Osteosarcoma : searching for new treatment options

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Summary and General Discussion

Osteosarcoma is the most common primary malignant bone sarcoma occurring predominantly in children and adolescents, and a second peak at middle age. It is characterized for being highly metastatic and resistant to chemotherapy, which gives these patients very poor prognosis.[1,2]

Before the introduction of chemotherapy, patients with osteosarcoma had a low chance of surviving this tumor. Once it was introduced, their prognosis increased dramatically, however, it has reached plateau. To address this challenge, there have been many clinical trials with the goal to find the best combination of chemotherapeutic agents that can increase the overall survival rate.[3,4] However, until now, there has been no further improvement. New efforts are being made to find new drug targets such as kinases or signaling pathways of the immune system. Many clinical trials testing these new molecules have shown that single agent therapies are not effective.

The aim of this thesis was to find new strategies to reduce osteosarcoma viability or that could potentiate the effect of doxorubicin. These findings, if translated to the clinic, would allow the use of lower doses of doxorubicin and avoid serious side effects that compromise the patient's life. I used a variety of techniques including high-throughput screening using siRNA and inhibitor libraries, which led to the discovery of new potential treatments.

Cell lines

Cancer cell lines derived from tumors are the most common tumor used in cancer research, and it has been of tremendous value in the field. There are doubts about how representative they are of the tumors they came from, and many research groups have made efforts to identify cell lines which are most represent the type of tumor they come from.[5–7] However, it has been also been shown for 127 cancer cell lines, that when injected in nude mice, they all formed a tumor which resembled histologically the cancer type.[8] Additionally, cancer cell lines retain the genotype of the original tumor such as mutations and expression of a characteristic gene.[9]

In this thesis I used osteosarcoma cell lines that have fully characterized by Mohseny A, et al. All of the cell lines had the capacity to differentiate *in vitro* into at least one of the three histological subtypes of osteosarcoma. However, not all of them had the capacity to form tumor in nude mice.[10] The cell lines employed in these studies, were chosen based on the genetic profile of p53 and CDKN2A, which are two of the well-known altered genes in osteosarcoma.[11] Additionally, the identity of cell lines was confirmed using the Cell ID

GenePrint 10 system (Promega Benelux BV, Leiden, The Netherlands) before and after completion of the experiments, and mycoplasma tests were performed on a regular basis.

Targeting the cell cycle

The DNA Damage Response (DDR) is evolutionary conserved and essential to ensure the faithful maintenance and replication of the genome. This signaling cascade senses DNA damage and triggers repair, cell cycle arrest and, in case of severe damage, cell death.[12] Chemotherapeutic drugs such as doxorubicin cause DNA double strand breaks, DNA alkylation, topoisomerase inhibition II among many other mechanisms.[13] This type of DNA lesions activate the DDR which lead to cell cycle arrest allowing the tumor cells to repair the damage and continue dividing. In **Chapter 2**, I proposed Aven to be a new regulator of DNA damage response showing that it is a key regulator of ATR-Chk1 axis. Subsequently, I investigated the effect of CHK1 inhibition in combination with doxorubicin. For the first time in osteosarcoma, these findings indicate that abrogation of Chk1 signaling using clinically relevant drugs may be combined with chemotherapy to treat osteosarcoma more effectively. Cancer cell cycle deregulation is often caused by altered CDK activity.[14] Furthermore, osteosarcoma is characterized by alterations in Rb protein and CDK4, which leads to uncontrolled cell cycle progression. In **Chapter 3** a screen of kinase inhibitors revealed that osteosarcoma cells are sensitive to inhibitors targeting kinases that regulate the cell cycle among others. Overall, I show that osteosarcoma is highly dependent on the cell cycle kinases to proliferate, and this signaling network is a potential therapeutic target.

3D cultures

Tumors are a complex disease that is governed by many intracellular signals such as gain of function of oncogenes, loss of function of tumor suppressors and mutations in key proteins. However, tumor cells are also influenced by the extracellular environment such as cell-matrix and cell-cell interactions. 2D mono-layer cultures have been a powerful tool but it was shown that the cells divide abnormally, change shape and physiological behavior.[15,16] 3D culture models provide a platform in which the tumor cells can behave more like the real tumor, and can be used to study cell viability and metastatic behavior after treatment with inhibitors.

Throughout the whole thesis I set out to find approved or preclinical inhibitors, which were effective alone or in combination with doxorubicin. In **Chapter 2,4,5** I assessed viability

of the treated cells in 2D monolayer cultures, and validated these results in 3D culture models.

