Amplification and overexpression of genes in chromosome region 17p1.2-p12 in human osteocarcinoma
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Chapter 1:

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Chapter 1

1 Cancer and genetics

1.1 Introduction

In normal adult tissue there is a strict balance between cell division, differentiation and death, so that the number of cells in a particular tissue or organ does not change. Disruption of this balance, by increased cell proliferation or decreased cell death, leads to growth of cell mass and tumor development. There is overwhelming evidence that the disordered cell proliferation in cancer is initiated by genetic changes. For instance, almost all carcinogens induce mutations, chromosome instability is observed in many types of cancer, and deficiency in enzymes necessary to repair lesions in DNA is associated with increased risk of cancer. As cancer is characterized by growth of cell mass, it is evident that genetic changes associated with tumor development usually affect genes that control cell division or cell death.

There are two mutational routes towards tumor development. The first involves the inactivation of tumor suppressor genes. The gene products of tumor suppressor genes inhibit events leading toward cancer as they prevent cell cycle progression, stimulate cells to die by apoptosis, or prevent mutations by ensuring accurate replication and repair of DNA damage. Both alleles of a tumor suppressor gene must be inactivated to change the biological behavior of a cell. The second mutational route involves the activation of proto-oncogenes, encoding proteins whose normal function is to initiate cellular responses to external signals that elicit cell growth. Hyperactive proto-oncogenes can induce constitutive activation of signal pathways that stimulate cell proliferation without the presence of the correct external signal. Genetic changes by which proto-oncogenes are converted into oncogenes, involve aberrations by which the function of the gene is amplified. This can be caused by the production of a hyperactive gene product or by an increased production of an unaltered gene product. There are three types of chromosomal changes that can lead to oncogene activation: point mutation, chromosomal translocation, and gene amplification. All the changes are dominant and affect usually only one allele of the gene. (Tannock, 1992; Strachan and Read, 1999).
1.2 Genetic changes and oncogene activation

**Point mutations**

In the case of a point mutation, a single nucleotide is replaced for another nucleotide, which may lead to a change in amino acid sequence of the encoded protein. In the case the alteration occurs in a nonessential region of the protein, the mutation can exist without consequence. However, when the mutation occurs in an essential part of the protein, it can lead to inactivation but also to hyperactivation of the protein. In cancer, inactivating point mutations are found in tumor suppressor genes whereas activating point mutations are found in oncogenes. A family of proto-oncogenes that is frequently found to harbor an activating mutation in human tumors is that of the RAS genes. The RAS proteins are involved in the signal transduction pathways involved in cell growth and differentiation. The RAS proteins become activated upon external stimuli, by binding of a GTP, after which it transmits the signal onwards in the cell. Through the GTP-ase activity of RAS proteins, active GTP-RAS is rapidly converted into inactive GDP-RAS. Mutated RAS proteins have lost their GTPase activity, by which it is slowly inactivated and the cellular responses to the external stimuli are extended (Bos et al. 1989). Point mutations in RAS genes are found in many different tumors including colon, lung, breast and bladder.

Most point mutations arise during DNA replication due to copy errors of DNA polymerases. Although the error rate of DNA polymerases is significantly reduced by its ability to proofread a newly synthesized DNA strand for copy errors, replication errors resulting in mismatched bases pairs occur frequently. Correction of the replication errors by the mismatch repair system (MMR) is important for the maintenance of the genome stability.

Defects in MMR lead to genetic instability (Kolodner et al. 1995; Lengauer et al. 1998). Although MMR dysfunction not directly enhances cell growth, it results in an accumulation of mutations in oncogenes and tumor suppressor genes by which it indirectly stimulates tumor development (Lengauer et al. 1998). Therefore, genes encoding proteins involved in MMR themselves may act as tumor suppressor genes. Inactivation, of both alleles of these genes by deletion or mutation may stimulate tumor development. Examples of genes encoding proteins involved in MMR that have a tumor suppression function are MSH2 and MLH1. Mutations in these genes are found in families with hereditary nonpolyposis colon cancer (HNPPC) (Leach et al. 1993; Papadopoulos et al. 1994), whose members develop most commonly colon cancer on an early age (Kolodner, 1995). The tumors of HNPPC patients contain frequent mutations in microsatellite repeat sequences like (CA)$_n$ or (A)$_n$, which is characteristic for mismatch repair deficiency (Modrich, 1997).
Translocations

In the case of chromosomal translocation, chromosomal regions of two different chromosomes are exchanged, which leads to a rearrangement of DNA segments. Activation of oncogenes by translocation can occur when, as a consequence of the translocation, a hyperactive fusion gene appears. This is the case with the translocation between chromosomes 9 and 22, which is frequently detected in myeloid leukemia patients. This translocation leads to a fusion between the ABL oncogene on chromosome 9 and the BCR gene on chromosome 22, resulting in the production of a new protein with enhanced tyrosine kinase activity, and abnormal capacities to transform normal cells into tumor cells (de Klein et al, 1982). Alternatively, chromosomal translocation can lead to oncogene activation when due to the translocation the expression regulation of a proto-oncogene is altered. This is seen in translocations involving the C-MYC proto-oncogene. In almost all Burkitt’s lymphomas the C-MYC gene is translocated to an immunoglobulin gene. Normally, the transcription of C-MYC is tightly regulated. In the case of C-MYC translocation to an immunoglobulin locus, its transcription is influenced by the regulatory elements of highly expressed immunoglobulin genes, resulting in an increase of C-MYC expression. Deregulation of C-MYC expression is thought to play a causal role in the development of lymphomas (Boxer and Dang, 2001).

