MOLECULAR GENETIC STUDIES

ON HUMAN SARCOMAS

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to by their Roman numerals in the text:

I Wolf, M., Aaltonen, L.A., Szymanska, J., Tarkkanen, M., Blomqvist, C., Berner, J-M., Myklebost, O. and Knuutila, S. Complexity of 12q13-22 amplicon in liposarcoma: Microsatellite repeat analysis. *Genes, Chromosomes and Cancer 18:66-70, 1997.*

II Wolf, M., Aaltonen, L.A., Szymanska, J., Tarkkanen, M., Elomaa, I. and Knuutila, S. Microsatellite markers as tools for characterization of DNA amplifications evaluated by comparative genomic hybridization. *Cancer Genetics and Cytogenetics 93:33-38, 1997.*

III Wolf, M., Tarkkanen, M., Hulsebos, T., Larramendy, M.L., Forus, A., Myklebost, O., Aaltonen, L.A., Elomaa, I. and Knuutila, S. Characterization of the 17p amplicon in human sarcomas: microsatellite marker analysis. *International Journal of Cancer 82:329-333, 1999.*

IV Wolf, M., Hemminki, A., Kivioja, A., Sistonen, P., Kaitila, I., Ervasti, H., Kinnunen, J., Karaharju, E. and Knuutila, S. A novel splice site mutation of the *EXT2* gene in a Finnish hereditary multiple exostoses family. *Human Mutation, Mutation in Brief #197 (Online), 1998.* *http://humu.edoc.com/1059-7794/pdf/mutation/197.pdf*

V Wolf, M., El-Rifai, W., Tarkkanen, M., Kononen, J., Serra, M., Eriksen, E.F., Elomaa, I., Kallioniemi, A., Kallioniemi, O-P. and Knuutila, S. Novel findings in gene expression detected in human osteosarcoma by cDNA microarray. Submitted.

In addition, some unpublished data is presented.

ABBREVIATIONS

AI	allelic imbalance
A2MR/LRP1	gene for α2-macroglobulin receptor / low density lipoprotein receptor-related protein
BAC	bacterial artificial chromosome
bp	base pairs
CDK4	gene for cyclin-dependent kinase 4
cDNA	complementary deoxyribonucleic acid
CGH	comparative genomic hybridization
cM	centiMorgan
CHOP/DDIT3	gene for DNA-damage-inducible transcript 3
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EFNB3/EPLG8	gene for ephrin B3
EXT	hereditary multiple exostoses syndrome
FISH	fluorescence *in situ* hybridization
FN1	gene for fibronectin 1
GCN	gain in copy number
GLI	glioma-associated oncogene homolog
HME	hereditary multiple exostoses syndrome
HMGIC	gene for high-mobility group protein I-C
HSP90β	gene for heat shock protein 90β
kb	kilobase
lod	logarithm of odds
LOH	loss of heterozygosity
Mb	megabase
MDM2	human homolog of murine double minute 2 gene
MKK4/SERK1	gene for mitogen-activated protein kinase kinase 4
p	short arm of the chromosome
PABPL1	gene for polyadenylate-binding protein-like 1
PCR	polymerase chain reaction
q	long arm of the chromosome
RB1	retinoblastoma 1 gene
RNA	ribonucleic acid
SAS	gene for sarcoma amplified sequence
THBS1	gene for thrombospondin 1
TOP3	gene for topoisomerase III
TP53	gene for tumor suppressor protein p53
YAC	yeast artificial chromosome

INTRODUCTION

Cancer is a genetic disease caused by changes in expression of genes that take part in the regulation and control of normal cellular functions. The expression of these genes can be altered by changes in DNA copy number, point mutations, chromosomal translocations, or by abnormal transcription and / or translation. The development of cancer is a multistep process requiring several genetic changes to develop and progress into a full-blown disease [184]. In most cases the changes are acquired during the lifetime of an individual caused by different carcinogenic factors. However, a minor proportion of cancers develop due to inherited predisposing mutations.

Sarcomas are a heterogeneous group of malignancies of primarily mesenchymal origin. They are rare solid tumors, often highly malignant, and can be divided into malignancies of soft tissues and bone, of which soft tissue tumors account for the majority of cases. Although information on the biological basis of sarcomas is accumulating, knowledge of the genetic factors in their pathogenesis remains limited. Analysis of sarcoma specimens by conventional cytogenetic methods is often restricted by low mitotic yields, poor chromosome morphology and complex karyotypes. Recent techniques, including comparative genomic hybridization, have had a significant value on revealing DNA copy number aberrations, and thus pinpointing putative loci for genes, that may be important to the development and / or progression of these tumors.

Formation of fusion proteins by tumor-specific gene translocations is a common aberration in a subset of sarcomas, and recently, some of the functions of the specific fusion proteins have been determined. In addition, specific DNA amplifications are frequently found in mesenchymal tumors indicating the important role of proto-oncogene amplifications in their tumorigenesis.

A detailed understanding of the underlying molecular genetic defects in sarcomas, including the specific genetic aberrations in the DNA, RNA and protein levels, is necessary to improve the characterization of different histological tumor types and increase the number of markers for tumor progression and patient follow-ups. Identification of these molecular aberrations provides also valuable information for the development of cancer therapy. The aim of this thesis is to characterize some of the molecular genetic events that are involved in the pathogenesis of sarcomas.

REVIEW OF THE LITERATURE

1. SARCOMAS

1.1. General characteristics

Sarcomas originate from cells arising primarily from the mesenchyme, which gives rise to the supporting tissues of the body. They form a separate entity of tumors, distinct from neoplasms arising from the epithelium, hematopoietic cells, or the central nervous system. Sarcomas are rare tumors. The annual incidence of new cases in Finland, with a population of 5.1 million was 174 during 1990-1994, which is approximately 1% of all new cancer cases diagnosed (data obtained from the Finnish Cancer Registry). Of these, 129 cases were classified as soft tissue sarcomas and 45 cases as sarcomas of the bone.

The survival rate of a sarcoma patient is dependent on several factors, including histology, grade, location and size of the tumor, occurrence of necrosis, vascular invasion, and the presence of metastases [141, 142]. Sarcomas metastasize to different organs, most frequently to the lungs [181]. Metastatic spread to lymph nodes, which is common in carcinomas, is a rare event [32]. Although patients with metastases have in general a very poor prognosis, resection of pulmonary metastases can lead to improved survival in some patients [181]. In addition, information has been obtained on the prognostic values of specific genetic aberrations [6, 74, 117, 169, 171]. However, the amount of genetic markers remains limited.

Although the etiology of sarcomas is still mostly unknown, some risk factors have been identified. Ionizing radiation, exposure to phenoxy herbicides, and some hereditary syndromes have been suggested to predispose individuals to sarcomas [38, 111, 123, 145, 191]. Infection with simian virus 40, a DNA tumor virus, has been shown to be associated with some malignancies, including sarcomas, especially osteosarcoma [25, 49]. In addition, putative roles for viral infections by human herpesvirus type 8 and human immunosuppressive virus in the etiology of Kaposi's sarcoma are under intense research [24, 48].

The genetic background of sarcomas differs from that of most solid tumors by their tumor-specific translocations [202]. These recurrent chromosomal translocations are crucial for the characterization of different histological tumor types and their subtypes, and have diagnostic value. Other frequent changes are amplifications of DNA sequences clustered to specific regions in the genome, suggesting that some of the genes involved in these amplicons are important for the pathogenesis of the disease.

1.2. Soft tissue sarcomas

Soft tissue sarcomas include malignant tumors derived from connective tissues other than bone and cartilage. The classification of different histological tumor types is done according to their resemblance to various adult tissue types, and altogether more than 50 different types have been identified [141]. The most common histological types according to the Scandinavian sarcoma group register during 1986-1997 were malignant fibrous histiocytoma (40%), liposarcoma (13%), leiomyosarcoma (8%), synovial sarcoma (8%), malignant peripheral nerve sheath tumor (5%), and fibrosarcoma (4%) [10].

Soft tissue sarcomas can occur at any site of the body but the majority of cases are located in the extremities, the trunk, the regions of the head and neck, and the retroperitoneum [10, 32, 133]. In general, soft tissue sarcomas do not show a predilection for any age group, however, some histological types are more common among children and young adults (e.g. rhabdomyosarcoma) and some are more prevalent during old age (e.g. malignant fibrous histiocytoma). The most important prognostic factors for patients with soft tissue sarcomas are the histological grade and size of the tumor, and the presence of metastases [105, 141]. The primary treatment of most cases is surgery [10]. The 5-year metastasis-free survival rates vary between 80% and 85% in patients with low-grade tumors, and drop to 43-48% in patients with high-grade tumors [141].

1.3. Bone sarcomas

The main bone sarcomas are osteosarcoma (31%), chondrosarcoma (31%) and Ewing's sarcoma (14%) [10]. Osteosarcoma is, together with chondrosarcoma, the most common malignant primary bone tumor. It is defined as a malignant mesenchymal tumor in which the cancerous cells, malignant osteoblasts, produce bone matrix [112]. Chondrosarcoma is less aggressive and has a better prognosis than osteosarcoma and Ewing's sarcoma. Ewing's sarcoma and other related tumors, primitive neuroectodermal tumor, neuroepithelioma, and Askin's tumor, form a distinct entity of malignancies belonging to the Ewing family of tumors. Like osteosarcoma, Ewing's sarcoma is highly malignant and rapidly fatal if left untreated. A minor part of other bone sarcomas include malignant fibrous histiocytoma, fibrosarcoma and giant cell sarcoma.

The average time of diagnosis for osteosarcoma and Ewing's sarcoma is during the second decade of life, while chondrosarcoma and malignant fibrous histiocytoma become more prevalent with advancing age. The most common sites of primary bone sarcomas are the femur, tibia and humerus [10]. The most important prognostic factors are the size of the tumor, its location, and the presence of metastases [142]. In addition, response to pre-operative chemotherapy has important prognostic value for patients

with osteosarcoma and Ewing's sarcoma [142]. The primary treatment of bone sarcomas is surgery. In addition, the introduction of chemotherapy, and chemotherapy and radiation, have clearly improved the survival rates in patients with osteosarcoma and Ewing's sarcoma, respectively, whereas they have only little to add in the treatment of chondrosarcoma [112]. The average 5-year disease-free survival rates in patients with primary osteosarcoma and Ewing's sarcoma are 45-70% [47, 142], while in metastatic patients the survival rates are only 20-30% and 15-20%, respectively [142].

2. GENETIC ABERRATIONS IN SARCOMAS

2.1. Cytogenetic alterations

In general, sarcomas contain multiple karyotypic changes including both numerical and structural aberrations. These changes are heterogeneous, with different cell populations containing distinct cytogenetic alterations.

