707 genes were used as probes.

To estimate the statistical significance of the overlap between differentially expressed genes in cell line 402-91 cells versus differentially expressed genes in the pFUS-DDIT3-EGFP and pDDIT3-EGFP transfected cell lines, two sets of genes corresponding to the up- and down-regulated genes in 402-91 cells were selected at random from the total of 23,707 genes. Similarly, two sets of genes now corresponding to up- and down-regulated genes in one of the transfected lines were randomly selected. The number of genes that were identical in the randomly selected groups was then recorded. This experiment was repeated $10^7$ times for each pair of up- or down-regulated gene sets.

**Quantitative reverse transcription-polymerase chain reaction**
Quantitative RT-PCR analysis of total RNA was performed with SYBR green as fluorophore.
**Study in female FOX CHASE severe combined immune deficient**

Female FOX CHASE severe combined immune deficient (SCID) mice were used as recipients of transfected and non-transfected HT1080 cells. Ten and 2 millions cells, respectively, were injected subcutaneously into the flank of SCID mice. Animals with palpable tumours were sacrificed and the tumours were excised, measured, fixed in formalin and embedded in paraffin for histological examinations.

Tissue sections were stained with monoclonal antibodies rat anti-mouse CD34 and mouse anti-human CD34 (DAKO) specific for mouse and human CD34, respectively.

**Paper IV**

**Tissue samples and cell lines**

Formalin-fixed tissue from MLSs/RCLSs was embedded in paraffin using routine procedures. Human skin fibroblasts, the fibrosarcoma cell line HT1080, the MLS cell lines 402-92 and 2645-94 and HT1080 pTLS-CHOP-EGFP were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 with 8% foetal calf serum.

**Immunohistochemistry**

Series of 5 μm tissue sections were cut from each biopsy, deparaffinized, rehydrated and stained with primary antibodies. Several protocols were tested for each antibody. The stained sections were examined under a light microscope and fields that were dominated by tumour cells were marked in each section. Immunohistochemistry (IHC)-stained cells were counted within the marked fields at 630 x magnification by two independent investigators. Between 300 and 700 cells were counted, the number depending on the frequency of stained cells.

**Serum starvation experiments**

For serum starvation experiments, the cells were grown to near confluence and then cultured at 0.5% serum for 14 hours before harvest for Western blot analysis.

**Western blot analysis**

Western blot analysis of total proteins from cultured cells was performed by electrophoresis and transfer membranes. Procedures for antibody
binding and detection were performed as described by the suppliers of antibodies.

**Microarray analysis**

Microarray analysis of cDNA from two MLS/RCLS tumours and tumour-derived cell lines was performed using Research Genetics GF201 gene filters containing approximately 4,000 genes.
RESULTS

Paper I

There were 128 men and 109 women and the median age at diagnosis was 54 years (range 13–90 years). As many as 84% of the lesions were located in the thigh. Seventy-nine per cent were deep-seated. Sixty-seven per cent were low-grade (grade I-II). The largest histological group was WDLSs (36%), followed by MLSs (27%) and mixed MLS/RCLSs and PLSs (both reaching 11%). The median tumour size was 11 cm. A total of 78% were primarily operated at a sarcoma centre, 45% with wide margins, while the 22% operated before referral to a sarcoma centre all had non-wide margins. Only 8% underwent all surgical treatment for primary tumour outside a sarcoma centre. The final surgical margins were intralesional in 10%, marginal in 43% and wide in 47%.

Altogether 29% of an expected 53% received postoperative radiotherapy. Radiotherapy was applied in 33% of lesions with intralesional margins, in 28% of lesions with marginal margins and in 4% of lesions with a wide surgical margin. The relation between histological grade and local recurrence and metastases is summarized in Table 2.

Table 2. Relation between histological grade and local recurrences and metastases.