Translocation may appear as consequence of double strand breaks, caused by the inevitable exposure of DNA to damaging agents of exogenous sources, such as ionizing radiation and certain chemicals, or of endogenous sources, such as free radicals generated during essential metabolic processes. Repair of double strand breaks is difficult, as in this type of DNA damage both strands of the double helix are affected, and by that no complementary DNA strand, which could be used as template, is available. There are two main pathways for DNA repair of double strand breaks: homologous recombination (HR) and non-homologous-end-joining (NHEJ) (Khanna and Jackson, 2001; van Gent et al. 2001). HR can repair double strand breaks by using the undamaged sister chromatid of the homologous chromosome as a template, usually resulting in accurate repair of the double strand break. Upon double strand breaks, the repair response is initiated by ATM protein. Incorrect initiation of the repair systems is seen in patients with ataxia telangiectasia (AT), who have a mutation in the ATM gene. AT disorder is characterized by a high incidence of chromosomal translocations and the patients are predisposed to cancer (Rotman and Shiloh, 1998). After initiation, HR is carried out by a group of proteins including NBS1, MRE11 and various RAD proteins like RAD51 and RAD52. Deletion of RAD51 in mouse embryonic cells is lethal (Lim and Hastly, 1996; Tsuzuki et al.1996). Mice lacking another RAD protein, RAD54, are viable and show no increase of spontaneous tumor development. However, RAD54-deficient embryonic stem (ES) cells and neonatal animals are hypersensitive to double strand break-inducing agents and have defects in HR (Essers et al, 1997). In human, mutations of RAD54 have been observed in different types of cancer like lymphoma, colon cancer and breast cancer suggesting a causative correlation (Matsuda et al, 1999; Hiramoto, 1999). Although the mechanisms are still unknown, besides the RAD proteins also the breast-
cancer-susceptibility proteins BRCA1 and BRCA2 are involved in HR. BRCA1- and BRCA2- deficient cells are hypersensitive for DNA-damaging agents and develop spontaneous chromosomal aberrations. Moreover, mutations in these genes are found in familial breast tumors, suggesting that a defect of HR can be at least partly responsible for the induction of breast cancer (Welcsh et al. 2000).

In the absence of a sister chromatid during the G1 phase of the cell cycle or when the HR system does not function properly, double strand breaks are repaired by NHEJ (Karran, 2000). In the case of NHEJ two broken chromatid ends are joined together without the use of an undamaged sister chromatid or other homologous sequences. As a result, NHEJ is, in contrast to HR, error prone. NHEJ is not only used for the repair of double strand DNA breaks caused by DNA damaging agents, but also in V(D)J recombination during differentiation of lymphocytes. The key component of NHEJ is the DNA-dependent protein kinase (DNA-PK) encoded by the genes KU70 and KU80. Mice with deficiencies in the NHEJ system have an impaired development of their immune system, are hypersensitive to ionizing radiation and have a high incidence of lymphomas (Smith and Jackson, 1999).

**Amplifications**

Oncogene activation by DNA amplification is caused by the generation of extra copies of a proto-oncogene, resulting in increased expression of that gene. The amplified DNA segments are usually several hundred kilo-bases in size and contain many genes. DNA amplification can be involved in the development and progression of cancer, when at least one of the genes located in the amplified region becomes overexpressed. Furthermore, overexpression of the amplified gene should result in a selective advantage for tumor cells like an increase of proliferation or a resistance to drug.

Regions of DNA amplification can exist as small separated chromosomes (double minutes), or integrated in normal chromosomes forming a homogeneously staining region (HSR) (Schwab, 1999). Cells of neuroblastoma often contain HSRs that harbor sometimes hundreds of extra copies of the proto-oncogene MYCN (Schwab et al. 1983).
1.3 Mechanisms of gene amplification

Broadly, two models have been proposed to explain the mechanism through which amplifications are generated. The first model suggests the involvement of breakage-fusion-bridge (BFB) cycles in the amplification of human oncogenes (Coquelle et al., 1997; Debatisse et al., 1998; Smith et al., 1992). In this model, DNA amplification is triggered by double strand breakage (or telomere dysfunction) during mitosis (see figure 1).

