# Osteosarcoma : searching for new treatment options

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# 1

# General introduction and outline of the thesis

## 1. What is Osteosarcoma?

Osteosarcoma is the most frequent high-grade primary malignant bone tumor that is thought to arise from mesenchymal stem cells with the capacity to produce osteoid.[1,2] The overall incidence is of three cases per million annually. Osteosarcoma occurs predominantly in children and adolescents, and in people over 50 years of age. It is located primarily in the metaphyseal region within the medullary cavity of long bones of the extremities (Fig. 1),[2,3] specifically in the knee area.[4] Other locations are the pelvis, ribs and spine, which are associated with worse outcome.[5,6]

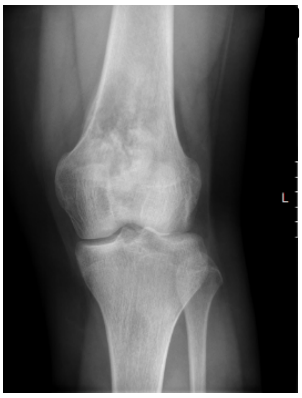

**Figure 1**. Osteo-sarcoma of the distal femur

Osteosarcoma is classified into various histological subtypes: conventional, telangiectatic, small cell and other rare types. Conventional osteosarcoma is the most frequent, which originates in the medullary cavity of the metaphyseal region of long bones, and is mainly high grade. Telangiectatic represent less than 4% of the osteosarcomas. It is similar to conventional osteosarcoma in terms of clinical presentation, treatment and prognosis.[7] Small cell osteosarcoma is a rare entity with 1%-2% prevalence, and it resembles morphologically an Ewing sarcoma. However, small cell osteosarcoma has different genetic characteristics such as the absence of EWSR1 and FUS gene rearrangements, and the production of osteoid.[8–10]

Osteosarcoma diagnosis is only confirmed by the presence of osteoid in the biopsy. However, these malignant cells also have the capacity to produce cartilage matrix or fibrous tissue, which divides osteosarcoma in three categories: osteoblastic, chondroblastic and fibroblastic. Usually, a tumor shows all three matrix types, making it difficult to categorize it. The tumor will fall into one of these categories when it presents more than 50% of one of the histological types.[9]

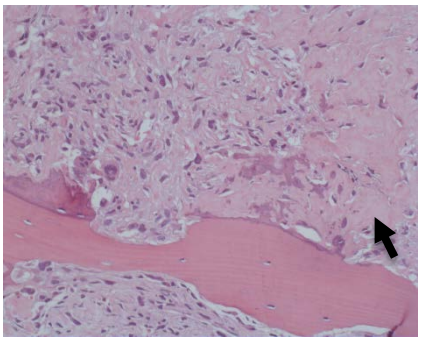

**Figure 2**. Primary osteosarcoma with osteoid (black arrow).

## 2. Etiology

*2.1 Bone growth and turnover*

Adolescent growth spurt coincides with the peak onset of osteosarcoma: girls show an earlier peak than boys which could be caused by their earlier growth spurt.[11] Research shows that there is higher incidence in boys (56%) compared to girls (42%).[12–14]

*2.2 Predispositions*

Paget's disease is characterized by a metabolic bone disorder leading to increased and disorganized bone formation.[15] It affects mainly people older than 50 years of age, and people with this disease present a 2% probability of developing osteosarcoma. Other predisposing factors are genetic disorders such as Li-Fraumeni syndrome, Rothmund-Thomson syndrome and Beckman-Wiederman syndrome.[11]

Li-Fraumeni syndrome is a hereditary disorder characterized by germline mutations in the *TP53* gene. This syndrome is characterized by the occurrence of sarcomas, among other cancers, in persons under the age of 45 years old.[16] These patients have a high risk of developing osteosarcoma. In fact, mice with $p53^{R172H/+}$ mutation showed 2 times increase in number of osteosarcomas compared to $p53^{+/-}$ mice,[17] and *p53*-null heterozygous mice present high numbers of osteosarcomas.[18,19]