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>Local recurrence n (%)</th>
<th>Metastasis n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>9 (12)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Grade II</td>
<td>7 (9)</td>
<td>7 (9)</td>
</tr>
<tr>
<td>Grade III</td>
<td>4 (13)</td>
<td>10 (32)</td>
</tr>
<tr>
<td>Grade IV</td>
<td>12 (26)</td>
<td>18 (38)</td>
</tr>
</tbody>
</table>

In the low-grade group with intralesional and marginal surgery the local recurrence rate was 13% with radiotherapy and 18% without, while in the high-grade group this rate was 19% and 47%, respectively.
The estimated 10-year overall and disease-specific survival was 64% and 84%, respectively. The estimated 10-year local recurrence-free and metastasis-free survival is summarized in Table 3.

Table 3. Estimated 10-year local recurrence-free and metastasis-free survival.

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>Local recurrence-free survival</th>
<th>Metastasis-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>83</td>
<td>100</td>
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<tr>
<td>Grade II</td>
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<td>Grade III</td>
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<tr>
<td>Grade IV</td>
<td>67</td>
<td>60</td>
</tr>
<tr>
<td>Grade III+IV</td>
<td>75</td>
<td>61</td>
</tr>
<tr>
<td>Grade I–IV</td>
<td>84</td>
<td>84</td>
</tr>
</tbody>
</table>

Surgery outside a sarcoma centre, and DDLS were adverse prognostic factors for local recurrence in the multivariate analysis and no other variable had any significant importance above these. Factors that influenced the risk for metastasis were age, tumour size, grade and histopathology RCLS (RCLS+MLS/RCLS). In the univariate analysis MLS had a lower risk of metastases than the other subtypes analysed.

Paper II

Thirty tumours were evaluated with MRI or CT both before and after radiotherapy. The median time period between radiological examination and initiation of radiotherapy was 48 days; between termination of radiotherapy and radiological re-examination it was 13 days.

Four tumours showed complete remission, eight tumours showed a >50% reduction in tumour volume, eleven tumours had <50% reduction and seven tumours showed an increase in tumour volume. The FUS-
DDIT3 fusion gene was detected in all six non-irradiated and six of the seven irradiated tumours. There was no correlation between radiological response and histopathological appearance and the presence of round cells did not correlate to either radiological response or post-irradiation morphology. Histopathology showed paucicellularity, fibrosis, hyalinization and, in 21/27 lesions, lipomatous appearance with mostly univacuolated adipocyte-like cells that varied in size.

**Paper III**

*Tumour growth in severe combined immunodeficient mice*

In SCID mice the original HT1080 grew faster than did the three transfected cell lines. Microscopic examination of the tumours revealed that the original HT1080 cells and the pFUS-EGFP-transfected cells grew as poorly differentiated sarcomas. The pFUS-DDIT3-EGFP and pDDIT3-EGFP-expressing cells developed liposarcomas containing lipoblasts of different sizes. Increased proportions of extracellular matrix and small myxoid pools were observed in tumours from pFUS-DDIT3-EGFP-transfected cell lines.

*Effect on vascularization*

Tumours that developed from HT1080 and pFUS-EGFP-carrying cells contained sinusoid vessels, while tumours from pFUS-DDIT3-EGFP and pDDIT3-EGFP-carrying cells developed a plexiform capillary network that was similar to the characteristic plexiform capillary network in MLS.

The staining for CD34⁺ endothelial cells with antibodies to mouse endothelial cells revealed a rich abundance of mouse CD34⁺ vessels in all tumours. The number of vessels was also considerably higher in tumours from HT1080 and pFUS-EGFP transfectants.

*In vitro adipogenesis induction experiments*

Adipogenic factors induced a morphological change of pDDIT3-EGFP-transfected cells. They developed large vacuoles, and some cells resembled the “signet ring cell” type of adipocytes. Oil Red O staining showed increased fat accumulation.
pFUS-DDIT3-EGFP transfectants had no increased fat accumulation in adipogenic medium and no morphological response or formation of large vacuoles was seen.