![Diagram of gene amplification via breakage-fusion-bridge (BFB) cycles](image)

Fig. 1 Scheme illustrating gene amplification via breakage-fusion-bridge (BFB) cycles. Oncogenes, yellow triangles; telomeres, orange (p-arm) or black (q-arm) circles; centromeres, orange rectangles. A. Interphase- an initial break gives rise to an uncapped chromatid. B. Metaphase- fusion of the 2 uncapped sister chromatids results in a dicentric chromosome. C. Anaphase-the dicentric chromosome forms a bridge between the opposite poles. A break of this chromosome at a fragile site leaves one daughter cell with 3 copies of the oncogene and only one copy in the other cell. Under selection for the oncogene, recurrent BFB cycles will occur, resulting in many copies of the oncogene in inverted orientation. (Taken from Hellman et al., 2002)

Repair of the double stand breakage could result in fusion of sister chromatids, forming dicentric ring chromosomes. During anaphase of the mitosis, fused chromatids form a bridge between the opposite poles. If this structure breaks asymmetrically, the daughter cell will receive either a chromatid with duplication of the oncogene or a chromatid with deletion of the gene. Most often the anaphase bridge breaks at common fragile sites (CFS), which are regions in mammalian chromosomes that are prone to breakage and rearrangements. Several recurrent cycles of chromosomal fusion and breakage under appropriate selection would eventually lead to DNA amplification with a ladder-like structure and inverted repeat organization of the amplified segment. In vivo, BFB cycles proved to drive amplification of the MET oncogene in human gastric carcinoma (Hellman et al., 2002). In different solid cancers with high intra-tumor genetic heterogeneity, anaphase bridges are detected, indicating that BFB cycles might be involved in the generation of genetic instability including DNA amplification (Gisselsson et al, 1999; Gisselsson et al, 2002).
The other mechanism that may be involved in DNA amplification events is unequal but homologous recombination (Smith et al., 1990). During mitosis, sister chromatids may become unequally paired due to the presence of repeating homologous DNA sequences. The misalignment of the sister chromatids may lead to unequal but homologous sister chromatid exchange causing deletion of the exchanged DNA sequence in one of the participating chromatids and duplication of the same DNA sequence in the other participating chromatid. Repeating cycles of unequal sister chromatid exchange could result in high-level amplification. Repeats in amplicons arising by unequal sister chromatid exchange would have a direct tandem arrangement.
2 Methods of genetic analysis in cancer research

2.1 DNA analysis

Polymerase chain reaction

The Polymerase chain reaction (PCR) has many applications in the analysis of DNA. In this technique, a selective DNA sequence is amplified by the DNA polymerase enzyme Taq (Saiki et al., 1988). For selective DNA amplification, two oligonucleotide primers flanking the DNA segment to be amplified are used. Repeating cycles of DNA denaturation, annealing of the primers to complementary DNA sequences and synthesis of new DNA strands by Taq polymerase, can provide selective DNA amplification by a factor of $10^6$.

By selective amplification of DNA sequences containing polymorphic micro-satellite markers, which are short tandem repeated sequences from one to four nucleotides, allelic variations in the genome can be determined. In tumor biology, this allelic variation of micro-satellite markers is used for determination of DNA deletions (Weber et al., 1990). Due to the highly polymorphic character of micro-satellite markers, each person is heterozygote for many of the micro-satellite markers. In tumors, deletion of a DNA segment causes loss of heterozygosity (LOH) of the micro-satellite markers located in the deleted region. LOH of micro-satellite markers can be visualized by PCR followed by separation of the PCR product by electrophoresis. The PCR technique can also be used for the determination of DNA amplification. In this application of PCR it is presupposed that the amount of product synthesized during the PCR cycles correlates with the amount of template at the start of the amplification reaction.

Fluorescence in situ hybridization

Many studies of genetic aberrations use the technique of fluorescence in situ hybridization (FISH). The technique is simple: a specific DNA sequence (probe) is labeled with a fluorescent molecule. The labeled probe and metaphase or interphase target chromosomes are denatured. The fluorescent probe and the target chromosomes are incubated together, allowing complementary sequences to anneal. The probe will hybridize to its complementary DNA sequence at the target chromosomes. The location in the target chromosomes, at which the probe is hybridized, can be visualized using a fluorescence microscope. Simultaneous hybridization of different multiple color fluorescent probes makes it possible to determine the position of DNA sequences relative to each other. For example, by simultaneous hybridization of different colored chromosome specific centromere probes with a probe of unique DNA sequence, one can determine at which chromosome the DNA sequence of the probe is located (Trask, 1991; Van Ommen et al., 1995). In cancer research, FISH is used for the detection of DNA deletions, amplifications and translocations.
Comparative genomic hybridization

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique, by which the total genome is screened for copy-number changes, providing a map of the chromosomal regions that contain deletions or amplifications (Kallioniemi et al., 1992). In tumor research, it is widely used to identify chromosomal regions that may contain novel tumor suppressor genes or oncogenes.