Rothmund-Thomson syndrome is an autosomal recessive genodermatosis characterized by poikiloderma, short stature, premature aging and skeletal abnormalities among other features. Patients with this rare disease have a predisposition to develop osteosarcoma.[20,21] It was found that 60-65% of patients present mutations in the RECQL4 helicase gene suggesting a possible role of this gene in osteosarcoma development.[20]

Retinoblastoma is a hereditary disease that causes eye tumors in children. It is caused by mutations in *RB1* gene. Osteosarcoma is the most common secondary tumor that arises in these patients.[22,23]

Other rare genetic diseases such as Bloom, Werner, Rapadilino and Diamond blackfan are known for development of osteosarcoma among other malignancies.[22]

Finally, osteosarcomas arise secondary to radiation affecting mainly older patients. Studies show that sarcomas associated with radiation are uncommon. However, osteosarcomas are the main secondary tumor and represent 2.7-5.5% of osteosarcomas.[2,24,25]

## 3. Prognostic factors

There are several clinical characteristics that are predictors of clinical outcome. The outcome for patients that at the moment of diagnosis present with metastasis is still poor, and no improvement was observed with chemotherapy.[11,26,27] Additionally, the most common sites of recurrence are local and lung, presented in 20% and 62% of the cases respectively, and metastasis is correlated with poor survival.[28,29] Another important prognostic factor is the tumor site. Several studies report that tumors located in the axial skeleton have particularly poor outcome.[4–6,30] One of the requisites for a better control of the tumor is to achieve surgical excision with clean margins, and this is difficult for most of the axial tumors.[11] Response to chemotherapy is another variable that affects the outcome of these patients. Good responders are described as those with more than 90% of necrotic tissue after pre-operative chemotherapy. Several studies show that there is a correlation between good response to chemotherapy and prognosis.[31,32] Furthermore, tumor size is also considered a prognostic factor as indicated in a retrospective study of 331 osteosarcoma patients.[33–35] Finally, age is another prognostic factors. Older patients with osteosarcoma tend to have a worse prognosis than younger patients.[4,36,37]

## 4. Tumor Biology

Osteosarcoma cells are pleomorphic, anaplastic and hyperchromatic.[38] They are also characterized by complex karyotype as a result of chromosomal abnormalities that are different from cell to cell and from tumor to tumor (Fig. 2).[39,40] It was reported that copy number gains range from 7 to 190 and loses from 7 to 170 per sample. Gains are mainly located in chromosomes 6p, 8q/9p and 17p, and loses are in chromosomes 3q, 6q, 8p/9p, 11p, 15q and 17q among other aberrations.[41–43] These studies also show that genomic instability is correlated with poor prognosis and could be a cause of tumor initiation.[44] The fact that there is abundant genetic instability in osteosarcoma, makes it difficult to pinpoint genes involved in tumor progression, metastasis or response to chemotherapy. However, it is well established that genetic alterations in the tumor suppressor genes *Rb1* and *TP53* are consistent across osteosarcoma tumors.

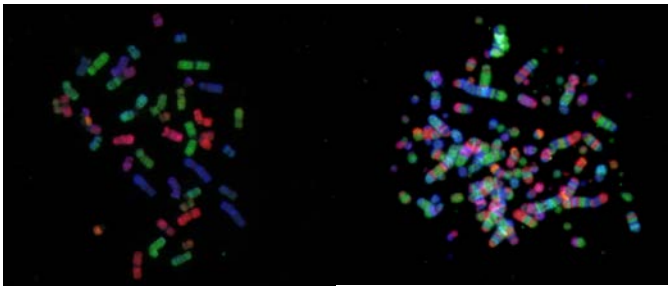

Figure 3. COBRA-FISH karyotype. Left) Normal human cell. Right) Osteosarcoma cell. Courtesy of Dr. Karolv Szuhai.