**Microarray expression analysis**

HT1080 cells, pFUS-DDIT3-EGFP and pDDIT3-EGFP-transfected HT1080 cells and MLS-derived cell line 402-91 were analysed. Only small numbers of genes were differentially expressed.

1. In pDDIT3-EGFP-transfected HT1080 cells 36 genes were up-regulated and 98 were down-regulated three times or more compared with the HT1080 cells.
2. In pFUS-DDIT3-EGFP-transfected HT1080 cells 48 genes were up-regulated and 82 were down-regulated three times or more compared with the HT1080 cells.
3. In MLS line 402-91, 364 genes were up-regulated and 309 were down-regulated compared with the HT1080 cells.

**Overlapping high expressed genes**

Thirteen high expressed genes were overlapping between MLS line 402-91 and pDDIT3-EGFP-transfected HT1080 cells and seven genes (e.g. SERPINB2) between MLS line 402-91 and pFUS-DDIT3-EGFP-transfected HT1080 cells. Only one of these genes (IL-6) was similarly highly expressed in all three cell lines.

**Overlapping low expressed genes**

Thirty low expressed genes were overlapping between MLS line 402-91 and pDDIT3-EGFP transfectants and nine genes between MLS line 402-91 and pFUS-DDIT3-EGFP transfectants. Thirteen genes (among others, VEGF and DSIPI) were low expressed in all three cell lines.

The probability of obtaining a similar overlap in up- and down-regulated genes by random selection among 23,707 genes on microarray is $<10^{-7}$ in each of the comparisons.
Paper IV

Immunohistochemistry

The results of expression of the different cell cycle-regulating factors are presented in Table 4.

Table 4.

<table>
<thead>
<tr>
<th>Cell cycle regulatory factors in six non-irradiated and two irradiated human MLS tumours</th>
<th>Stained cells %</th>
<th>Low expression</th>
<th>Stained cells %</th>
<th>Variable expression</th>
<th>Stained cells %</th>
</tr>
</thead>
<tbody>
<tr>
<td>High expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1* in cytoplasm</td>
<td>68-92</td>
<td>Cyclin A</td>
<td>&lt;1-3.9</td>
<td>P53</td>
<td>0-60</td>
</tr>
<tr>
<td>CDK4 in cytoplasm</td>
<td>60-90</td>
<td>P21</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P16*</td>
<td>87-98</td>
<td>Ki 67</td>
<td>5-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin E*</td>
<td>86-98</td>
<td>pRB</td>
<td>0 (or weak)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK2</td>
<td>82-99</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P27</td>
<td>55-82</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P57</td>
<td>63-91</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>43-86</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCNA</td>
<td>74-96</td>
<td></td>
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</table>

* tested in eleven irradiated and six non-irradiated human myxoid liposarcoma (MLS) tumours
PCNA=proliferating cell nuclear antigen; pRB=retinoblastoma protein; Ki-67=proliferating nuclear antigen

Starvation experiments and microarray

The serum-starved MLS-derived tumour cell line showed no decreased expression of MYC or cyclin D1, as tested with Western blotting. The original HT1080 cell line responded to serum starvation by down-regulation of cyclin D1 but MYC and cyclin E and A expression remained stable. The pFUS-CHOP-EGFP-transfected HT1080 cells showed a similar response to serum starvation as did the original HT1080.
Results from microarray-based expression analysis of two MLS tumours showed that platelet-derived growth factor receptor (PDGFR)-β and epidermal growth factor receptor (EGFR) were strongly expressed.
DISCUSSION AND CONCLUSIONS

The liposarcoma study presented in Paper I can be regarded as population-based in contrast to most other large institution-based series. This was also evident in the fact that low-grade liposarcoma constituted 2/3 of the tumours, a high fraction compared with that reported from the majority of single sarcoma institutions (9, 52, 54, 56, 119). In Sweden 90% of the patients with STS from the Swedish National Cancer Register are recorded in the SSG Register (113). All centres in Norway and Sweden report their data to the SSG Register. There is a possibility, however, that the number of WDLS patients is underestimated in the SSG Register. Tumours interpreted as lipoma may be treated outside a sarcoma centre and will therefore not be reported. The other subtypes are more likely to be recognized as STSs, and to be referred. Furthermore, the SSG Pathology Board has reviewed the other STSs, such as MFHs (118), and the tumours reclassified as liposarcoma were included in our study.