Unlike most solid tumors and similar to many hematological malignancies, several sarcoma types are characterized by tumor-specific reciprocal translocations [202]. Table 1 summarises the known translocations in sarcomas and the genes involved in the fusions. These chromosomal abnormalities are found in the majority of the particular tumor types; e.g. 90% of Ewing's sarcomas show t(11;22)(q24;q12) or other variant translocations involving either the 11q24 or 22q12 region, 75% of extraskeletal myxoid chondrosarcomas have been reported to contain t(9;22)(q22;q11.2), t(2;13)(q35;q14) is seen in 70% of alveolar rhabdomyosarcomas, and the formation of fusion protein by translocations t(12;16)(q13;p11), t(12;22)(q13;q12) and t(X;18)(p11.2;q11.2) is seen in most cases of myxoid liposarcomas, clear cell sarcomas of the soft parts and synovial sarcomas, respectively [23, 42, 70, 84].

Translocations usually result in the fusion of a transcriptional activating domain from one gene (e.g. *EWS*) and a DNA binding domain of another gene (e.g. *FLI1, ERG, ETV1, ETV4* and *FEV* in Ewing's sarcomas), creating an oncogenic fusion protein. The role of some the fusion proteins in tumorigenesis has been revealed, but in most cases their specific functions remain unknown. *TGFBR2,* encoding the TGF-β type II receptor, has been shown to be a likely direct target for the EWS/FLI1 fusion protein [66]. By binding to the *TGFBR2* promoter region EWS/FLI1 suppresses its expression, leading to loss of the TGF-β growth-inhibitory signalling pathway. TLS/CHOP fusion protein, found in the majority of myxoid liposarcomas, has been shown to lead to unscheduled expression of the megakaryocyte stimulatory factor DOL54, normally produced during the early phases of adipocytic differentiation [95].

Recently, cDNA microarray studies have indicated that the PAX3/FKHR fusion protein, specific in alveolar rhabdomyosarcoma, activates a myogenic transcription program, including the induction of the muscle-specific transcription factors *MYOD* and myogenin, together with several other genes involved in muscle function [85].

Other cytogenetic features of malignant mesenchymal tumors are double minutes and homogeneously staining regions manifesting gene amplification, and ring chromosomes.

Table 1. Tumor-specific chromosomal translocations in sarcomas

Tumor type	Translocation[1]	Genes involved in the fusion[2]	References
Chondrosarcoma, extraskeletal myxoid	**t(9;22)(q22;q11.2)**	***TEC;EWS***	[23, 72]
	t(9;17)(q22;q12)	*TEC;TAF2N*	[153]
Clear cell sarcoma of the soft parts (Malignant melanoma)	t(12;22)(q13;q12)	*ATF1;EWS*	[22, 201]
Dermatofibrosarcoma protuberans	t(17;22)(q22;q13)	*COL1A1;PDGFB*	[128]
Ewing family of tumors			
Askin's tumor	t(11;22)(q24;q12)	*FLI1;EWS*	[190]
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*WT1;EWS*	[97, 146]
Ewing's sarcoma	**t(11;22)(q24;q12)**	***FLI1;EWS***	[41]
	t(21;22)(q22;q12)	*ERG;EWS*	[157]
	t(7;22)(p22;q12)	*ETV1;EWS*	[77]
	t(17;22)(q21;q12)	*ETV4;EWS*	[178]
	t(2;22)(q33;q12)	*FEV;EWS*	[130]
Fibrosarcoma, congenital	t(12;15)(p13;q25)	*ETV6;NTRK3*	[89]
Liposarcoma, myxoid and round cell	**t(12;16)(q13;p11)**	***CHOP;TLS***	[37, 175]
	t(12;22)(q13;q12)	*CHOP;EWS*	[127]
Rhabdomyosarcoma, alveolar	**t(2;13)(q35;q14)**	***PAX3;FKHR***	[57, 176]
	t(1;13)(p36;q14)	*PAX7;FKHR*	[42]
Synovial sarcoma	**t(X;18)(p11.2;q11.2)**	***SSX1;SYT***	[29, 84, 174]
	t(X;18)(p11.2;q11.2)	*SSX2;SYT*	[39, 174]
	t(X;18)(p11.2;q11.2)	*SSX4;SYT*	[154, 174]

[1] The most recurrent translocations are marked in bold text in tumor types with many translocation variants.

[2] Abbreviations for genes:

ATF1, activating transcription factor 1 gene; *CHOP/DDIT3,* DNA-damage-inducible transcript 3 gene; *COLIA1,* collagen type I alpha 1 gene; *ERG,* v-ets avian erythroblastosis virus E26 oncogene related gene; *ETV1,4,6,* ets variant genes 1, 4 and 6; *EWS/EWSR1,* Ewing's sarcoma breakpoint region 1 gene; *FKHR,* forkhead homolog 1 gene; *FLI1,* Friend leukemia virus integration 1 gene; *NTRK3,* neurotrophic tyrosine kinase receptor type 3 gene; *PAX3,7,* paired box homeotic genes 3 and 7; *PDGFB*, platelet-derived growth factor beta polypeptide gene; *SSX1,2,4,* synovial sarcoma, X breakpoint genes 1, 2 and 4; *SYT,* synovial sarcoma translocation gene; *TAF2N,* TATA box binding protein-associated factor 2N gene; *TEC/CHN/NR4A3,* nuclear receptor subfamily 4, group A, member 3 gene; *TLS/FUS,* translocated in liposarcoma; *WT1,* Wilms tumor 1 gene.

2.2. Molecular genetic changes

2.2.1. Structural alterations of cellular proto-oncogenes

Cellular proto-oncogenes are genes, which by genetic alterations can transform into cancer-causing oncogenes [79]. Protein products encoded by proto-oncogenes have growth-promoting functions, which in their oncogenic forms are produced or function without normal control. Activated proto-oncogenes have a dominant effect on cell phenotype, manifested by the disruption of growth control due to one aberrant allele.

In addition to those rearrangements that are involved in the tumor-specific fusion genes (Table 1), structural alterations of other cellular proto-oncogenes have been reported in sarcomas. However, these aberrations appear to be relatively infrequent, and include alterations of *MYC, MYCN* and *FOS* [110, 132]. In addition, mutated members of the *RAS* proto-oncogene family have been detected, especially in rhabdomyosarcomas (35%) [162]. Frequent rearrangements of the high mobility group protein I-C gene (*HMGIC*), encoding a small nuclear architectural factor, have been reported in benign mesenchymal tumors [149] and, more recently, in a subset of sarcomas, leading to truncation of the HMGIC protein [15, 94]. The biological role of the alteration is not yet known.

DNA amplification is one mechanism for activating cellular proto-oncogenes, leading to overexpression of oncogenic proteins. The specific gene amplifications detected in sarcomas will be discussed in the next chapter in more detail.

2.2.2. Gene amplifications

Gene amplifications are common in malignant mesenchymal tumors. Amplifications of DNA sequences in sarcomas show clustering to specific regions in the genome like 1q, 8q, 12q and 17p, which are considered to harbor (proto-onco)genes important for the development and / or progression of these tumors [62, 92].

Recurrent amplifications of 1q21-q23 have been reported both in soft tissue and bone sarcomas, especially in osteosarcoma (36-58%), liposarcoma (23-32%), leiomyosarcoma (10-25%) and the Ewing family of tumors (8-25%), detected by comparative genomic hybridization (CGH) [5, 53, 167, 169, 170]. In addition to sarcomas, 1q21-q23 amplifications have been found in other tumors, particularly in desmoid tumors (39%) [100]. Studies on the affected region suggest a complex structure consisting of at least two amplicons with frequent amplification of *FLG* (filaggrin gene), and less frequent amplifications of *SPRR1, SPRR2* and *SPRR3* (small proline-rich protein genes 1, 2 and 3), *S100A6/CACY* (calcyclin gene) and *S100A2 /S100L* (calcium-binding protein A2 gene) [51]. It remains to be seen what are the precise target genes and their functions in tumorigenesis.

Common amplified regions in sarcomas also include 8q, which harbors the *MYC* proto-oncogene (8q24) [5, 169, 170]. *MYC* has been shown to be involved

either through amplification or rearrangement in several malignancies [9, 199]. The expression of *MYC* is highest in proliferating cells and drops as cells differentiate [122, 129]. It has been suggested that a decrease in the protein expression level is required for cessation of cell division during differentiation. The protein product of *MYC* is a DNA-binding transcription factor. It dimerizes with another protein, MYC-associated factor X (MAX), and as a heterodimer has been implicated in inducing the expression of several genes involved in cell proliferation [31, 125]. Amplification of the 8q region is a recurrent finding especially in embryonal rhabdomyosarcoma (60%), leiomyosarcoma (21-47%), osteosarcoma (10-52%), the Ewing family of tumors (35%), synovial sarcoma (30%), liposarcoma (29%) and chondrosarcoma (27%) [5, 44, 98, 165, 166, 189].

Amplification of 12q13-q15 region has been reported in 8-36% of sarcomas, and is particularly common in liposarcoma (50%), synovial sarcoma (30%), alveolar rhabdomyosarcoma (50%) and Ewing family of tumors (25%) [5, 165, 166, 189]. Besides sarcomas, amplification of the 12q13-q15 region has also been reported in malignant glioma (15%), but rarely in other tumors [114, 115, 137]. Several genes from the region, including *MDM2* (human homolog of murine double minute 2 gene), *SAS* (sarcoma amplified sequence gene), *CDK4* (cyclin-dependent kinase 4 gene), *CHOP/DDIT3* (DNA-damage-inducible transcript 3 gene), *GLI* (glioma-associated oncogene homolog), *HMGIC* (high-mobility group protein I-C gene) and *A2MR/LRP1* (α2-macroglobulin receptor / low density lipoprotein receptor-related protein gene) have been shown to be amplified in various sarcoma types [15, 52, 86, 126, 139, 155]. In addition, an amplification of *PRIM1* (DNA primase 1 gene), located at 12q13, was recently reported in osteosarcoma [200]. In most cases tumors exhibit coamplification of several genes, but based on studies revealing discrete amplicons around *MDM2* and *CDK4*, these two are considered to be the main candidates for target genes [14, 86, 124]. However, not all amplicons have been found to contain amplification of either *MDM2* or *CDK4*, and therefore the existence of a yet unknown target gene cannot be excluded [14].

Information about the functions of the genes located at 12q13-q15 is accumulating. Most information has been obtained for the *MDM2* gene product. It has been shown to interact with tumor suppressor p53, inactivate its function, and thereby promote cell proliferation [55, 118]. Sarcomas usually contain either an *MDM2* amplification or a mutated *TP53* gene, indicating two mutually exclusive mechanisms for escaping growth control through the same regulatory pathway [103]. However, a subset of tumors has been reported to contain both overexpression of MDM2 and mutated *TP53*. These patients have a worse prognosis than those containing only one of the two aberrations [33]. Further evidence supporting the proposal that activated *MDM2*

may have additional functions as an oncogene, have been obtained from studies showing that MDM2 can interact with the tumor suppressor protein pRB, and furthermore, it inhibits the TGF-β-induced growth arrest [164, 197].