More than 70% of osteosarcomas have loss of heterozygosity (LOH) of the *TP53* gene, 20% present rearrangements, and 30% harbor mutations in *TP53*.[41,44,45] The *Rb1* gene was found to harbor LOH and mutations in more than 35% of osteosarcomas.[44,46,47] The establishment of a murine model with mutant or deleted p53 that leads to development of osteosarcomas spontaneously in more than 50% of the mice, confirms the role of p53 in osteosarcoma development.[19] Furthermore, MDM2 and COPS3, which are negative regulators of p53 that facilitate its proteasomal degradation, are amplified in 10% and 25% of osteosarcomas respectively.[48,49] The Rb protein binds to the E2F transcription factor, and this complex represses the transcription of genes necessary for cell cycle transition from G1 to S-phase.[50] The Rb protein is regulated by CDKN2A/p16 and CDK4/CDK6. CDK4/CDK6 phosphorylates Rb, thereby driving cell cycle progression, and CDKN2A/p16 inhibits the activation CDK4/CDK6.[51] It has been shown that in osteosarcoma *CDK4* is amplified in 10% of the tumors, and *CDKN2A/p16* is deleted in tumors that lack *Rb* mutations.[47,52–54] Deletion of *CDKN2A/p16* is correlated with poor prognosis.[1] Furthermore, a genome-wide expression study on a series of high-grade osteosarcomas compared to mesenchymal stem cells and osteoblasts, revealed significantly altered pathways in osteosarcoma such as upregulation of genes involved in mitosis and DNA replication.[55]

c-Myc and c-Fos are two proto-oncogenes that are regulators of cell cycle progression by modulating the cyclin-Cdk complex activity.[56,57] Expression of the *c-Myc* and *c-Fos* genes is increased in osteosarcoma.[58] One of the frequent genomic gains found in 34% of the cases is chromosome arm 8q, which contains the *c-Myc* proto-oncogene,[39,59] and its amplification is associated with poor overall survival and event-free survival.[47] In a genetic mouse model, 100% of transgenic mice overexpressing *c-Fos* were found to develop osteosarcoma.[58]

Receptor Tyrosine kinases (RTKs) are transmembrane receptors that are activated upon extracellular ligand binding such as growth factors, hormones and cytokines. They are mediators of the these environmental signals that lead to normal cellular processes like growth, proliferation, survival, differentiation and migration.[60] If these receptors are mutated or abnormally activated, they can be effective oncoproteins driving tumorigenesis.[61] The RTK family is composed of 58 members classified into 20 subfamilies[62] which include: epidermal growth factor receptors (EGFR), platelet-derived growth factor receptors (PDGFR), fibroblast growth factor receptors (FGFR), hepatocyte growth factor receptor (Met), insulin receptor (INSR), among others.[63]

The EGFR family is composed of EGFR, ERBB2, ERBB3 and ERBB4.[62] The *EGFR* gene was found to be amplified in 82% and expressed in 50% of osteosarcoma.[64,65] However, inhibition of EGFR in vitro had no effect on cell viability in vitro, and in osteosarcoma patients high EGFR expression is correlated with good prognosis.[66,67] There are contradicting results with respect to ERBB2 expression and its correlation with osteosarcoma prognosis, which could be due to study methodologies.[68–70] Inhibition of ERBB3 expression *in vitro* and *in vivo* reduces cell growth and invasiveness of osteosarcoma cells[71]. Studies on ERBB4 in osteosarcoma are limited but it was found to be paired with ERBB2 for its activation.[72]

PDGFR family is composed of CSF1R, KIT, FLT3, PDGFRα and PDGFRβ.[62] The *KIT* gene was found to be amplified in 57% of osteosarcoma patients.[73] PDGFRα/β were found to be expressed in osteosarcoma cell lines,[74] however, in osteosarcoma patients expression of PDGFRα is not correlated with overall survival.[75] No information is available on FLT3 and CSF1R in osteosarcoma.

The FGFR family includes FGFR1/2/3/4.[62] *FGFR1* gene has been reported to be amplified in 17% of the osteosarcoma cases, and it was significantly correlated with poor response to chemotherapy.[76] No studies have reported on the relation between FGFR2/3/4 expression and osteosarcoma.