CGH is based on modified in situ hybridization, by which equal amounts of tumor DNA and normal reference DNA are labeled with different colored fluorescent dyes and co-hybridized to normal metaphase spreads. Copy number difference between tumor and normal reference DNA can be detected due to unequal emission of the fluorescent dyes on the metaphase chromosomes. DNA deletions are seen as regions with a relatively increased intensity of the fluorescent dye used to label normal DNA, while amplifications are seen as regions with a relatively increased intensity of the fluorescent dye used to label tumor DNA (Kallioniemi et al., 1992; Kallioniemi et al., 1995; Forozan et al., 1997). By CGH, single copy deletions can be detected when the size of the deleted region is in the range of 10-20 Mbp (Bentz et al., 1998). The resolution of CGH for high copy number amplification is such that the amplified segment must be at least 2 Mbp (Joos et al., 1995).

Array comparative genomic hybridization

Classic CGH enables the screening of the total genome for amplifications and deletions. However, due to the limited resolution of this technique, copy number changes that involve small chromosomal regions cannot be detected by CGH. This limitation of CGH can be resolved by the use of array-CGH, by which tumor DNA and reference normal DNA, labeled with two different fluorescent dyes, are not hybridized to metaphase chromosomes but to sequenced and mapped DNA segments, spotted on a glass slide. The human DNA segments are mostly isolated from bacterial artificial chromosome (BAC) clones containing large human DNA inserts. Like in classic CGH, amplifications and deletions of DNA are detected by differences in emission of the fluorescent dyes hybridized to a specific clone. The resolution of a clone-based CGH, is determined by the chromosomal distance of the spotted DNA sequences or the length of the DNA segments in the spotted clones (Pinkel et al., 1998). Array-CGH can be used to screen the whole genome or specific chromosomes for DNA copy number changes. A genome-wide array with an average resolution of 1.3 Mb has been constructed by Snijders et al. (2001). Buckley et al. (2002) constructed a chromosome-specific array, which covers 95% of chromosome 22 and has an average resolution of about 75 kb. This chromosome 22 array is an important tool to identify tumor genes involved in cancers that are associated with chromosome 22 such as glioblastoma, ependymoma, meningioma, breast and colon cancer.
2.2 RNA analysis

Microarray

Large-scale analysis of gene expression became possible by the introduction of the microarray technology (Schena et al., 1995). Microarrays are glass slides on which unique DNA sequences of known and predicted genes are printed. The printed DNA sequences are cDNA inserts of IMAGE clones synthesized by PCR or oligonucleotides. One microarray may contain thousands of different DNA sequences. For simultaneous analysis of the relative expression of each printed DNA sequence, total RNA is isolated from two samples, for instance from tumor and from corresponding normal tissue. Of both RNA samples cDNA is made, which is labeled using different fluorescent dyes for each of the two samples. The labeled cDNA samples are mixed and co-hybridized to a microarray. After hybridization, fluorescence measurements are made with a scanner that illuminates each DNA spot and measures the intensity of fluorescence for each dye separately. These intensities are used to determine for each spot at which ratio tumor cDNA and normal tissue cDNA are hybridized to the DNA sequence. This hybridization ratio between cDNA of the tumor and cDNA of normal tissue correlates with the relative abundance of the spotted DNA sequence, and by that with the relative expression of the gene represented by the DNA sequence (Brown et al. 1999).

Macroarrays are an alternative for microarrays that can be analyzed by conventional radioactive procedures. In the macroarray procedure, cDNA inserts of IMAGE clones are spotted onto nylon filters (Cox, 2001) and the cDNA probes used for the hybridization are radioactively labeled. The cDNA probes of normal tissue and tumor sample are hybridized to separate filters. It is not possible, as in the microarray procedure, to directly compare the hybridization intensity of the two probes for each spot and, in this way, determine their relative expression levels. Instead, one has to correct for each sample the measured hybridization intensities for differences in hybridization and labeling efficiency, using house-keeping genes that are supposed to have the same expression levels in all samples.

Real time PCR

Real time PCR enables quantification of expression levels of individual genes using gene-specific primers. For this technique total RNA is isolated from tumor and normal tissue samples, and converted into cDNA. The cDNAs are used as template for gene-specific real time PCR (Higuchi et al, 1992). During real time PCR, the amount of DNA present in the reaction volume is measured after each PCR cycle. In the real time PCR on the LightCycler system, double strand DNA is stained with fluorescent dye SYBR Green, which can be determined and quantified by a camera and computer. Through amplification of the DNA during the PCR, the amount of double strand DNA will increase and, by that, the fluorescent emission of SYBR Green. By plotting this increase in fluorescent emission to the number of cycles, the complete amplification process can be visualized. The number of cycles that are necessary to reach a stated fluorescent emission is representative for the initial cDNA concentration and, thus, the expression level of the tested gene. This initial cDNA concentration can be quantified by extrapolating the data obtained for the tested samples to a standard curve, at which the number of cycles necessary
to reach the stated fluorescent emission is plotted to the concentration of template at the start of the PCR. The standard curve is made by, performing parallel to each experiment, several PCRs by which different known amounts of template are added to the PCR mix.
3 Origin, presentation, and clinical aspects of osteosarcoma