The protein product of *CDK4*, the other main candidate gene, participates in the phosphorylation of pRB when activated by cyclin D1 [68]. pRB functions as a cell cycle regulator. By binding to transcription factor E2F in a hypophosphorylated state, pRB inhibits the growth-promoting actions of E2F. At the end of the G1 phase of the cell cycle pRB becomes hyperphosphorylated by cyclins and cyclin-dependent kinases, and releases E2F. Released E2F recovers its activity and permits the cell cycle to proceed through activation of genes involved in cell proliferation. A simplified overview of the cell cycle regulation by CDK4 is given in Figure 1.

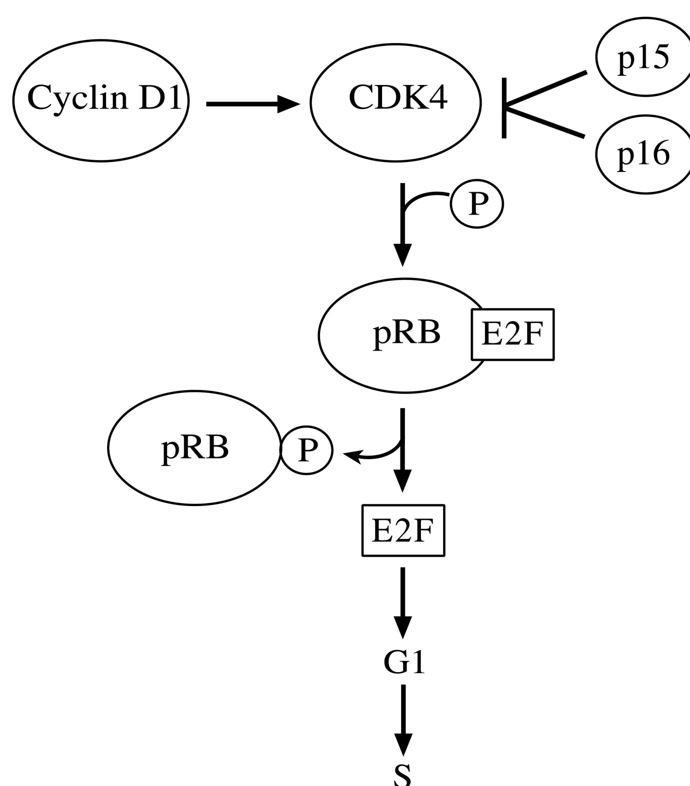

Figure 1. The CDK4-mediated cell cycle regulation. pRB phosphorylation is mediated by the kinase subunit of CDK4 after binding with cyclin D1. The phosphorylated form of pRB releases the bound transcription factor, E2F, and enables the cell cycle to proceed. p15 and p16 interact with CDK4 inhibiting its activation by cyclin D1.

Less is known about the other 12q13-q15 genes. *SAS* encodes a protein member of the transmembrane 4 superfamily functioning in signal transduction

pathways [75]. The product of *GLI* belongs to the Kruppel family of zinc finger proteins. It acts as a transcription factor during embryogenesis, and is not normally expressed in adult tissues [87]. The product of *CHOP/DDIT3* is induced by cellular stress, including DNA-damage, and functions in growth inhibition at the G1/S checkpoint phase [8, 140]. A2MR/LRP1 acts as a receptor for apo E enriched lipoproteins [11]. The protein product of *HMGIC* has a role in the assembly of transcriptional complexes. It has been shown to be abundant in transformed cells, and its increased expression levels to correlate with more malignant phenotypes. Interestingly, also gene amplifications of its rearranged form have been reported in sarcomas [15, 94]. *PRIM1* encodes for DNA primase polypeptide 1, which is required for the initiation of DNA synthesis.

In addition to the 12q13-q15 region, a gain of DNA sequences in a more distal, q21-q22, region has been reported in liposarcomas [163], indicating another target area for DNA amplification. The target gene(s) remains unknown.

Amplification of the 17p11-p12 region was first detected by Southern blotting analyses in a subset of astrocytoma samples and subsequently, more frequently in leiomyosarcoma (24-59%), osteosarcoma (13-29%), chondrosarcoma (21%) and malignant fibrous histiocytoma (16%) using CGH [17, 44, 54, 73, 98, 99, 169, 170]. The role of 17p amplification is emphasized by studies showing a gain of 17p11-p12 as the only detectable amplification in some osteosarcoma cases, suggesting that it is an early event [54, 170]. No known target genes have been assigned to the 17p amplicon so far. However, amplification of *PMP22* (peripheral myelin protein gene) has been reported in cell lines derived from osteosarcoma and glioma [107]. The exact function of the protein product is not yet known. It is expressed at high levels in Schwann cells of the peripheral nervous system, and a duplicated form of the gene has been found in a portion of patients with Charcot-Marie-Tooth type 1A disease, a hereditary motor-sensory neuropathy syndrome [180]. In addition, the 17p11-p12 region harbors several interesting genes, like *MKK4/SERK1* (mitogen-activated protein kinase kinase 4 gene), *MAPK7* (mitogen-activated protein kinase 7 gene), *TOP3* (topoisomerase III gene), *EFNB3/EPLG8* (ephrin B3, EPH-related receptor tyrosine kinase ligand 8 gene) and *EIF5A* (eukaryotic translation initiation factor 5A gene) that could be considered in further studies which aim to identify the target gene(s).

Correlation with clinical outcomes has been obtained for DNA copy number gains at 1q and 8q. Copy number increase at 1q21 or 1q21-q22 show a trend toward poor overall survival and worse distant disease-free survival in patients with osteosarcoma and Ewing's sarcoma, respectively [169, 171]. Furthermore, DNA copy number gains at 8q24.1-qter, and 8q21.3-q22 and / or 8cen-q13 have been reported to correlate with shorter overall survival and poor distant disease-free survival rates in individuals with chondrosarcoma and high-grade osteosarcoma, respectively [98, 169].

In addition to the amplifications discussed above, several other chromosomal regions have been reported to show gains in DNA copy number in sarcomas. However, the gains of 1q, 8q, 12q and 17p are common characteristics of many sarcoma types, and amplifications at 1q21-q23, 12q13-q15, and 17p11-p12 seem more common in sarcomas than other tumor types. Therefore, identification of the target genes in these regions is of major importance in understanding the basic molecular defects in these tumors.

2.2.3. Alterations of tumor suppressor genes and loss of DNA sequences

Genes which through inactivation lead to unconstrained cell growth, are called tumor suppressor genes [104]. They encode proteins which normally have a negative effect on cell proliferation. Close to thirty years ago, Alfred Knudson proposed his well-known model for the development of retinoblastoma, a malignant tumor arising in the eyes of affected children [90]. According to this "two-hit hypothesis", children with the sporadic form of retinoblastoma acquire retinal tumors due to two somatic mutations of a gene, later named the retinoblastoma 1 gene *(RB1)*, whereas in the hereditary form, one of the two alleles is inactivated due to a somatic mutation while the other is inherited inactive. Whereas only one mutation is sufficient to cause abnormal function of a proto-oncogene, the lack of both functional alleles is required for a tumor suppressor gene to lose its ability to constrain malignant behavior in a cell. Inactivation often occurs through a mutation of one allele and a deletion of the other. Repeated observations of losses of DNA sequences in tumor samples can thus give information of putative loci harboring tumor suppressor genes.

Alterations in the *TP53* gene, located at 17p13.1, are frequent in human cancers. The protein product, p53, is a transcription factor which is expressed ubiquitously in all tissues. In its wild-type form it functions as a negative growth controller at the G1/S checkpoint, inducing growth arrest after DNA damage [76, 159]. In addition, p53 can suppress malignant transformation by inducing programmed cell death after exposure to DNA-damaging agents [131]. p53 abnormalities have been reported in sarcomas with varying frequencies (8-33%) [132, 173], and allelotyping studies have revealed loss of heterozygosity at polymorphic marker loci mapped to the 17p13 region in 30-60% of sarcoma cases studied [4, 33].

Deletions and structural alterations of the *RB1* gene are found frequently in various types of cancers including sarcomas, and especially, in osteosarcoma (11-72%) [56, 132, 187, 198]. The mutated / deleted form of pRB is unable to bind the transcription factor E2F allowing a constant activation of genes involved in cell proliferation (Fig. 1). In addition to losses of 13q14, harboring the *RB1* gene, more distal regions, 13q21-q22 and 13q21-qter show deletions in leiomyosarcoma (45%),

malignant fibrous histiocytoma of bone (42%) and soft tissue (21%), osteosarcoma (23-26%), liposarcoma (21%) and synovial sarcoma (15%) [91]. The target gene for the deletion remains unknown, however, putative candidate genes like *ING1* (inhibitor of growth 1 gene) and *PCDH9* (protocadherin 9 gene) have been mapped to the region.

Other commonly lost chromosomal regions include 9p, frequently deleted in malignant fibrous histioocytoma of bone (23%), liposarcoma (21%) and chondrosarcoma (17%), and 18q12-qter, which has been found to be affected in malignant fibrous histiocytoma of bone (27%), leiomyosarcoma (17%) and osteosarcoma (13%) [91, 109, 121]. One of the target genes for chromosome 9p losses is *CDKN2A* (cyclin-dependent kinase inhibitor 2A gene), located at 9p21 and encoding p16, an inhibitor of CDK4- and CDK6-mediated cell cycle progression (Fig. 1) [109, 152]. In addition, gene for another CDK4/CDK6 inhibitor, *CDKN2B* (cyclin-dependent kinase inhibitor 2B gene), encoding p15, is mapped to the same region. Chromosome 18 harbors candidate genes like *SMAD2*, *SMAD4* (human homologs of *MAD* gene) and *DCC* (deleted in colorectal carcinoma gene). Further studies are needed to reveal the exact target genes of these losses and their roles in sarcoma pathogenesis.

2.3. Hereditary predisposition

In addition to the somatic aberrations discussed above, a minor fraction of mesenchymal malignancies arises due to inherited predisposition.

Li-Fraumeni syndrome is associated with germ-line mutations in *TP53* and *CHK2* (checkpoint kinase 2 gene) genes [12, 111]. The syndrome is inherited as an autosomal dominant trait with high penetrance, and is characterized by predisposition to several malignancies, including sarcomas of bone and soft tissues, already in early childhood [18].

Hereditary retinoblastoma is, as already mentioned, a malignant tumor arising in the eyes of affected children, due to the loss of function of pRB. Patients with hereditary retinoblastoma are also predisposed to various secondary malignancies, and have, in particular, a several hundredfold increased risk of developing osteosarcoma [123].

Neurofibromatosis type I, also called von Recklinghausen's syndrome, is an autosomal dominant disorder caused by germline mutations in the *NF1* gene, located at 17q11.2. The clinical features of patients with this disease include café-au-lait spots on the skin, pigmented iris hamartomas, and the development of multiple benign peripheral nerve sheath tumors, neurofibromas [138]. In addition, 2-3% of the patients develop malignant peripheral nerve sheath tumors [134, 195].