Primary surgery outside a sarcoma centre was performed in 22% of the patients. The analysis exposed the problem of getting wide surgical margins when the patients had surgery outside a specialized centre. No wide surgery was achieved in this group while surgery at a sarcoma centre led to wide surgical margins in 45% of the patients. This explains that surgery outside a sarcoma centre was an independent adverse prognostic factor for risk of local recurrence.

The group of patients with non-wide surgery without re-resection (84/115) was predominantly composed of the WDLS and MLS histological type. A majority of cases both had WDLS and had surgery at a centre (49/56). As mentioned previously, the WDLS subtype occurs in older patients, is usually large and does not metastasize, which explains the restrictive surgery.

The grade I tumours was of the WDLS histological type. There were no metastases in this group, which is in good agreement with the literature (10, 59, 61). This entity is to be recognized as locally growing mesenchymal neoplasm and, according to the latest WHO classification, the preferred terminology is “atypical lipomatous tumour” for lesions arising at extremities and superficial trunk (10, 64), which our results support. The SSG members will discuss this proposal of changing the nomenclature, which if introduced, will lead to not registering this entity as STS in the SSG Register.
The analysis of how the SSG treatment guidelines for radiotherapy were implemented showed that only 29% of the expected 53% patients with non-wide surgery (intralesional or marginal) were treated with radiotherapy. In the low-grade group the local recurrence rate was 13% with and 18% without radiotherapy and in the high-grade group, these figures were 19% and 47%, respectively. Presumably this difference is underestimated since those referred for radiotherapy were probably more advanced than those with deferred radiotherapy. Radiotherapy significantly reduced the risk of local recurrence as tested together with grade and margin. In the group of WDLS patients 84% with non-wide surgery and without postoperative radiotherapy did not experience local recurrence. In spite of the statistically protective effect of radiotherapy it is questionable whether postoperative radiotherapy should be routinely recommended for WDLS. Radiotherapy should, however, be considered if a subsequent local recurrence may lead to amputation or other major loss of function. Yang et al. (38) showed in a prospective randomized study that radiotherapy significantly decreased the risk of local recurrence for both high- and low-grade STSs. It should be noted, however, that there were 40% MLS patients in the low-grade group, which is a much more radio-responsive group in comparison with the rest of low-grade STSs.

In the high-grade group the metastatic rate was 36%. The prognostic factors vascular invasion, necrosis, and growth pattern were reviewed by the SSG Pathology Board in this patient material. There were 110 lesions with pushing growth pattern, 93 with infiltrative, and 31 undefined. Only 8 lesions had vascular invasion and 43 had necrosis. The investigation of whole-tumour section, as suggested by Engelau et al. (57) was not performed and therefore the identification of the prognostic factors may be underestimated. However, Gustafson et al. (12) found no correlation between growth pattern and survival among 43 patients with liposarcomas. Furthermore, he found that vascular invasion impaired metastasis-free survival only in univariate analysis.