Importance of inhibiting migration

Ostesarcoma is a highly metastatic tumor and at the moment of diagnosis, 10-20% of the patients already present with metastasis. About 30-40% of the patients with localized osteosarcoma will relapse mainly by presenting lung metastasis. Patients with recurrence have very poor prognosis with 23-33% 5-year overall survival.[17,18]

Tumor cell migration to distant locations has already occurred in patients with metastases implicating that cell migration is not a therapeutically relevant aspect of tumor progression. However, it has been shown that short range-migration (dispersal) to adjacent sites affects tumor topology and growth rates. This is the case in primary tumors and metastatic tumors. Although the tumor origin is genetically homogeneous, clonal variations arise that change the fate of these cells leading to resistance to treatment, and regrowth of the tumor after months of the treatments. Recent modeling approaches have shown that short-range dispersal contributes to cell mixing inside the tumor and targeting cell migration could in fact considerably suppress tumor growth.[19] In **Chapter 5** I used two 3D models to study the inhibition of migration using dasatinib, saracatnib and bosutinib. Using the spheroid collagen injection model, dasatinib was the only inhibitor capable of containing the cells in the spheroid; it inhibited migration completely. In the other 3D model employed here, the cells were re-suspended as single cells in a collagen-matrigel mix allowing me to study their morphology under treatment conditions. In this case I exposed these cells to doxorubicin in combination with the inhibitors mentioned above. Strikingly, only the combination of dasatinib and doxorubicin induced the retraction of branches and a round shape morphology. These two experiments confirm each other, and indicate that dasatinib in combination with doxorubicin is an effective targeted therapy that may avoid recurrence. Doxorubicin together with dasatinib is a potential candidate for further clinical studies.

Signaling pathways involved in osteosarcoma cell survival

PI3K-Akt-mTOR pathway is a network that controls many cellular processes such as cell proliferation, survival, metabolism and genomic integrity.[20] The expression of mTOR is correlated with event-free survival and cancer progression in osteosarcoma[21]. A kinase

inhibitor screen described in **chapter 4** indicated PI3K/mTOR pathway as crucial: 37% of the hits were inhibitors that targeted this pathway. Another relevant signaling network for osteosarcoma is the cell cycle with 37% of the hits inhibiting kinases in this pathway, such as aurora kinases, Chk1, CDKs and Plk1.

The Ras-Raf-MEK-ERK mitogen activated protein kinase cascade is known to be involved in cell proliferation, survival, differentiation and development. It integrates signals from cell surface receptors that activate MEK, which will activate ERK. Once ERK is activated, it enters the nucleus and activates transcription factors such as c-Myc, c-Fos, Ets, and Elk-1.[22] In osteosarcoma, ERK pathway activity was reported to occur in 67% of the cases analyzed.[23] In **chapter 4**, I identified three MEK inhibitors in a screen, which led me to further investigate this pathway. Although no genomic or transcriptomic changes in the MEK pathway discriminated sensitive from insensitive cell lines, I could show that MEK inhibitors are only effective in cells where relatively high ERK activity can be detected. Thus, active, phosphorylated ERK (that may be detected by immunohistochemistry in clinical samples) may serve as a biomarker for treatment with MEK inhibitors.

In **Chapter 3** I investigated the anti-apoptotic protein Bcl-xL. The expression of Bcl-xL did not correlate with survival, but in osteosarcoma cells the inhibition of Bcl-xL did potentiate the effect of doxorubicin. Furthermore, it has been shown that Bcl-xL expression is dependent on ERK activity.[24,25] Strikingly, the osteosarcoma cell lines sensitive to MEK inhibition were also the ones with highest Bcl-xL expression and more sensitive to Bcl-xL inhibition. These results suggest that ERK expression could also be used as a marker for this strategy, pointing to a more personalized treatment.

Future perspectives

In this thesis I described several possible therapies to treat patients with osteosarcoma. These results come from *in vitro* studies, and must be tested in osteosarcoma animal models to be translated to the clinic. Mouse models are commonly used because of the close genetic and physiological resemblance to that of humans, and the ease with which they can be genetically modified to facilitate tumor formation.[26,27] Genetically engineered mouse models with *p53* and *Rb* deletions in the osteoblasts effectively induce osteosarcoma formation that resembles human osteosarcomas.[28] Another possible mouse model involves overexpression of *c-fos* and *c-jun* proto-oncogenes, which induces the formation of osteosarcoma.[29] This model allows the spontaneous formation of osteosarcomas that can be

used to validate novel treatments. Alternatives to genetic mouse models for further investigation of drugs and drug combinations used in this thesis include mouse or zebrafish xenografts with patient materials or patient-derived osteosarcomas cultured in a 3D collagen matrix. Such strategies allow testing of treatment options for specifically for a given patient.

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