The clinical features of patients with Beckwith-Wiedemann syndrome include pre- or postnatal overgrowth, facial abnormalities, and hypoglycemia [46]. In addition, patients are characterized by an increased risk of developing specific tumors at a young

age, including rhabdomyosarcoma. The syndrome is caused by genetic changes of the 11p15 region, and one of the molecular key features includes the overexpression of IGF2 (insulin-like growth factor 2) [106, 119].

Hereditary multiple exostoses syndrome (HME/EXT) is a rare autosomal dominant skeletal disorder characterized by multiple exostoses in the juxtaepiphyseal regions of the long bones. The exostoses are cartilage-capped osseous projections called osteochondromas that gradually develop and are evident during the first decade of life in more than 80% of the patients [156]. The prevalence of HME ranges between 1/50 000 and 1/100 000 and the penetrance is estimated to be 100% [191]. Several complications are associated with the disease. One of the most serious complications is the malignant degeneration of the osteochondromas either to chondrosarcomas, or rarely to osteosarcomas, in 0.9-2.9% of the patients [186, 191]. Studies on HME have shown it to be genetically heterogeneous with at least three susceptibility loci; *EXT1* (8q24.1), *EXT2* (11p11-p12) and *EXT3* (19p) [1, 102, 160, 196]. Loss of heterozygosity at polymorphic marker loci linked to the *EXT* genes in tumors from HME patients and in sporadic chondrosarcomas suggests that these genes function as tumor suppressor genes [69, 135]. Studies on the *EXT2* mouse homolog suggest a role in limb development in early embryogenesis [161]. Furthermore, *EXT1* and *EXT2* have been shown to encode glycosyltransferases, enzymes participating in heparan sulphate biosynthesis [108]. Several other genes have been found with related structures, suggesting the existence of a distinct *EXT*-gene family [143, 182, 194].

In addition, other disorders, including Gardner syndrome, Werner's syndrome and nevoid basal cell carcinoma syndrome, have been implicated in the sarcomatous degeneration [58, 64, 67, 179].

3. DNA AMPLIFICATION

Amplification increases the copy number of DNA sequences and usually results in the overexpression of some of the genes included in the amplicon. This can lead to the aberrant activation of a proto-oncogene or increase the level of a protein involved in drug-resistance. Besides the selected driver gene usually a much larger region is amplified, leading to the coamplification of nearby DNA sequences and genes.

Gene amplification was first detected in drug resistant cell lines after increasing the concentration of cytotoxic drug in the culture medium [16]. An increase in the copy number of a gene encoding the protein that is the target of the drug results in overexpression of the protein, resistance to the drug, and cell survival.

DNA amplification is one mechanism for activating proto-oncogenes [2, 120, 151]. *MYCN* was the first proto-oncogene whose amplification in human

neuroblastoma was associated with aggressive tumor growth [150]. Nowadays, several malignancies are known to harbor amplified genes encoding proteins with growth promoting functions. Amplification provides neoplastic cells with a growth advantage over the strictly controlled normal cells, leading to overgrowth of the transformed cell population.

3.1. Structure and mechanisms of DNA amplification

Cytogenetically DNA amplification can be seen in metaphase spreads as an extra chromosome resulting from amplification of one chromosome (trisomy), or as many extra chromosomes following the amplification of several chromosomes (polysomy). In addition, amplified DNA can be seen to be integrated within chromosomes, forming homogeneously staining regions, or as extrachromosomal structures called double minutes [35].

Homogeneously staining regions are chromosomal segments that stain abnormally when treated with trypsin-Giemsa; instead of forming a clear dark or light band the region stains with intermediate intensity. They were first described in antifolate-resistant Chinese hamster cell lines, and were proposed to contain amplified sequences from a gene encoding dihydrofolate reductase [16]. Since some chromosomal regions harboring amplified segments do not display trypsin-Giemsa bands, the term extended chromosomal region has also been used to describe the phenomenon. Double minutes appear in a metaphase spread as small (0.3-0.5 μm in diameter), paired, acentric elements [113]. They replicate autonomously and characteristically the number of double minutes varies in different cells due to their unequal distribution into daughter cells. Usually, only one of the two abnormal structures, homogeneously staining regions or double minutes, can be seen within the same cell [28].

The exact mechanism of DNA amplification is not fully understood. However, several possibilities for the origin of homogeneously staining regions and double minutes have been suggested. One model proposes that homogeneously staining regions arise from unequal crossing over between sister chromatids and that double minutes arise from episomes, submicroscopic precursors originating from a deletion of the corresponding chromosomal sequence [26], or as a result of DNA uptake from lysed cells [148]. Evidence has been obtained that double minutes may also arise from the breakdown of homogeneously staining regions and vice versa [34], but the exact mechanisms are still unknown.

A schematic model for DNA amplification by the so called onion skin (overreplication) model is shown in Figure 2. According to the model, multiple initiations of DNA replication occur during one S phase of the cell cycle, creating a

replication bubble, which is then resolved by recombination into a linear sequence or alternatively, into extrachromosomal DNA circles [19, 144]. Based on this model an amplification gradient is formed, with the highest copy number for those DNA sequences that are located closest to the origin of replication, and which gets smaller for more distant sequences included in the amplicon. However, amplified regions have been reported that do not contain such a gradient, leaving the issue still unresolved [3]. As was already mentioned, several models for DNA amplification have been proposed, and it seems likely that amplification can occur through several mechanisms [158, 193].

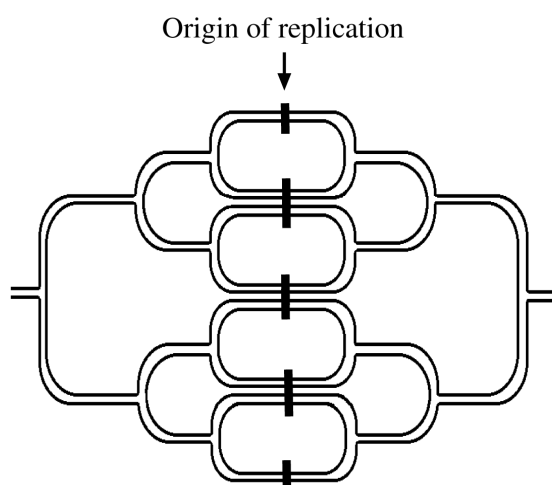

Figure 2. Schematic presentation of the onion skin model for DNA amplification. Modified from Botchan et al., 1979 [19] and Sambrook et al., 1975 [144].

4. METHODS FOR DETECTING ABERRATIONS IN DNA COPY NUMBER AND GENE EXPRESSION

4.1. Comparative genomic hybridization

Comparative genomic hybridization (CGH) is a genome-wide screening method used for the detection of DNA copy number changes [82]. Since the introduction of the method in 1992, it has revolutionized the genetic studies of DNA copy number aberrations in human neoplasms [91, 92]. Solid tumors are characterized by complex, often chaotic karyotypes which in many cases are difficult or impossible to analyze using conventional cytogenetic techniques but can be analyzed by CGH.

CGH is based on the labelling of tumor DNA and normal reference DNA with different fluorochromes, and simultaneous hybridization of equal amounts of labelled DNAs to normal metaphase chromosomes on a microscope slide. Labelled DNA

sequences compete for the correct target sequences fixed on the microscope slide and thus the hybridization efficiency between the different fluorochromes in optimal conditions reflects the proportion of the homologous sequences of the labelled DNAs. Analysis of the hybridization is done with automated digital image analysis software. Unequal intensity of the two fluorochromes reveals an aberration in the DNA copy number in the tumor sample. Gains are detected when the intensity of the fluorochrome that was used to label the tumor DNA is stronger than the other fluorochrome, and losses are discovered when the intensity of the fluorochrome that was used to label the normal DNA prevails over the other.

The sensitivity of the method allows detection of DNA copy number gains and losses of approximately 10-20 Mb in size [83]. Smaller areas, even down to 1 Mb in length, are detectable if they are highly amplified, whereas losses as small as 10-12 Mb in size have been reported [13, 50]. The sensitivity of CGH depends highly on the proportion of the abnormal cells in the sample. In general, at least 50% of the cells should contain the aberration in order to be detectable by CGH [83].

When used together with conventional cytogenetic analysis, CGH enables identification of the origin of genetic material from marker chromosomes, homogeneously staining regions, double minutes and ring chromosomes. Furthermore, it can be used as a screening method for identification of novel chromosomal regions with recurrent DNA copy number aberrations from a specific tumor entity. These regions can then be investigated with other, more sensitive methods for unidentified genes that may play a role in tumor pathogenesis. Studies where CGH has helped to pinpoint loci for new disease genes are the most convincing evidence of the power of the technique [71, 183].

4.2. Microsatellite marker analysis

Microsatellite markers are genomic loci containing tandem repeats of 1-6 bp nucleotide motifs [172]. These loci are often polymorphic as they exhibit variations in the number of motifs. Highly informative polymorphic markers can be used for the detection of allelic imbalances at specific chromosomal sites of a tumor sample, indicated by the unequal intensity of the two alleles in the tumor DNA compared to the allele intensities in the normal, reference DNA, obtained from the same patient [80]. Polymerase chain reaction (PCR) can be used for efficient analysis of the alleles of a marker locus [188].

Based on the hypothesis that, in addition to one mutated allele of a tumor suppressor gene, the wild type allele is often lost by a deletion, frequently observed loss of heterozygosity in tumor samples indicates the presence of a tumor suppressor gene at a nearby locus. Similarly, consistent allelic imbalance can be indicative of the activation of a proto-oncogene due to a gain in copy number of a specific chromosomal region.

4.3. The array technique and cDNA microarrays

The rapidly developing array-based technology has had a significant impact on studies on the variations in DNA, RNA and protein levels (for reviews, see the Nature Genetics supplement issue for volume 21, 1999). Different applications of array-based assays include oligonucleotide, DNA, cDNA and tumor tissue arrays. Glass- and membrane-based arrays offer miniaturized tools for detecting aberrations of several hundred or thousand target sequences / tissues simultaneously in one hybridization.

One of the most popular applications of array-based techniques are studies on RNA expression levels in various cell populations, including cancer cells [43, 147]. Arrays printed with cDNA sequences, expressed in different target tissues, and compatible software for analysing the data are commercially available [21]. In addition, close to two million human expressed sequence tags are available due to the massive efforts of the Human Genome Project to sequence the whole human genome, producing a huge resource of human cDNA clones for arraying and screening purposes.

In practice, an array is hybridized with a fluorescent- or radioactively-labelled cDNA probe obtained from test tissue after RNA extraction and reverse transcription. The amount of total RNA required for one hybridization ranges from a few micrograms up to hundreds of micrograms, being in general higher for fluorescent-based detection. A phosphoimager is used for the detection of filter-based arrays and a fluorescence reader for the detection of glass slides. Analysis is performed by software especially designed for this purpose. The rapidly accumulating data from cDNA array studies has made it necessary to establish specific databases containing information obtained from different projects. Recent studies and the future prospects for the utilization of cDNA arrays in cancer research include, besides the molecular profiling of different malignancies, cancer classification based on gene expression patterns thus providing help for diagnostics and distinctions relating to clinical outcomes [63].