In this material, 15 of 35 patients with a diagnosis of MLS/RCLS had a tumour size >10 cm. Thirteen of these developed metastases. In contrast, only two of 20 with tumour size <10 cm metastasized. Necrosis and infiltrative growth pattern was seldom present in the primary tumours of the metastatic MLSs/RCLSs and vascular invasion was absent. Applying the SSG inclusion criteria for adjuvant chemotherapy in this material, only 50% of the metastatic MLS/RCLS would be offered adjuvant chemotherapy. The MLS/RCLS is reported as a chemo-sensitive entity (120, 121) and it is important to recognize the high-risk group of patients that may benefit from adjuvant chemotherapy. In our patient material prognostic factors tumour size >10 cm and round cell component >5% identified 90% of the high-grade MLS
patients that later developed metastases as high-risk patients. Further prognostic studies are needed to better identify high-risk MLS patients. To improve the outcome it is crucial to co-operate internationally and to report patient data to a central register since country populations are small and STS is a rare disease.

Myxoid liposarcoma has an unusually high radio-responsiveness, as shown in Paper II and other reports (42-45, 114). The SSG do not recommend pre-operative radiotherapy. This study, however, shows a >50% reduction of tumour volume in 40% of lesions and supports the use of pre-operative radiotherapy whenever a non-wide surgery is overhanging. One problem with pre-operative radiotherapy is that areas with a round cell component could be eliminated and missed and adjuvant chemotherapy for high-grade lesions may not be offered. It was shown by Antonesco et al. that areas with round cells >5% have an impact on survival and such small areas could be missed with pre-treatment fine needle or core needle biopsy techniques.

The morphology displayed a remarkable change after radiotherapy, with lipoma-like areas, hyalinization and paucicellullarity. The primitive, non-lipogenic mesenchymal cells were to a large extent eradicated in our tumours. According to Kuroda et al. (95), FUS deficient mice exhibited increased radio-sensitivity and it may be the translocated FUS gene that contributes to the high radio-responsiveness in MLSs. The lipoma-like appearance after radiotherapy could be due to leaving the more differentiated lipoblasts unaffected or it could be due to radiation-induced cell cycle arrest leading to maturation of the lipoblasts. In the literature it is suggested that a mixed MLS with WDLS is very rare (76) and in our material 21 out of 27 lesions had areas with lipoma-like appearance.

It was shown in human liposarcoma cells that induction of terminal differentiation was possible with treatment with the PPAR-γ synthetic ligand pioglitazone (106). Unpublished data from our group showed an induction of P21 expression after irradiation of cultured MLS cells. The possible mechanism behind the lipoma-like appearance in the irradiated MLSs could be an induction of P53 which activates transcription of P21, and the P21 protein in turn inactivates the cyclin E/CDKs – pRB pathway and arrests the cell at the G1-S checkpoint. TP53 is not frequently mutated in MLS tumours but there are reports in the literature that P21 can be induced independently of P53 (122, 123). Ultraviolet B (UVB) irradiation of keratinocytes induces C/EBPα by direct binding of P53 to the C/EBPα promoter (124). C/EBPα is a strong inhibitor of cell proliferation cf Johnson P F (92) and if the ionizing radiation of liposarcomas induces the same signalling pathway this could contribute to the adipocyte-like morphology seen in our irradiated lesions.
Further support for our hypothesis was found in the work of Ventura A (125). He showed that mice carrying reactivable TP53 knockout allele developed lymphomas and sarcomas. By reactivating the TP53 gene the lymphomas underwent apoptosis. However, regression of sarcomas was delayed and no extensive apoptosis was evident. Two cell lines derived from the mice sarcomas were further investigated and no apoptosis was induced, consistently with the results in vivo. Instead, restoration of P53 expression suppressed proliferation, induced cell cycle arrest, and the cells changed morphology and expressed senescence-associated β-galactosidase, P15 and P16. The same markers were also induced in the in vivo model. This report supports our hypothesis that radiation-induced expression of P53 may lead to cell cycle arrest in MLS and terminal differentiation of lipoblasts.