Met is part of the Met family together with MST1R. Met is highly expressed in osteosarcoma, and it has been implicated in osteosarcomagenesis by inhibiting the differentiation of the osteo-progenitor cell population.[77,78] Additionally, Met expression was associated with ostesarcoma progression and aggressiveness.[79]

The INSR family groups the insulin receptor (IR) and the insulin-like growth factor 1 receptor (IGF-1R). IGF-1R is known to be expressed in osteosarcoma and its downstream signaling pathway was found to be altered in osteosarcoma.[80,81] However, IGF-1R expression is not proven to be a predictive marker for response to therapy with IGF-1R inhibitors.[82]

All these RTKs are activated by many different ligands, and to exert their effect they must activate downstream signaling pathways converting ligand binding into gene expression alterations. The pathway from cell surface to nucleus is mainly governed by: 1) the Ras/Raf/MEK/ERK cascade, 2) the PI3K/AKT pathway and 3) the Jak/STAT pathway.[83]

The Ras/Raf/MEK/ERK cascade is known to be involved in cell proliferation, apoptosis, differentiation and development. Activated cell surface receptors lead to ERK activation, which activates transcription factors such as c-Myc, c-Fos, Ets, and Elk-1.[84] This pathway is often deregulated in tumors caused by mutations or overexpression of upstream signaling components. *B-Raf* and *Ras* are frequently mutated in melanoma, colorectal cancer, ovarian cancer, lung cancer and pancreatic cancer among others.[85,86] In osteosarcoma, the ERK pathway was reported to be active in 67% of the cases analyzed, and mutations in B-RAF were only found in 13% of the cohort.[87] The PI3K pathway regulates processes such as proliferation, metabolism, apoptosis and cytoskeletal rearrangements.[88] In osteosarcoma, genetic screens have identified this pathway to be upregulated.[89–91] Recently, AKT2 was found to be overexpressed in osteosarcoma samples compared to normal tissue, and there was a positive correlation with shorter overall survival time.[92] Furthermore, it has been reported that STAT3 is overexpressed and constitutively active in osteosarcoma, and contributes to tumor progression.[93,94] Upstream of these three pathways is Src, a non-receptor tyrosine kinase that belongs to a family of 11 members.[95] It was shown that in osteosarcoma, Src expression and activity correlates with clinical stage and survival time.[96]

Finally, another important pathway involved in osteosarcoma development is Wnt/β-catenin. Active Wnt/β-catenin signaling stimulates osteogenic differentiation. This pathway was found to be inactive in osteosarcoma, thus facilitating dedifferentiation.[97]

## 5. Metastatic behavior

Osteosarcoma is a highly metastatic cancer. Approximately 20% of the patients present with pulmonary metastasis at the moment of diagnosis and when patients present with recurrence around 90% of the cases is in the lungs.[98] Ras/Raf/MEK/ERK activation downstream from IGF-1R has been shown to drive lung metastasis in an orthotopic mouse model.[99] The PI3K/AKT pathway is also involved in osteosarcoma metastasis. Several studies showed that this pathway is active in cell lines capable of forming metastatic lesions in mice[100] and that AKT activity is upregulated in anoikis-resistant cells.[101] As mentioned before, Src kinase activity can stimulate these pathways. Src regulates a variety of cellular processes such as cell morphology, migration, adhesion, survival and proliferation.[95] In cell-

matrix adhesions where integrin receptors connect the intracellular cytoskeleton to the extracellular matrix; Src forms a complex with focal adhesion kinase (Fak). Src phosphorylates Fak at multiple positions thereby creating a cell adhesion signaling platform that regulates cell-matrix adhesion dynamics and downstream signaling.[102] Fak is overexpressed in osteosarcoma, and it was shown to be involved in metastasis.[103] Another cytoskeleton-associated protein that influences the metastatic behavior of osteosarcoma is ezrin. Ezrin links the cytoskeleton to the plasma membrane allowing the cell to interact with the environment. In osteosarcoma, ezrin is necessary for initial survival once the cells metastasize, and this effect is dependent on ERK activity. Moreover, high expression of ezrin is correlated with poor survival.[104] Lastly, increased expression of vascular endothelial growth factor (VEGF), a factor that binds VEGF-R on endothelial cells and stimulates angiogenesis, has been reported as a prognostic marker in osteosarcoma[105].