AIMS OF THE STUDY

The general aim of this study was to characterize genetic changes in sarcomas using molecular genetic methods. More precisely the aims of the projects were:

- to test the applicability of microsatellite marker analysis for characterizing amplicon structures, and to characterize more precisely the 12q amplicon from liposarcoma samples, previously screened by CGH. (Studies I and II)

- to characterize the 17p amplicon in sarcomas by microsatellite marker analysis. (Study III)

- to study the molecular defect of a large Finnish family with hereditary multiple exostoses. (Study IV)

- to define changes in gene expression in osteosarcoma cell lines and primary tumors. (Study V)

MATERIALS AND METHODS

5. MATERIALS

Studies I and II: Eight liposarcoma samples from seven patients were selected for the microsatellite marker analysis (Table 2). All tumor samples had shown a gain or high-level amplification of the 12q region in previous CGH studies. In addition, one sample consisting 80% of neoplastic B cells from a patient with B-cell chronic lymphocytic leukemia was included as a control. Previous studies had shown that a great proportion of the neoplastic B cells contained trisomy for chromosome 12 [93], and a gain of whole chromosome 12 had been detected in the sample by CGH. Blood obtained from the same patient as the tumor sample was used as the normal reference for the liposarcoma patients. DNA extracted from skin fibroblasts was used as the normal reference for the control patient.

Study III: The material included samples from 11 osteosarcomas, four malignant fibrous histiocytomas, three chondrosarcomas, one parosteal osteosarcoma and one synovial sarcoma (Table 3). All cases had been analyzed previously by CGH. Ten of the cases had shown a gain or high-level amplification of 17p, two a loss of DNA copy number and nine a normal copy number of 17p. Microsatellite marker analysis was performed with 20 specimens. Blood obtained from the same patient as the tumor sample was used as the normal reference for all cases. Five tumor samples including one sample that was not included in the microsatellite marker analysis, all showing a high-level amplification of 17p11-p12 by CGH, were further analyzed by Southern blotting.

Study IV: Linkage and mutation analysis was done using 35 patient samples from a Finnish family with hereditary multiple exostoses including samples from 21 affected and 14 unaffected individuals (Fig. 3). Epstein-Barr virus-transformed lymphoblastoid cell lines were established from the blood samples obtained from one affected family member and from a healthy individual not belonging to the family. Blood cell lysates obtained from 63 healthy individuals, living in the same geographical regions as the analyzed family members, were used as control samples.

Study V: Three osteosarcoma cell lines; IOR/OS9, IOR/OS10 and IOR/OS15, obtained from the Rizzoli Institute (Bologna, Italy), were selected for cDNA microarray analysis. IOR/OS9 was established from a bone metastasis of a high-grade osteosarcoma. The corresponding patient had received pre-operative chemotherapy with adriamycin, cisplatin and methotrexate before the establishment of the cell line. IOR/OS10 was established from a fibroblastic osteosarcoma (grade 4), and OS15 from

an osteoblastic osteosarcoma (grade 4). Patients who gave rise to cell lines IOR/OS10 and IOR/OS15 had not received chemotherapy before establishments of the cell lines. All cell lines were mycoplasma negative. Human nontransformed osteoblasts derived from bone marrow stromal cells were included in the study as a normal reference.

Three primary osteosarcoma samples, obtained from the Rizzoli Institute, were analyzed by RT-PCR to further validate the array data. All samples were high-grade (grade 4), osteoblastic osteosarcomas, and none of the patients had received any chemotherapy before the sample was taken.

Table 2. Sarcoma samples included in study I (samples 1a-3) and study II (samples 1a-7).

Case no.	Histological type (subtype) and grade of the tumor	P/R[1]	Gain or amplification by CGH[2]
1a.[3]	Liposarcoma (dedifferentiated) gr. III	R	+12q/++12q15
1b.[3]	Liposarcoma (well-differentiated) gr. II	R	+12q12-q23/++12q14-q21
2.	Liposarcoma (well-differentiated) gr. I	R	+12q13-q23/++12q14-q21
3.	Liposarcoma (dedifferentiated) gr. III	P	+12q12-q23/++12q13-q22
4.	Liposarcoma (dedifferentiated) gr. IV	P	+12q13-q21
5.	Liposarcoma (well-differentiated) gr. I	R	+12q14-q21
6.	Liposarcoma (well-differentiated) gr. II	P	+12q14-q22
7.[4]	Liposarcoma (well-differentiated, atypical lipoma) gr. I	P	+12cen-q22/++12q14-q21

[1]P, primary tumor; R, recurrent tumor. [2]+ indicates gain in copy number of the marked area that has been obtained by using a cut-off value 1.15, ++ indicates amplification of the marked area that has been obtained by using a cut-off value 1.5. [3]Samples 1a and 1b were obtained from the same patient with an interval of one year. [4]Sample 7 was obtained after chemotherapy and radiotherapy.

Table 3. Sarcoma samples included in study III.

Case no.	Histological type (subtype) and grade of the tumor	P/R/M[1]	Gain, amplification or loss by CGH[2]
1.[3]	Osteosarcoma (osteoblastic) gr. III	P	Normal 17
2.	Osteosarcoma (osteoblastic, with some features of parosteal osteosarcoma) gr. III	P	Normal 17
3.[3]	Osteosarcoma (osteoblastic) gr. IV	P	Normal 17
4.	Osteosarcoma (osteoblastic) gr. IV	P	Normal 17
5.	Parosteal osteosarcoma gr. I	P	Normal 17
6.	Chondrosarcoma gr. II	P	Normal 17
7.	Chondrosarcoma gr. II	R	Normal 17
8.	Malignant fibrous histiocytoma of bone gr. IV	P	Normal 17
9.	Synovial sarcoma (monophasic) gr. IV	M	Normal 17
10.	Osteosarcoma (osteoblastic) gr. IV	P	+17p
11.	Malignant fibrous histiocytoma gr. IV	P	+17p
12.	Malignant fibrous histiocytoma (myxoid) gr. III	P	+17cen-p12
13.	Malignant fibrous histiocytoma (myxoid) gr. II	R	++17p
14.[4]	Osteosarcoma (osteoblastic) gr. III	P	+17p/++17p11.2-p12
15.	Osteosarcoma (osteoblastic) gr. IV	P	+17/++17p11.2-p12
16.	Osteosarcoma (osteoblastic) gr. IV	P	+17p/++17p11.2-p12
17.[3]	Osteosarcoma gr. IV	P	++17p11.2-p12
18a.[5]	Chondrosarcoma (extraskeletal myxoid)	P	+17pter-q12/++17p11.2-p12
18b.[5]	Chondrosarcoma (extraskeletal myxoid)	P	+17pter-q12/++17p11.2-p12
19.	Osteosarcoma (osteoblastic) gr. IV	P	–17p11.2-pter
20.	Osteosarcoma (chondroplastic) gr. IV	P	–17pter-q21

[1]P, primary tumor; R, recurrent tumor; M, metastases. [2]+ indicates gain in copy number of the marked area that has been obtained by using a cut-off value 1.17, ++ indicates amplification of the marked area that has been obtained by using a cut-off value 1.5, – indicates a loss of the marked area. [3]Samples 1, 3, and 17 were taken after chemotherapy. [4]Sample 14 was included in the Southern blotting analysis only. [5]Samples 18a and 18b were obtained from the same patient with an interval of 2 weeks.

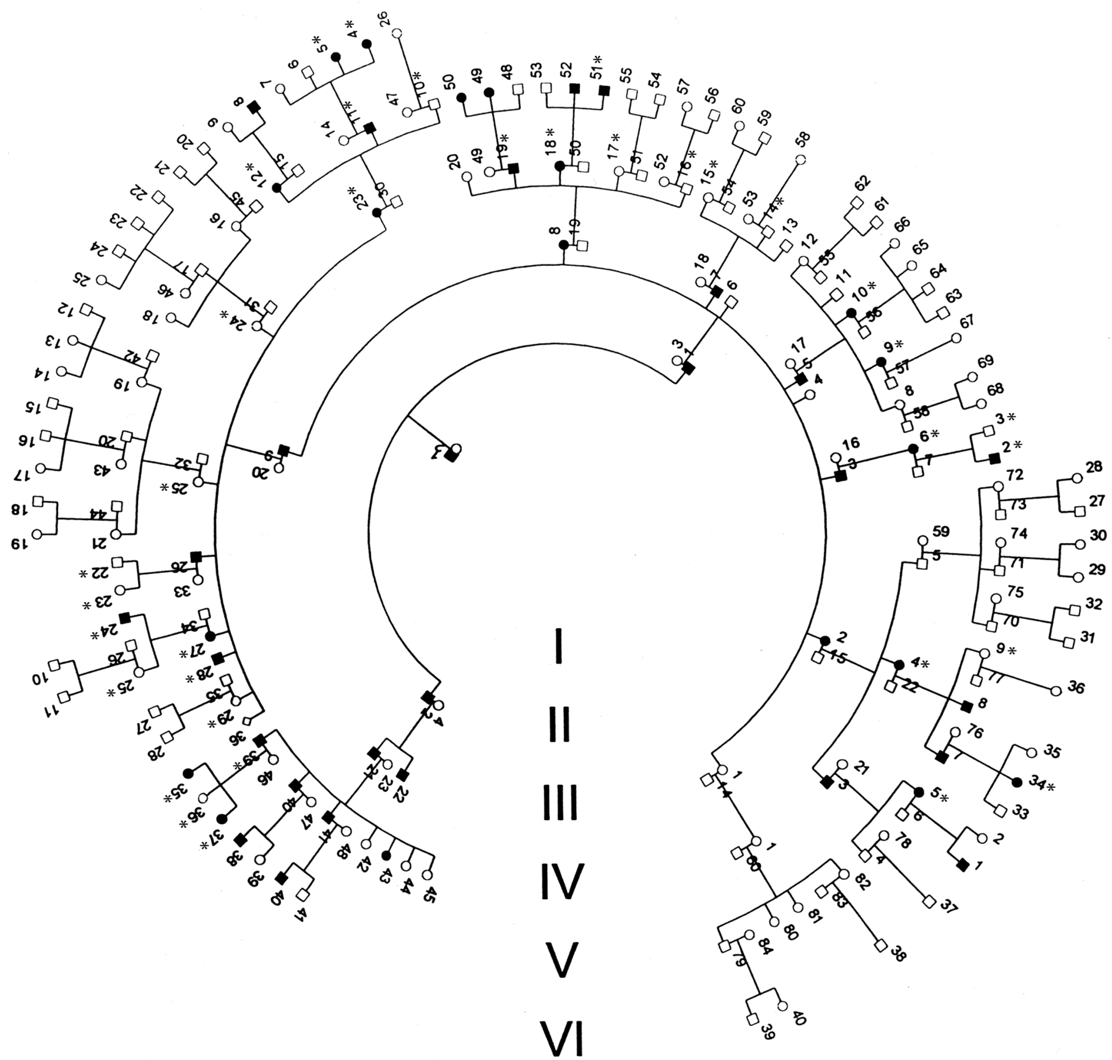

Figure 3. Pedigree of the Finnish family with hereditary multiple exostoses (study IV). Symbols: squares, males; circles, females; filled symbols, affected individuals; empty symbols, unaffected individuals. Roman numerals refer to the generations, Arabic to the individuals. Individuals from whom blood samples were obtained are marked with asterisks.