The question is whether the gene fusion in MLSs arises in normal mesenchymal stem cells or whether it occurs at a later differentiation stage and also, whether the fusion gene is sufficient for neoplastic transformation. It has been shown that the fusion gene can block terminal differentiation of pre-adipocytes in vivo and in vitro (126: Kuroda, 1997 #104). Also, transgenic mice that expressed FUS-DDIT3 in all tissues developed MLS/RCLS-like tumours that arose in adipose tissue (116). These reports led to the hypothesis that MLS/RCLS develops from pre-adipocytes carrying FUS-DDIT3 incapable of terminal differentiation (106, 115, 117, 126, 127). Myxoid liposarcomas in humans, however, preferentially develop in or between the large muscles of the thigh and rarely in adipose tissue (10), which is why the hypothesis was called into question. We observed in study III that the pFUS-DDIT3-EGFP and pDDIT3-EGFP-transected, xenografted HT1080 cell line did induce a switch from a poorly differentiated sarcoma to an MLS/RCLS-like morphology. Microarray expression profiling supported the switch in morphology by showing a changed expression pattern of transfected HT1080 cells toward an MLS/RCLS-like profile. These findings support the idea that FUS-DDIT3 can drive a primitive mesenchymal tumour cell toward an MLS/RCLS phenotype. Further support for this hypothesis came when Riggi et al. (128) reported that mesenchymal progenitor cells, isolated from bone marrow of mice, transformed and grew as MLS-like tumours in SCID mice when transfected with the FUS-DDIT3 fusion gene. Pérez-Losada et al. (104) showed that transgenic mice that expressed high levels of DDIT3 did not develop liposarcoma tumours. In our experiments DDIT3, when expressed in a primitive fibrosarcoma cell, induced a switch in morphology and expression pattern toward an MLS/RCLS type. This supports the hypothesis that it is the DDIT3 gene that determines the tumour entity when fused with the FUS gene.
Barone et al. have reported that micro-injection of FUS-DDIT3 expression plasmids into NIH-3T3 cells inhibited the growth arrest, and consequently adipocyte differentiation (127). We have shown the same effect. The HT1080 cells transfected with pFUS-DDIT3-EGFP did not accumulate fat on treatment with adipogenic factors while the DDIT3-transfected HT1080 cells accumulated large amount of fat. We speculate that forced expression of DDIT3 triggered the first steps of an adipocyte differentiation program that resulted in responsiveness to adipogenic factors.

The microarray analysis showed that only a small number of genes were differentially expressed in the original HT1080 cell line and the transfected variants. Only IL-6 was upregulated in both pFUS-DDIT3-EGFP and pDDIT3-EGFP transfected cell lines and MLS cells. Interleukin 6 is an anti-adipogenic factor and plays a role also in angiogenesis (85, 129). In the pFUS-DDIT3-EGFP transfectants and MLS cell lines the gene SERPINB2 (130) that encodes a protein with multiple roles in cell cycle control, e.g. inhibition of degradation of retinoblastoma protein, was strongly upregulated. Vascular endothelial growth factor (VEGF), a potent angiogenic factor was down-regulated in the transfected HT1080 cells and MLS cells. It may contribute to the differences in vascular pattern between HT1080 cells and the transfected cell lines and MLS cells. The gene DSIPI, which encodes an anti-adipogenic transcription factor, was also down-regulated (131). This may be an important factor behind the morphological shift in the SCID mouse tumours.

The above results show that forced expression of FUS-DDIT3 and DDIT3 induces switch in HT1080 cells toward MLS/RCLS-like morphology and gene expression. The results also indicate that the transcription factor partner DDIT3 of FUS-DDIT3 is the tumour type- determining part of this EWS-group fusion oncogene.