Sarcomas are a heterogeneous group of tumors largely arising from derivatives of the embryonic mesoderm, which gives rise to the supporting tissues of the body. They can be divided into malignancies of soft tissues and of bone. Soft tissue sarcomas, which include all tumors derived from connective tissues other than bone and cartilage, account for the majority of the sarcomas. The most common histological types of soft tissue sarcomas are malignant fibrous histiocytoma, liposarcoma, leiomyosarcoma, synovial sarcoma, malignant peripheral nerve sheath and fibrosarcoma (Weiss and Goldblum, 2001). The main bone sarcomas are conventional osteosarcoma and Ewing’s sarcoma (Bell and Siegal, 2002). Ewing sarcomas are round-cell tumors of neuroectodermal origin (Lizard-Naclot et al., 1989; Navarro et al., 1994). All conventional osteosarcomas are osteoid or bone producing tumors (Ragland et al., 2002). They are subdivided based on the primary differentiation of the mesenchymal component present. The most commonly recognized subtypes are osteoblastic tumors (osteosarcoma), chondroblastic tumors (chondroblastoma) and fibroblastic tumors (fibrosarcoma). Less commonly observed subtypes are giant-cell tumors (osteoclastoma), small-cell tumors and telangiactatic tumors (Dahlin and Unni, 1977; Schajowicz and Sobin, 1993). About 35% of all bone sarcomas are of the osteoblastic subtype (Dorfman and Czerniak, 1995). Although by this it is the most common primary tumor of the bone, osteosarcomas are very rare with only 35-45 newly diagnosed patients each year in The Netherlands (Voûte et al., 1997).

The peak incidence of osteosarcoma occurs in the second decade of life in patients 10 to 20 years of age. A second but smaller peak incidence is seen after age 50 (Dorfman and Czerniak, 1998). Osteosarcomas in the older age groups may be associated with Paget disease, which is a bone disorder characterized by rapid bone remodeling resulting in abnormal bone formation (Huvos, 1986; Klein and Norman, 1995). Osteosarcoma in older patients may also be induced by earlier therapeutic radiation of the patient (Mark et al., 1994).

Although osteosarcoma can develop in any bone, the preferential sites of origin are the metaphyseal regions of the distal femur, proximal tibia and proximal humerus (Campanacci, 1999). An osteosarcoma grows in a radial manner, forming a ball-like mass. The tumors frequently penetrate through and destroy the cortex of the bone and extend into the surrounding soft tissue (Kumar et al., 1987). Areas of necrosis in the tumor are common, especially when the patient is preoperatively treated with chemotherapy. Depending on cellularity, pleomorphism, anaplasia and number of mitoses, osteosarcomas that present without metastases are divided into low-grade (stage I) and high-grade (stage II) variants. Most osteosarcomas present at stage IIB, which are high-grade tumors associated with soft tissue mass. In patients with a stage III tumor, metastases are detected at the time of diagnosis (Enneking et al., 1980).

Before 1970, osteosarcomas were only locally treated by amputation of the affected extremity. Although in most of these patients no metastases could be detected at the time of diagnosis, over 80% developed metastases and died most commonly from pulmonary metastases within 2 years after surgery (Marcove et al,
Over the past three decades management of osteosarcoma has dramatically changed. The introduction of adjuvant chemotherapy has resulted in an increase of the long-term survival from 10-15% to 50-60% (Link et al, 1986). The current treatment of osteosarcoma involves both post- and preoperative chemotherapy. Preoperative chemotherapy induces tumor necrosis and reduction of the tumor size, which enables in most patients limb-sparing tumor resections instead of amputations (Bacci et al, 1993). The chemotherapy regimen generally includes high-dose methotrexate, doxorubicin, cisplatin and ifosfamide given in various combinations (Bacci et al, 2001).

The degree of chemotherapy-induced necrosis is a very powerful indicator for prognosis of patients with an osteosarcoma. Poor responders (most often defined as less than 90% histological tumor necrosis) have a significantly worse prognosis than good responders (90% or more histological tumor necrosis) (Meyers et al, 1992). Other important prognostic factors are the staging of the tumor, its initial location and the presence of metastases. Age of the patient is probably a less significant prognostic factor, however some studies reported that patients aged 5-25 had poorer prognosis than young adults of 25-30, while the prognosis worsened again with advancing age (Davis et al. 1994).

At this moment, the response to chemotherapy is the strongest prognostic parameter. It is not possible to determine this at the time of diagnosis when the treatment is planned. There are no prognostic factors that accurately predict the outcome of treatment in patients with a non-metastatic osteosarcoma before onset of the therapy (Davis et al. 1994, Saeter et al. 1997). Additional prognostic markers that can be used to determine clinical behavior of osteosarcoma would be helpful in the choice of therapy. In recent years, much research is done aiming at identifying genetic aberrations that are of value in the diagnosis, prognosis and biology of osteosarcoma.
4 Genetic aberrations in osteosarcoma

4.1 Introduction

Cytogenetic studies revealed that osteosarcomas are characterized by complex genomic changes, including changes in chromosome number and structure, often with pronounced cell-to-cell variation. Using several molecular genetic techniques such as FISH, PCR and CGH the genetic aberrations in osteosarcomas were investigated in detail. The following chromosomal bands or regions were most commonly involved in structural abnormalities: 1p11~p13, 1q10~q12, 1p21~p22, 11p15, 12p13, 17p12~p13, 19q13 and 22q11~q13. Chromosome arms 3q, 13q, 17p and 18q proved to be most frequently deleted. DNA amplification was most frequently detected in chromosome regions 1q21, 3q26, 6p, 8q, 12q12~13, 14q24~qter, 17p11~p12, Xp11.2~21 and Xq12 (Sandberg and Bridge, 2003).