6. METHODS

6.1. Microsatellite marker analysis (studies I–IV)

Microsatellite marker loci from paired tumor and normal tissue DNA samples obtained from the same patient were analyzed to detect allelic imbalances. The samples were amplified by PCR in the following conditions: 1 x PCR buffer, 1.5 mM $CaCl_2$, 200 μM each dATP, dGTP and dTTP, 2.5 μM dCTP, 0.7 μCi of α-^{32}P-dCTP (3000 Ci/mmol), 30 ng of each primer, 50 ng of DNA, 0.3 U Dynazyme Taq (Finnzymes,

Espoo, Finland) or AmpliTaq polymerase (Perkin Elmer, Norwalk, CT) in a volume of 10 μl. The thermal profile consisted of 27 cycles of 94°C for 30 seconds, 55°C for 75 seconds, 72°C for 15 seconds with an additional stage at 72°C for 6 minutes after the last cycle. Subsequently, the PCR products were subjected to electrophoresis in 6% polyacrylamide gel. After electrophoresis the gels were dried and exposed to Kodak XAR film.

PCR results were evaluated visually by two researchers. Patients were designated uninformative when homozygous for the locus in question and informative when heterozygous. Allelic imbalance (AI) was detected from the heterozygous samples when one of the two alleles in the tumor DNA was either increased (gain in copy number, GCN) or reduced (loss of heterozygosity, LOH) in intensity relative to the remaining allele and those in the lymphocyte DNA. The imbalance was interpreted as LOH or GCN when one allele was close to being totally missing or when a major increase in the intensity of one allele compared to the alleles in the normal tissue was observed. Any change of an unidentified nature and changes that were inconsistent were interpreted as an allelic imbalance. In some cases one of the alleles in the tumor sample showed an increase and the other a decrease in intensity. This situation was acknowledged as a concomitant gain and loss of alleles in studies I and II, but thereafter, due to limitations of the technique, it was considered merely as an allelic imbalance in study III. All the reactions interpreted as GCN, LOH or concomitant gain and loss of alleles (studies I and II) were repeated to confirm the nature of the imbalance.

6.2. Southern blotting analysis (studies I and III)

Southern blotting was used to analyze the copy numbers of probes for genes *MDM2* and *CDK4* (study I) and probes for markers 745R and D17S67 (study III). The studied DNA samples were digested with HindIII (study I) or MspI (study III) and the DNAs were separated on 0.8% agarose gel. After electrophoresis, the products were transferred to nylon filters (Amersham Pharmacia Biotech, Uppsala, Sweden) and hybridized with corresponding probes. Autoradiography was used for detection of the results. The signals were quantified by two-dimensional image densitometry and normalized by calibration with a control probe for apolipoprotein B *(APOB)* located at 2p (study I) or D17S115 located at 17q (study III).

6.3. Linkage analysis (study IV)

Linkage analysis was used to find the corresponding disease locus in the Finnish family with hereditary multiple exostoses. Two-point linkage analysis was carried out using the LINKAGE 5.1 package program MLINK [101]. A dominant form of inheritance

with full penetrance was assumed and the disease frequency set to 1/50 000. Microsatellite markers were selected from each known *EXT* locus using marker allele frequencies as published in the CEPH (Centre d'Etude du Polymorphisme Humain) database (http://www.cephb.fr). At first, markers D8S522, D11S903 and D19S413 from 8q24.1 (*EXT1*), 11p11-p12 (*EXT2*) and 19p (*EXT3*), respectively, were analyzed. After linkage was suggested to 11p *(EXT2)*, it was confirmed by markers D11S1785 and D11S4103.

6.4. Genomic PCR and sequence analysis (study IV)

Genomic PCR and subsequent sequence analysis were used to identify the disease-related mutation of the linked *EXT2* gene from the Finnish family. The coding region and the exon-intron boundaries of the *EXT2* gene were analyzed using the available information of the genomic structure of the gene (GenBank accession nos. U67353-U67368 and U67837) [30]. Primers were designed by using the Primer3 program available at the www-server of the Whitehead Institute (http://www-genome.wi.mit.edu). PCR was performed in 50 μl reactions containing 100 ng of genomic DNA, 1 x PCR buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 μM each primer and 2 U of AmpliTaq Gold polymerase (Perkin Elmer). The thermal profile consisted of 95°C for 10 minutes and thereafter 40 cycles of 95°C for 45 seconds, 55°C for 30 seconds, 72°C for 60 seconds with an additional stage at 72°C for 6 minutes after the last cycle. Following the amplification, the PCR products were subjected to electrophoresis in 1.5% ethidium bromide stained agarose gel. The corresponding band was cut from the gel and purified using the QIAquick gel extraction kit (Qiagen, Chatswoth, CA). An ethidium bromide stained agarose plate was used to estimate the DNA concentrations. 40 or 75 ng of the PCR product, depending on the size of the product, was sequenced using an automated sequencing machine. The sequencing reactions were performed using either ABI PRISM Dye Terminator or ABI PRISM dRhodamine cycle sequencing kits (Perkin Elmer) according to the manufacturer's instructions, and the reactions were run on ABI 373 A or 377 sequencers (Perkin Elmer), respectively.

6.5. Restriction enzyme analysis (study IV)

Restriction enzyme analysis was adopted to screen *EXT2* sequences for the detected DNA sequence variation. A computer-based search using the MAP program in the Wisconsin Package, Genetics Computer Group (Madison, Wisc.) was performed to identify possible added or lost restriction enzyme cleavage sites from the altered sequence. In the case of the detected G to T transversion, the variant allele was shown to have lost a restriction site for the MnlI enzyme. This information was used to screen

63 reference samples for the transversion, in order to exclude the possibility that the change was due to polymorphism. 10 μl of the PCR product obtained by using primers for exon 6 was digested in 30 μl reaction volume with MnlI enzyme, following the instructions given by the manufacturer (BioLabs, Beverly, MA). After digestion, the samples were run in 3% ethidium bromide stained agarose gel.

6.6. cDNA microarray analysis (study V)

cDNA microarray analysis was used to reveal changes in gene expression between osteosarcoma cell lines and normal human osteoblasts. cDNA array filters (GF200, GeneFilters, Research Genetics, Huntsville, AL) containing 5,184 cDNA sequences were used for the hybridizations. Prehybridization was performed with 5-7 ml of Hybrizol I mixture (Oncor) for 3-4 hours while 250 ng of mRNA was labelled with α^{33}P-dCTP according to the supplied protocol. The probe was purified by passage through a Micro Bio-Spin chromatography column (Bio-Rad, Hercules, CA). After purification the probe was denatured and added to the prehybridization mixture. Hybridization was performed overnight at 42°C and thereafter the filters were washed twice at 50°C in 2x SSC, 1% SDS for 20 minutes and once at room temperature in 0.5 x SSC, 1% SDS for 15 minutes. The filters were exposed to imaging plates and after the exposure the plates were scanned with phosphoimager (Fuji, Kanagawa, Japan). Pathways software (Research Genetics) was used for the analysis of the hybridization pictures. Analysis was done by comparing the images obtained using osteosarcoma cells as probes to the image that was obtained using osteoblasts as a probe for hybridization. The average intensity of all control positive data points was used for normalization to avoid problems associated with differences in the efficiency of probe labelling and hybridization.

6.7. Reverse transcriptase polymerase chain reaction (RT-PCR) (studies IV and V)

RT-PCR was adopted for studies on the RNA level; for analyzing alterations in the mRNA transcript of the *EXT2* gene from affected individuals with the detected splice site mutation (study IV) and for confirming the gene expression alterations detected by cDNA microarray analysis (study V). Total RNA or messenger RNA samples were extracted from cell lines and tissue samples using the RNeasy and Oligotex kits (Qiagen), respectively. In study V, total RNA extraction was supplemented with DNase treatment (Qiagen) to avoid contamination with genomic DNA. Reverse transcription was accomplished using 0.8 μg of total RNA or mRNA, with M-MLV reverse transcriptase and RNAse inhibitor in standard conditions (Perkin Elmer) in a total volume of 20 μl. The consecutive PCR was done using 1-5 μl cDNA as template in a

50 μl reaction volume containing 1 x PCR buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.32-0.6 μM each primer and 2 U of AmpliTaq Gold polymerase. In study IV, primers flanking the exon 6 from *EXT2* were used. In study V, two primer pairs were used in each reaction, with the additional primer pair being used for the human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, selected as an internal reference. In addition, the primers were designed to span an intron to avoid problems associated with possible genomic DNA contamination. The thermal profile consisted of 95°C for 10 minutes and after that 40 (study IV) or 23-25 cycles (study V) of 95°C for 45 seconds, 55°C for 30 seconds, 72°C for 90 seconds and 72°C for 6 minutes after the last cycle. After PCR, the amplified products were subjected to electrophoresis in 1.5-2% agarose gel. The ISIS image analysis system (Metasystems) was used to quantitate the results in study V, and the intensity ratios between *GAPDH* and the test genes were determined.

RESULTS

7. MICROSATELLITE MARKERS AS TOOLS FOR THE CHARACTERIZATION OF DNA AMPLIFICATIONS — APPLYING THE METHOD TO STUDY THE 12q AMPLICON (studies I and II)

The applicability of highly polymorphic microsatellite markers for the characterization of DNA amplifications was studied by adopting microsatellite marker analysis to investigate the structure of the 12q amplicon, detected in sarcomas. 55 microsatellite markers, located at the 12q12-q24 region, were analyzed from eight liposarcoma samples from seven patients.

The patients were informative for at least 32 loci. Allelic imbalance (AI) was detected in every sample tested and was interpreted as a gain in copy number (GCN) at one or more loci in six of the eight (75%) samples. Interestingly, AI interpreted as loss of heterozygosity (LOH) was found in five of the eight (63%) samples, all but one of them (sample 2) displaying GCN at other loci. In addition, in five cases, simultaneous amplification of one allele and loss of the other was detected. Samples 1a and 1b, obtained from the same patient at a one-year interval, displayed similar changes by microsatellite marker analysis. In addition, in sample Ib, AI was detected at 11 marker loci, interpreted normal in 1a.