The fusion oncprotein FUS-DDIT3 or EWS-DDIT3 is believed to induce abnormal expression of growth-controlling genes, especially those exerting their effect at the G1/S cell cycle checkpoint (108). The broad span of over-expression of P53 in study IV is similar to that reported by Dei Tos. We have not performed mutation analysis but Dei Tos showed that the pattern of P53 expression was unrelated to the TP53 gene status. He found mutation of the TP53 gene in 28% of cases. Different results on TP53 mutations are presented in the literature, raising from a few per cent to 30% (108-111) Dei Tos also reports only 10% of cases with immunopositivity for cyclin D1, which is in contrast to our results with high expression of cyclin D/CDK4 in cytoplasm in all 17 tumours. However, Dei Tos also reports CDK4-positive immunoreactivity in 56% of cases, with 60-80% of cells staining. Our results
indicate, together with high nuclear expression of cyclin E/CDK2, that most of the cells were in the late G1 or S-phase of the cell cycle. P21 was not over-expressed in our tumours but P27, P57 and P16 were over-expressed in both MLSs and MLSs/RCLSs. Dei Tos found P21-positive immunoreactivity in 60% of cases, especially in patients with a round cell component and even in TP53 mutated cases, which could support the view of P53-independent P21 induction. The discrepancy between the two studies concerning the staining level of P21 may be due to a more sensitive method in Dei Tos study, leading to detection of the low P21 expression present in all cells. In Oda et al. (111), P16 expression was found to be significantly reduced in the round cell components in comparison with the myxoid components. Oliveira et al. (112) found that P27 expression <75% was significantly associated with decreased metastasis-free and overall survival. In our material the proliferation marker Ki67 was low and together with the low expression of cyclin A it indicated low mitotic activity. The paradoxical combination of high cyclin E expression and slow growth rate may be explained by the strong expression of CDK2 inhibitors P27 and P57.

The surprising results in our study are above all the weak or absent expression of pRB, in spite of the high expression of cyclin D/CDK4 and cyclin E/CDK2, and the high expression of cyclin B, despite low Ki67 and normal cyclin A. The high expression of cyclin D and CDK4 indicates that the majority of the cells have received stimuli to enter the cell cycle. This would in a normal cell also lead to expression of pRB. The weak expression of pRB found in our study may have been due to methodological problems but it may also indicate a malfunction in the control of pRB expression. While cyclin A was expressed at normal levels in MLS/RCLSs, cyclin B was strongly expressed in most of the cells. This anomaly could possibly be explained by a defective degradation of cyclin B at mitosis.

Microarray analysis suggested that PDGFRβ and EGFR were strongly expressed. In normal cells, activated growth factor receptors induce the transcription of the proto-oncogene MYC and the MYC protein induces the transcription of a number of growth-associated genes including CCND1 (cyclin D1). We have shown that the MYC protein and cyclin D1 were stable in serum starved MLS/RCLS-derived cell lines and independent of the FUS-DDIT3 fusion oncogene.

The high expression of the growth factor receptors led us to test tyrosine kinase inhibitors specific for these receptors. Preliminary results suggest that inhibition of PDGFRβ in cultured MLS cells with imatinib gave no response while gefitinib (epidermal growth factor receptor-tyrosine kinase inhibitor) gave a modest response and ZD6474, a tyrosine kinase inhibitor with
activities against VEGFR2 and RET, gave a strong response. The RET oncogene has been identified to be expressed in MLS but not in fat tissue (132).

We conclude that the strong cyclin E expression, the P27 and P57 expression and the few cyclin A and Ki67 positive cells suggest that the vast majority of the tumour cells are accumulated in late G1 phase. Abnormal expression of the G1 cyclins D1 and E and their kinases seems to be a recurrent pattern in MLS/RCLS. Whether the FUS-DDIT3 fusion oncogene is involved in the deregulation of the G1 cyclin activity or expression remains to be shown.

In conclusion liposarcoma should be treated at specialized centres and postoperative radiotherapy is indicated for high-grade lesions, at least after non-wide surgery. Low-grade MLSs have high radio-responsiveness, and radiotherapy is indicated after non-wide surgery or in a pre-operative setting. The fusion oncogene FUS-DDIT3 and DDIT3 may induce a liposarcoma phenotype, with DDIT3 being the tumour type-determining part of this fusion oncogene. Deregulation of G1-controlling proteins is common and indicates that MLS cells accumulate in the late G1 phase.
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