4.2 Loss of tumor suppressor genes

Loss of the retinoblastoma gene

Deletion of chromosome arm 13q probably reflects the inactivation of the retinoblastoma gene (RB1) (Toguchida et al, 1988). Deletion of the RB1 locus is seen in about 70% of the osteosarcomas (Belchis et al, 1996). An association of osteosarcoma with the RB1 gene is well recognized, with patients affected by hereditary retinoblastoma having up to 1000 times the incidence of osteosarcoma compared with the general population (Abramson et al, 1984; Kitchin and Ellsworth, 1974). The RB1 gene product (RB) has a fundamental role in the regulation of cell division (see figure 2). In normal cells, active RB protein binds to the transcription factor E2F and blocks cell cycle progression. For transition from the G1 to the S phase of the cell cycle, RB protein has to be inactivated by phosphorylation, after which E2F is released and activated resulting in transcription of S-phase genes and promotion of DNA synthesis. Abnormalities in either the RB1 gene or in genes encoding proteins that regulate the activity of the RB protein result in loss of the G1-S cell cycle checkpoint. The RB protein is phosphorylated by cyclin-dependent kinase 4 (CDK4) in complex with cyclin-D1. Amplification and overexpression of CDK4 or the cyclin-D1 gene (CCND1) may result in an increase of phosphorylation of the RB protein and therefore a decrease in RB activity and a functional inactivation of RB signaling pathway. In turn, the p16 protein, which is the product of the INK4A gene, inhibits the activity of CDK4 (see figure 2). By negatively regulating the activity of CDK4, the p16 protein may promote the activity of the RB protein. Deletion of the INK4A gene and, consequently, loss of functional p16 protein will thus also result in the functional inactivation of RB protein. CDK4 amplifications and INK4A gene alterations have been found in osteosarcomas and proved to be mutually exclusive events (Benassi et al., 1999; Wei et al., 1998). LOH at the RB1 locus in osteosarcoma has been associated with a poor prognosis for the patient (Feugeas et al, 1996).
G1 → S-phase: Transcription of S-phase genes

Fig. 2 The retinoblastoma pathway. See text for details. (Adapted from Baker and McKinnon, 2004)
Loss of the TP53 gene

A chromosomal region frequently identified as abnormal in osteosarcomas is 17p13 harboring the tumor suppressor gene TP53. In osteosarcoma, genetic alterations affecting the TP53 locus have been reported in many different studies with frequencies up to 50% of the cases (see for an overview Ragland et al, 2002). Alterations in the TP53 gene include allelic loss, gene rearrangement, and point mutation. The TP53 gene product is a transcription factor that regulates the expression of many genes of which some are involved in cell growth control, DNA damage response, and apoptosis. Loss of the function of the TP53 protein is thought to contribute to the development of many tumors including osteosarcoma. Evidence of the association of TP53 with osteosarcoma is further provided by the high risk of bone sarcomas in patients with the Li-Fraumeni syndrome who have a germ-line mutation of TP53 (Nichols et al, 2001). No correlation has been found between expression of the mutated TP53 gene product and clinical variables. The TP53 mutation status did not differentiate between localized osteosarcoma and metastases. TP53 mutations are evident before the development of metastases, indicating that these are not a late event in the tumorigenesis of osteosarcoma (Gokgoz et al, 2001).

The TP53 protein has a central role in mitotic checkpoints. Aberrations in mitotic checkpoints are thought to result in chromosomal instability (Cahill et al, 1998). Cytogenetic studies have shown that osteosarcomas are characterized by a high frequency of structural and numerical alterations, compounded by gross changes in ploidy. Most high-grade osteosarcoma have many atypical mitotic figures (Fechner and Mills, 1993), suggesting that the chromosomal instability in these tumors may be a consequence of aberrant mitosis, leading to unequal segregation of sister chromatids during cell division. Al-Romaikh et al (2003), have found an increased frequency of atypical mitotic figures and numerical aberrations of centrosomes in osteosarcoma cell lines with TP53 mutations. A relationship between TP53 mutation and genomic instability was also found by Overholtzer et al (2003), who observed by that the number of genetic changes was significantly higher in osteosarcomas with TP53 mutation than in those without TP53 mutation.

Loss of other tumor suppressor genes

Allelic losses on the chromosome arms 17p and 13q are respectively associated with the loss of the tumor suppressor genes TP53 and RB1. Beside these regions harboring known tumor suppressor genes, deletions of the chromosome arms 3q and 18q are also frequently detected in osteosarcoma, suggesting that at least two other tumor suppressor genes might be involved in the tumorigenesis of osteosarcoma (Yamaguchi et al, 1992).