Of the analyzed marker loci, indications of DNA copy number gain were most often detected at locus for D12S335. For that marker, five out of six (83%) informative samples were shown to contain GCN, and in one sample a concomitant gain and loss of the alleles was observed. In samples 1a, 1b, 3, 6, and 7, loci with normal DNA copy number between the amplified marker loci were detected, indicating a discontinuous structure for the studied region.

Southern blotting analysis of probes for *MDM2* and *CDK4* using samples 1a, 1b, 2 and 3 revealed an amplification of both genes in these samples. Compared to the reference probe *(APOB), CDK4* showed 9, 5, 12, and 6 fold copy numbers and *MDM2* 14, 11, 19, and 14 fold copy numbers, respectively.

The chromosome 12 trisomic control sample showed an allelic imbalance at 74% and a gain in copy number at 60% of the informative loci when approximately 80% of the cells had been estimated to contain an extra chromosome 12.

8. CHARACTERIZATION OF AMPLICON STRUCTURE FROM THE 17p REGION BY MICROSATELLITE MARKER ANALYSIS AND SOUTHERN BLOTTING (study III)

Microsatellite marker analysis on nine sarcoma samples showing a gain or high-level amplification of 17p by CGH revealed AI from all but one sample (sample 11) (89%) within the region. In seven of the eight samples (88%), the AI was determined at two or more loci as GCN. Nine cases, detected as normal by CGH, revealed consistent results by microsatellite marker analysis as they showed retained heterozygosity at all informative loci from all samples but one (sample 8), which contained several marker loci displaying imbalances. The discrepant sample was later revealed to be hypertetraploid by flow cytometry and thus the normal CGH result can be regarded as false-negative, since in such cases CGH is not sensitive enough to detect copy number changes. Furthermore, four cases displaying GCN at some of the informative loci were associated with LOH at other loci. In one of the two samples with loss of the 17p region, screened by CGH, only one marker locus with AI was detected, further interpreted as GCN, whereas the other sample contained several marker loci displaying AI, with GCN at one locus.

Samples 18a and 18b displayed identical allelotyping results. This was expected since the two samples were obtained from the same tumor within only a two week interval.

Intragenic marker at *TP53* locus did not display AI with the samples showing normal copy numbers of 17p by CGH. However, all informative samples with copy number gain by CGH showed AI at that locus, further determined as GCN in one sample (sample 13).

Southern blotting analysis revealed an amplification of probes for markers 745R and D17S67 in three out of five samples (60%) (samples 14, 15 and 16). One of the samples (sample 14) was included in the Southern blotting analysis only. The remaining two samples (samples 2 and 4) did not display copy number changes.

9. LINKAGE ANALYSIS AND MUTATION DETECTION OF THE *EXT2* GENE (study IV)

A Finnish family with hereditary multiple exostoses was analyzed for the disease-causing locus and mutation. A two-point LOD score value of 5.43 was obtained with marker D11S1785, revealing linkage to the *EXT2* locus at 11p11-p12 region. No recombination between the marker and the disease phenotype was observed.

Mutation analysis showed a G to T transversion within the conserved 5-prime splice site following exon 6. Furthermore, the change was found to delete a MnlI restriction site. 63 normal reference samples were included in the restriction enzyme analysis, and no individuals with the described transversion were found. RT-PCR revealed an altered transcript in addition to the normal 3.5-kb transcript from a cell line established from an affected individual, but not from a cell line originating from a healthy individual. A shortened transcript was sequenced and shown to be caused by the absence of exon 6.

10. CHANGES IN GENE EXPRESSION IN HUMAN OSTEOSARCOMA CELL LINES AND PRIMARY TUMORS (study V)

Comparison of gene expression profiles between osteosarcoma cell lines and normal human osteoblasts revealed 35 common aberrations for all three cell lines among the 100 most up- and down-regulated genes detected from each cell line. Of these 35, eight were up-regulated and 27 down-regulated when compared to normal osteoblasts. RT-PCR was used to verify the results for four selected genes displaying a clear change by microarray analysis. As suggested by the array results, up-regulation of a gene for heat shock protein 90 beta *(HSP90β)* and down-regulation of genes for fibronectin 1 *(FN1)* and thrombospondin 1 *(THBS1)* was detected in all three cell lines. Furthermore, two of the cell lines displayed up-regulation of a gene for polyadenylate-binding protein-like 1 *(PABPL1)*. Three primary osteosarcoma samples were included in the RT-PCR analysis. The results were the same as those detected from the cell lines. All primary tumors revealed overexpression for HSP90β and underexpression for FN1 and THBS1. In addition, two of the samples showed up-regulation for *PABPL1*.

Previous copy number analysis by CGH on the same osteosarcoma cell lines that were included in the cDNA microarray analysis, had indicated a high-level amplification of the 17p region in these cell lines (unpublished data). Only minor changes were discovered in the expression levels of genes located in that region. The array analysis suggested a modest increase in the expression level of MKK4, EIF4A1 (translation initiation factor 4A isoform 1), and ABR (protein for active BCR-related gene) in at least two of the three cell lines (unpublished data).

DISCUSSION

The studies included in this thesis provide information on the molecular genetic changes in human sarcomas. Studies I, II and III reveal the applicability of highly polymorphic microsatellite markers for the analysis of DNA amplifications, and give new information of the structure of the 12q and 17p amplicons. Furthermore, the genetic background of a Finnish family with hereditary multiple exostoses syndrome is characterized in study IV, and is shown to be caused by a previously unknown splice site mutation of the linked *EXT2* gene. Study V gives evidence of aberrantly expressed genes in human osteosarcoma and indicates their relevance in the tumor pathogenesis. These results will be discussed in more detail below.

11. APPLICABILITY OF MICROSATELLITE MARKER ANALYSIS IN STUDIES ON DNA AMPLIFICATIONS (study II)

Microsatellite marker analysis revealed amplified marker loci from all samples but two (samples 2 and 4) which did, however, display allelic imbalance for one or more loci. The control sample revealed the method to be sensitive enough to detect allelic imbalance at 74% of the informative loci as the studied material consisted of approximately 80% trisomic cells.

Based on these results microsatellite marker analysis can be regarded to be applicable to a more detailed characterization of an amplicon structure. Since PCR is a semiquantitative technique, previous knowledge of the DNA copy number changes obtained with other methods such as CGH is helpful, but not straightforward, in the determination of the nature of the imbalance.

As with conventional loss of heterozygosity-studies, microsatellite marker analysis can be used to narrow down the target region for amplification after preliminary data obtained by other methods, such as CGH. In addition, it gives supplementary information on the amplicon structure and, especially, reveals discontinuities in the amplification status of an area, which, due to differences in the resolutions of methods, often appear as a single amplicon in CGH analyses.

12. STRUCTURE OF THE 12q AND 17p AMPLICONS IN SARCOMAS

12.1. Complexity of the 12q amplicon in liposarcoma (studies I and II)

All samples that were analyzed by microsatellite marker analysis had shown a DNA copy number gain or a high-level amplification in the long arm of chromosome 12 using CGH. The red-to-green ratio profiles, depicted according to CGH analysis, implicated one large amplicon in the area. However, microsatellite marker analysis suggested a more complex structure of the region including DNA sequences with normal copy numbers located between amplified segments. Although the reason could be related to the insufficient proportion of tumor cells in the sample, results revealing discontinuity in the 12q13-q15 area, obtained by using Southern blotting analysis and fluorescence *in situ* hybridization (FISH), were reported at the same time, thus supporting our findings. The studies revealed coamplification of separate regions harboring genes for *MDM2* in one amplified segment, and *CDK4* and *SAS* in another, with no or reduced amplification of the intervening sequences [14, 45]. Similar reports on discontinuous amplicon structures have been made from the 11q13 area. This region is amplified in a variety of tumors and has been shown to contain discrete amplification units [60]. In addition, the 20q amplification, a common aberration in breast cancer, has been shown to underlie at least three independent regions as targets for DNA amplification [168]. A putative explanation for discontinuous amplicon structures is the exclusion of non-amplified sequences in the amplification process. Alternatively, the discontinuity could be due to simultaneous activation through coamplification of multiple genes important in tumorigenesis, promoting cell cycle progression in a synergistic fashion.

The complex structure of the 12q amplicon was further characterized by losses of DNA sequences. Support for this observation was obtained from a deletion mapping study on malignant glioma samples by Reifenberger and collaborators. They reported 50% of the studied tumor samples, containing amplification of the 12q13-q14 region, to demonstrate LOH at loci proximal or distal to the amplified loci, and to be more frequent in those samples with 12q13-q14 amplification than in the tumors without amplification [136]. The reason for this may be one or more potential tumor suppressor genes located in the area. Another and perhaps a more likely explanation for deletions accompanying amplified loci is the mechanism for DNA amplification. As described earlier, most models for amplification include chromosomal breakages and deletions of DNA sequences [192].

Furthermore, putative loci with concomitant gain and loss of alleles were observed from the 12q amplicon. However, due to the limitations of the PCR technique in obtaining quantitative results, these loci should be studied further with other, more quantitative methods. The possible existence of loci at 12q with loss of one allele and concomitant amplification of the other still remains and raises questions for the putative

biological functions of such loci. Allelotyping and cytogenetic studies have suggested the gain of one allele and loss of the other as a mechanism to unmask recessive mutations contributing to the inactivation of a tumor suppressor gene by mitotic recombination and loss of the wild type allele followed by duplication of the remaining mutant allele [27, 40]. On the other hand, a similar phenomenon is observed at loci displaying uniparental disomy, with two copies of one allele originating from one parent and none from the other. The detection of regions displaying uniparental disomy can reveal putative loci for genes, whose expression is under normal circumstances regulated by imprinting. Genomic imprinting characterizes an epigenetic form of gene regulation, whereby only the paternally or maternally inherited copy of the gene is functional. In addition to embryogenesis and behavioral development, abnormally imprinted genes have been shown to play a role in tumorigenesis [78, 81]. Whether our observation made on the 12q region were based on the technique that was used, or underlies a real biological function, like putative loci for tumor suppressor genes or genes that are regulated by imprinting, requires further research with other techniques.

12.2. The 17p amplicon in sarcomas (studies III and V)

Amplification of the 17p11-p12 in sarcomas has implicated a putative proto-oncogene in the region, which may be involved in the malignant transformation of connective tissues, especially into osteosarcoma and leiomyosarcoma. In order to characterize in more detail the structure of the amplicon, sarcoma specimens showing a gain or high-level amplification of 17p11-p12, detected by CGH, were studied by microsatellite marker analysis.

Allelotyping results indicate the involvement of a large region with a complex structure denoted by frequent association of amplification with losses of DNA sequences of nearby loci. Similar results have been obtained from astrocytoma samples showing amplification of the 17p12 region, where LOH was detected at loci proximal to, or flanking an amplified region [73]. In addition, three samples (8, 12, 13) displayed loci with retained heterozygosity between amplified loci, suggesting a discontinuous structure for the amplicon.

Southern blotting analysis revealed amplification of markers 745R and D17S67 in three samples showing an increase in the DNA copy number of the corresponding region by CGH, and normal copy number in two samples with normal DNA copy number of 17p by CGH. The results were consistent with the results obtained from the microsatellite marker analysis.