## 6. Treatment options

Historically, osteosarcoma was treated with amputation of the limb, and the maximum 5-year survival rate was 20%. However, the majority of the patients died 2 years after diagnosis because of metastasis.[106] As surgical techniques advanced, resection of the tumor was possible with limb-salvage techniques, and it was proven to be as safe as amputation.[107] After the introduction of chemotherapy the disease survival rate increased to >50% with patients surviving more than 5 years.[108,109] Today, the treatment consists of preoperative chemotherapy followed by resection of the tumor. The most effective systemic chemotherapeutics are cisplatin,[110] doxorubicin[108] and methotrexate.[111] Despite extensive studies aimed at finding optimal combined chemotherapeutic strategies, overall 5-year survival rates have not increased above 70%. Furthermore, around 35-45% of the patients have tumors that do not respond to chemotherapy.[112–114] The mechanisms underlying such resistance are not well understood but may include p53 mutation as well as overexpression, rewiring of signaling pathways including PI3K/AKT and Ras/MAPK, and expression of ABC transporters.[115,116]

There is a clear need for alternatives to conventional chemotherapy or to drugs that suppress the resistance to chemotherapy. Genome-wide RNA interference (RNAi) screening to identify new drug targets and screening of chemical compound libraries hold the promise of identifying new strategies for molecularly targeted therapy. RNAi screens in osteosarcoma have identified the mTOR pathway (downstream from PI3K/AKT), CDK11, WEE1 as candidate

drug targets among others.[89,117–119] Other studies have reported that inhibition of Aurora A/B or polo-like kinase 1 sensitizes osteosarcoma cells to doxorubicin.[120,121]

Some of the candidate therapeutic targets have entered clinical testing in osteosarcoma patients. Recently, a clinical trial studying the effect of Alisertib (Aurora A inhibitor) was completed (NCT01154816). There are several ongoing clinical trials that are studying the inhibition of VEGFR in solid tumors (NCT02389244, NCT02432274, NCT02357810, NCT02243605). Others are studying the possibility of inhibiting Src with saracatinib (NCT00752206) and dasatinib in combination with chemotherapy (NCT00788125). Besides inhibiting kinases, other trials are investigating the effect of targeting the immune system (NCT02470091, NCT00743496, NCT00134030).

## 7. Aim and outline of this thesis

The aim of the studies described in this thesis was to discover new therapeutic options for osteosarcoma patients. I focused on finding candidate targets and pharmaceutical inhibitors for killing human osteosarcoma cells or for sensitizing osteosarcoma cells to the chemotherapeutical, doxorubicin. **Chapter 2** describes the role of Aven in cell cycle control in osteosarcoma cells. It shows that silencing Aven causes cell cycle arrest through downregulation of the checkpoint kinase, Chk1. It further explores the efficacy of small molecules targeting Chk1 in combination with doxorubicin. In **chapter 3**, the role of Bcl2 family members in osteosarcoma cell survival is studied using an RNAi library targeting members of this family. Identification of Bcl-xL and validation of this hit using small molecules is described for a panel of human osteosarcoma cell lines. In **Chapter 4** identification of MEK inhibitors in a chemical kinase inhibitor library screen is described. Results are presented pointing to MEK inhibitors as a candidate therapeutic option for osteosarcomas showing high MEK activity. **Chapter 5**, focuses on elucidating the effect of three Src inhibitors on osteosarcoma viability and cell migration using 2D cultures and validation in 3D culture systems. Lastly, **chapter 6** provides overall conclusions of the studies described in this thesis and describes future perspectives.

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