The chromosomal region 18q21.3 has been linked to Paget disease. Paget disease is a heritable bone disorder leading to an increased risk of developing osteosarcoma. LOH analysis of osteosarcomas from patients with Paget disease revealed that these tumors undergo LOH in 18q21.3 (Nellissery et al.1998), suggesting that the association between Paget disease and osteosarcoma is the result of a gene located at that position. Recent LOH analysis of chromosome arm 18q in sporadic osteosarcoma has narrowed the putative tumor suppressor gene
containing region to a 500 kb DNA fragment in 18q21.33 (Johnsson-Pais et al., 2003). Two previous candidate genes in 18q21.3, RANK and BCL2, are no longer within this new critical region. This agrees with other data from osteosarcoma cell lines and sporadic Paget disease patients that suggested that somatic mutations in the RANK gene do not contribute to the development of osteosarcoma. (Sparks et al., 2001). Moreover, it could be demonstrated by immunohistochemical methods that there was no loss of BCL2 expression in the tumors (Johnsson-Pais et al., 2003).

Allele losses on chromosome arm 3q are found in about 75% of the osteosarcomas, suggesting the presence of a tumor suppressor gene on that chromosome arm (Yamaguchi et al., 1992). Kruzelock et al (1997) defined by detailed LOH analysis the minimal common region of deletion at 3q26.2-3q26.3, indicating that a putative tumor suppressor gene might be located at that region.

4.3 Amplification of oncogenes

Amplification of chromosome region 12q13~q15

Amplification of chromosomal region 12q13~q15 is detected in about 10% of the osteosarcomas (Tarkkanen et al, 1995). The chromosomal region 12q13~15 is large and contains numerous potential oncogenes, including MDM2, SAS, and CDK4. The MDM2 gene is located at 12q13 and encodes a protein that binds to and inactivates the TP53 gene product. Overexpression of the MDM2 gene caused by amplification of the gene may disrupt the normal TP53 pathway (Freedman and Levine, 1999). Amplification of MDM2 has been associated with tumor progression and metastasis in osteosarcoma (Ladanyi, 1993; Nakayama, 1995).

MDM2 is often co-amplified with the flanking SAS (sarcoma amplified sequence) gene. SAS is a member of a transmembrane-protein family. This family includes various tumor-associated antigens that are thought to be involved in growth-related cellular processes (Meltzer et al, 1991).

Another gene that might be an amplification target for the 12q13~q15 region is CDK4. In osteosarcoma, CDK4 amplification may occur in combination with or independently of MDM2 amplification (Berner et al, 1996; Elkahloun et al, 1996). As noted earlier, CDK4 forms a complex with cyclin-D1, which phosphorylates the RB protein resulting in release and activation of transcription factor E2F (see figure 2). These events drive cells through the G1-phase of the cell cycle and facilitate entry into S-phase. High CDK4 protein levels disrupt the balance between the dephosphorylated and phosphorylated form of the RB protein in favor of inactive phosphorylated RB protein. This will lead to impaired cell cycle control and enhanced cell proliferation.
Amplification of chromosome arm 8q

One of the regions most frequently amplified in osteosarcomas is chromosome arm 8q. The amplification of 8q is complex involving large genomic regions and multiple independently amplified targets (Ozaki et al, 2002; Bayani et al, 2003). Using CGH, Squire et al (2003) observed three different patterns of 8q genomic gain in osteosarcoma. First, overall gain of the whole 8q arm or chromosome. Second, gain of variable but extensive regions of 8q including 8q12~21.3 and 8q22~q24. Third, localized gain affecting only the proto-oncogene MYC (C-MYC) at 8q24.2. Gain of 8q22~q24 without the involvement of MYC has also been reported. Whereas gain of 8q22~q24 is observed in 24% of the osteosarcomas, MYC amplifications occur only in 7-12% of the tumors (Ladanyi et al, 1993; Pompetti et al, 1996). These results suggest the presence of an oncogene other than MYC at 8q22~q24 that might be involved in the pathogenesis of osteosarcoma. The osteoprotegerin (OPG) and BMP1 genes are mapped in that region (Tan et al, 1997; Tabas et al, 1991). Both genes are supposed to have a regulatory function in bone formation (Simonet et al, 1997; Scott et al, 1999), and may be considered as potential candidate oncogenes in osteosarcoma. Copy number increase of chromosome arm 8q was found to been associated with poor prognosis for the patient (Tarkkanen et al, 1999).