We have recently studied further the physical structure of the 17p amplification by FISH from human osteosarcoma cell lines showing a high-level amplification of 17p11-p12 by CGH. Our results using yeast artificial chromosomes (YACs) as probes, suggest similarly to the microsatellite marker analysis, amplification of a large area,

with a slight increase in the relative DNA copy number of YACs 961f10-916b4, covering approximately 11 Mbs (unpublished results). In addition, we have used three BAC clones as probes to examine the DNA copy numbers of *TOP3, EFNB3* and *MKK4* in one of the cell lines (IOR/OS9). The relative copy numbers, which take into account the chromosome 17 centromere copy numbers, were moderately low, indicating that they are probably less significant in the tumorigenesis (unpublished results).

As mentioned above, identification of the target gene for DNA amplification is difficult due to the involvement of a large area including, besides the actual driver gene, other nearby genes in the amplicon. This was also the case in our studies; based on microsatellite marker analysis, pointing out clearly the target region for DNA amplification on chromosome 17p11-p12 was not possible. Microsatellite marker analysis revealed DNA copy number changes in a large area, with no clear clustering of loci displaying a gain in copy number, and only a slight increase in the relative copy number of an 11 Mbs area has been suggested by our recent FISH studies.

cDNA microarray analysis on osteosarcoma cell lines did not suggest any clear aberrations in the expression of the 17p mapped genes spotted on the filter. This is most probably due to the limited number of target transcripts included in the array used.

In order to reveal the core of the amplification and most importantly, the target gene(s) itself, the region needs to be studied further. Recently, a putative locus for bone-specific telopeptide lysyl hydroxylase *(TLH)* was reported to map between markers D17S969 and D17S2196 (18 cM) at 17p12 [7]. The gene was found to be defective in Bruck syndrome patients, characterized by fragile bones, osteoporosis and short stature due to a reduction in the mineral content and increase in size of the hydroxyapatite crystals of their bones. The suggested role of *TLH* is involved in the regulation of collagen 1 crosslinking, which is important for correct bone mineralization. Further studies will show whether amplification of *TLH* has a role in tumorigenesis and whether the 17p amplification in various histological tumor types is driven by the same target gene(s).

13. GENETIC BACKGROUND OF A FINNISH FAMILY WITH HEREDITARY MULTIPLE EXOSTOSES (study IV)

Linkage to and a G to T transversion in a conserved splice site of the *EXT2* gene was found in a Finnish family with HME. The DNA alteration was found to cause aberrant splicing and mutated transcript due to skipping of exon 6, leading to a change in the reading frame and introducing a premature stop codon. This finding is in concordance

with other reported *EXT2* mutations. At the time of writing, 12 other *EXT2* mutations have been identified. Only one of the known *EXT2* mutations is a missense mutation, causing an amino acid change. Most of the described mutations in the *EXT1* and *EXT2* genes are either nonsense mutations, frameshifts or splice site aberrations, suggesting that a majority of the mutations lead to truncated protein products.

Four of the 46 (8.7%) family members affected with HME had by the autumn of 1997 developed chondrosarcoma, suggesting a slightly higher frequency for malignant transformation than indicated in other studies (0.9%-2.9%) [88]. However, the limited number of affected individuals in this family does not allow us to make any statistical conclusions, rather, it remains to be seen whether some mutations associate with a more frequent transformation into a malignant tumor than others.

Microsatellite marker analysis was performed on a tumor sample obtained from one family member with malignant degeneration into chondrosarcoma (unpublished data). LOH at marker D11S1785 locus, linked to *EXT2,* was observed. This data is consistent with previous studies on sporadic and exostosis-derived tumor samples, displaying LOH at loci linked to the *EXT* genes, suggestive of their role as tumor suppressors.

The mechanism of cancer development in families with hereditary multiple exostoses has been proposed to occur according to the Knudson's "two-hit hypothesis", and thus resemble other cancer predisposition syndromes. In addition to the inherited germ line mutation, inactivation of the wild type allele is required for the development of benign exostoses [20]. After other acquired genetic alterations, neoplastic proliferation can proceed into malignant chondrosarcoma or osteosarcoma. Since the clinical symptoms are usually evident already during the first decade of life, genetic screening for germ line mutations in HME families does not seem necessary for diagnostic purposes. However, it is important that family members with exostoses are informed and their exostoses followed for possible malignant growth, since the early detection of malignancy is associated with a significantly improved prognosis.

14. ABERRANT GENE EXPRESSION IN HUMAN OSTEOSARCOMA (study V)

Cell lines are important tools for *in vitro* studies of neoplasms. In order to investigate changes in gene expression that could be of importance in the pathogenesis of human osteosarcoma, three established human osteosarcoma cell lines were used as a model system for cDNA microarray analysis.

Several changes in the gene expression levels were detected, compared to normal human osteoblasts, by screening the cell lines with a cDNA microarray. The RT-PCR performed on four selected genes, based on the array results, were concordant, suggesting that at least the most striking changes in the RNA levels, detected by the cDNA microarray, were real findings. Three primary tumors included in the RT-PCR analysis further validate the significance of the obtained data for these genes. One of the clear aberrations observed from all the studied cell lines and primary tumor samples was the overexpression of heat shock protein 90β. By acting as a molecular chaperone, HSP90β binds to the exposed hydrophobic regions of a misfolded protein and helps the protein to refold. In addition, HSP90 molecules have been implicated in the regulation of tubulin assembly / disassembly and have been shown to prevent the progression of cellular division by inhibiting tubulin polymerization [59]. In addition to heat stimulated cells, overexpression of HSP90α, another member of the HSP90 family, has been reported to associate with poor prognosis in human breast cancer tissue [116]. Interestingly, in osteosarcoma, the overexpression of a related heat shock protein, HSP27, has also been reported to associate with poor prognosis [177]. Our findings further support these previous implications of the role of heat shock proteins in tumorigenesis, and as a novel finding, the overexpression of HSP90β in osteosarcoma.

The up-regulation of the polyadenylate-binding protein-like 1 gene was observed in two of the three cell lines and patient samples. The protein product of this gene is known to be involved in the regulation of translation and stability of mRNA molecules, and high levels of the protein have been found from actively growing HeLa cells [65]. Although, it must be regarded possible that our observation of the overexpression of PABPL1 in osteosarcoma cell lines is, as such, related to actively growing cell cultures, as the reference sample did not represent a cell line, results obtained from the primary tumors indicate that it may also have a role in tumorigenesis. No previous data on the up-regulation of *PABPL1* in osteosarcoma exists.

Underexpression of glycoproteins fibronectin 1 and thrombospondin 1 was observed from all three cell lines and primary tumors. FN1 has been shown to be involved in cell adhesion, cell motility, wound healing and the maintenance of cell shape, and similarly, THBS1 in cell-to-cell and cell-to-matrix interactions. The loss of function of these genes has been previously implicated in malignant transformation and metastasis, supporting our findings of the role of these genes in osteosarcoma tumorigenesis [36, 61, 96, 185].

SUMMARY AND CONCLUSIONS

The aim of this thesis was to identify genetic aberrations in sarcomas using molecular genetic methods. Special attention was paid to obtaining more detailed information of the structures of DNA amplifications at the 12q and 17p regions first detected by other methods, particularly comparative genomic hybridization (CGH). CGH is a modern, genome-wide screening method used for the analysis of DNA copy number changes. It is highly effective, revealing gains and losses of DNA sequences from a tumor sample in one single hybridization. However, the obtained results give only an approximate cytogenetic location for the aberration, and therefore other methods are required to reveal the exact position of the defect, and most importantly, to define the gene(s) in question.

Studies I and II tested the applicability of highly polymorphic microsatellite markers for the analysis of amplicon structures. Our results revealed the method to be sensitive enough for further characterization of DNA amplifications after preliminary screening by CGH. Application of the method for the analysis of the 12q amplicon, detected in liposarcoma, gave additional information about the structure of the region, including discontinuity of the amplification and losses of DNA sequences. Similar results of the complexity of the 12q amplicon have been obtained also by other researchers, thus supporting our findings. In addition to the region harboring the main candidate genes, amplification of a more distal region was observed. This finding is in concordance with a previous CGH study showing two chromosome 12-derived amplicons in well-differentiated liposarcoma, one at 12q14-q15 and another at 12q21.3-q22 [163].

Amplification of the 17p11-p12 region has been reported frequently in sarcomas, especially in osteosarcoma and leiomyosarcoma. In study III, the aim was to determine in detail the amplicon structure by using highly polymorphic microsatellite markers and to test marker sequences that have previously been found amplified in astrocytoma and osteosarcoma. Microsatellite marker analysis revealed the involvement of a large region and the association of losses of DNA sequences at nearby loci. Based on the obtained results, we were not able to narrow down the target region for amplification. Furthermore, the cDNA microarray technique, which was adopted to screen for aberrantly expressed genes in three human osteosarcoma cell lines in study V, implicated only modest up-regulation for the 17p-mapped genes that were included in the array. All the cell lines had been screened by CGH, and had shown a high-level amplification of the 17p region. The future prospects for revealing the target gene(s) on

the 17p11-p12 region should include more efficient cloning methods together with specific arrays containing the known transcripts from this region.

In study IV, we characterized the molecular defect of a Finnish family with hereditary multiple exostoses syndrome. Linkage and mutation analysis revealed a previously unknown splice site mutation leading to a truncated protein product of the *EXT2* gene. Loss of heterozygosity was observed from a chondrosarcoma sample obtained from one affected family member, supporting the previously published data.

In study V, the cDNA microarray analysis was used to reveal aberrations in gene expression in human osteosarcoma cell lines, and the obtained data was further validated from primary tumors by using RT-PCR. A variety of changes in gene expression were detected from the cell lines when compared to normal human osteoblasts, some of which were more common than others. The expression levels of genes for heat shock protein 90β *(HSP90β),* polyadenylate-binding protein *(PABPLI1),* fibronectin *(FN1)* and thrombospondin *(THBS1),* were analyzed from primary tumors and similar aberrations as in the cell line material were observed. Future studies with additional primary tumor samples will reveal whether the abnormal expression of these genes is specific for the development and / or progression of osteosarcoma, or related to tumorigenesis in general.

Defining the molecular background of sarcomas, including the target gene(s) for DNA copy number changes, has been proceeded by the development of new molecular and molecular cytogenetic methods and their applications. Especially, recent applications of the array technology have important roles in revealing the specific gene expression profiles in different histological tumor types. The results presented in this thesis provide, in addition to new data, also information for future studies, which are essential for obtaining a more complete knowledge of the DNA copy number aberrations, gene expression and protein levels in sarcomas. Identification of specific genetic changes in sarcomas is an important step in the development of more effective tools for diagnosis. In addition, new information will increase the number of distinctive markers for tumor progression and provides new molecular targets for more efficient cancer therapy.

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