Amplification of chromosome region 17p11.2~p12

A region that is frequently amplified in osteosarcoma and therefore is of particular interest is chromosomal region 17p11.2~p12. According to several CGH studies this region is amplified in 13-29% of the osteosarcomas (Forus et al, 1995; Tarkkanen et al, 1995; Tarkkanen et al, 1999; Stock et al, 2000; Zielenska et al, 2001; Ozaki et al, 2002; Lau et al, 2004). Besides in osteosarcoma, the amplification of this chromosomal region is also observed in a number of other tumors. Amplification of 17p was detected by Southern blot and microsatellite analysis in about 5% of high-grade gliomas (Bijlsma et al, 1994; Hulsebos et al, 1997, van Dartel et al., 2003). Using CGH, high-level amplification was also detected in 24% of leiomyosarcomas (El-Rifai et al, 1998), while gain of this chromosomal region was observed in 16% of malignant fibrous histiocytomas (Larramendy et al, 1997a), 38% of chondrosarcomas (Larramendy, 1997b), and 42% of oral squamous cell carcinomas (Weber et al, 1998). These data strongly suggest that chromosome arm 17p, in particular chromosome region 17p11.2~p12, harbors an oncogene or oncogenes that might stimulate the development and/or progression of these tumors. As amplification of the putative oncogenes located at 17p112~p12 probably affects the clinical behaviour of the tumor, identification of the amplification targets might be helpful to predict the prognosis and determine the treatment of patients with osteosarcoma.
General Introduction

5 Aim and outline of this thesis

Aim of the thesis

Osteosarcoma is a highly malignant bone tumor. The tumor is characterized by complex chromosomal aberrations. In earlier studies, others and we have shown that amplifications in chromosome region 17p11.2~p12 are frequently occurring in this tumor. This suggests the presence of one or more oncogenes of which the overexpression is causally related to osteosarcoma tumorigenesis. This study aims at identifying these oncogenes. The identified oncogenes can hopefully be used as genetic markers to more accurately assess diagnosis of the tumor and prognosis for the individual patient and for improvement of treatment decision. Moreover, these oncogenes and their product(s) might serve as potential targets in osteosarcoma therapy.

Outline of the thesis

Chromosomal CGH (comparative genomic hybridization) analysis revealed the frequent amplification of 17p11.2~p12 sequences in high-grade osteosarcomas, occurring in about 25% of cases. However, the resolution of chromosomal CGH is rather low. To determine a more exact location of the putative oncogenes causing the amplifications in this region, we established in Chapter 2 detailed amplification profiles by semi-quantitative PCR, using 17 microsatellite markers and 7 candidate genes in 19 tumors with chromosomal CGH-proven amplification of 17p11.2~p12. We detected frequent amplification of the TOP3A gene and, in some cases, very high-level amplification of genes PMP22 and MAPK7. The amplification profiles of a number of the investigated tumors could be explained by assuming the involvement of so-called low-copy repeats in the amplification events.

Originally, we detected the amplification of 17p11.2~p12 sequences in high-grade gliomas. However, this proved to be an infrequent event, occurring in only 3 of 60 (5%) investigated cases. To detect additional cases with 17p11.2~p12 amplification, we screened in Chapter 3 in total 104 gliomas of various type and grades. However, no new cases were found. To investigate whether the same oncogene(s) could be involved in osteosarcoma and glioma tumorigenesis, we also determined in this chapter detailed profiles in the 3 glioma cases with amplification using the same set of markers and genes as was applied in the study of the osteosarcomas. Distinct and high-level amplifications were found in these tumors. One of the tumors showed high-level amplification of PMP22.

Reasoning that amplification of a causative oncogene in a tumor should result in increased expression of that gene, we determined in Chapter 4 the expression status of genes and expressed sequence tags (ESTs) in 17p11.2~p12. We constructed a 17p11.2~p12-specific macroarray containing 40 genes and 21 ESTs from this region, which was used for the expression profiling of 11 osteosarcoma samples and of normal human osteoblasts. Genes PMP22 and COPS3 and 3 ESTs were found to be most frequently overexpressed and to be most consistently overexpressed after amplification in the tumors and where, therefore, considered to be potentially causative oncogenes. The expression status of PMP22 and COPS3 could be confirmed by real time reverse transcription-PCR.
Chapter 1

*PMP22* is highly expressed in peripheral Schwann cells and encodes an important constituent of the myelin sheath. It is also expressed at lower levels in other normal tissues, in which the protein is supposed to be involved in cell growth regulation. Because of its frequent overexpression (and amplification) in osteosarcoma, we studied the expression of *PMP22* in greater detail in Chapter 5. It is known that in normal Schwann cells *PMP22* transcription may start from three promoters, resulting in the synthesis of three alternatively spliced transcripts that all code for the same protein. We studied *PMP22*-promoter usage in osteosarcoma tumors and in normal osteoblasts by semi-quantitative reverse transcription PCR. In normal Schwann cells and in NIH3T3 cells, *PMP22* expression increases upon serum starvation-induced growth arrest. To characterize *PMP22* expression under comparable conditions in osteosarcomas, we determined *PMP22* expression in osteosarcoma cell lines after serum withdrawal. We found that the regulation of *PMP22* expression differed markedly between normal and osteosarcoma cells.

In Chapter 6 we discuss the results of our studies, compare these with recent data obtained by other groups and suggest new directions for future research.

In Chapter 7 we summarize our findings.
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Chapter 1


Chapter 1


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