Chondrosarcoma models : understanding chemoresistance mechanisms for use in targeted treatment

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Chapter 9

Summary and concluding remarks

I. Chondrosarcoma from bench to bedside

Chapter 1 emphasizes the need for translational research in chondrosarcoma. Model systems are needed to establish a bench to bedside pipeline and translate laboratory findings to new therapeutic strategies. The application of model systems and the importance of translational research in chondrosarcoma is illustrated in **chapter 2** which reviews recent advances in pre-clinical chondrosarcoma research unraveling the role of EXT and IDH mutations in tumorigenesis as well as the therapeutic potential of targeting apoptosis and survival pathways. This thesis has shown the development of two model systems, both *in vitro* (cell lines, 2D and 3D culture) and *in vivo* (orthotopic mouse models), to aid in the search for new therapeutic strategies in chondrosarcoma. These model systems were subsequently used to study the role of apoptotic and survival pathways in chemoresistance.
As translational research is focused around the patient (fig 9.1), the collection of tumor material for frozen tissue, paraffin embedded tissue and cell culture is crucial. Frozen tissue can be used for DNA, RNA, and protein analysis, and paraffin embedded tissue is used for immunohistochemical analysis. When tissue microarrays (**chapter 6- 8**) are created, immunohistochemical analysis of multiple tissues simultaneously is facilitated. Collection of cells for cell culture and the use of cell lines (**chapter 3**) are vital in chondrosarcoma translational research, as in vitro tumor cell behavior can be studied in 2D and 3D culture (**chapter 5**) , in response to drug treatment (**chapters 5-8**). Moreover, stable cell lines can be used for injection in mouse models (**chapter 4**), to generate xenografts. These in vivo models enable the evaluation of drug efficacy taking into account the microenvironment. If a drug proves to be safe and effective in the mouse models, the results can guide the design of clinical trials increasing the chance of a successful trial. Conducting large clinical trials is difficult, requiring large multicenter collaboration given the rarity of these tumors, and obtaining funding is challenging. Through combined European efforts, such as EuroBoNet and euroSARC, the study of larger patient groups and design of multicenter clinical trials has been made possible, significantly advancing chondrosarcoma research.

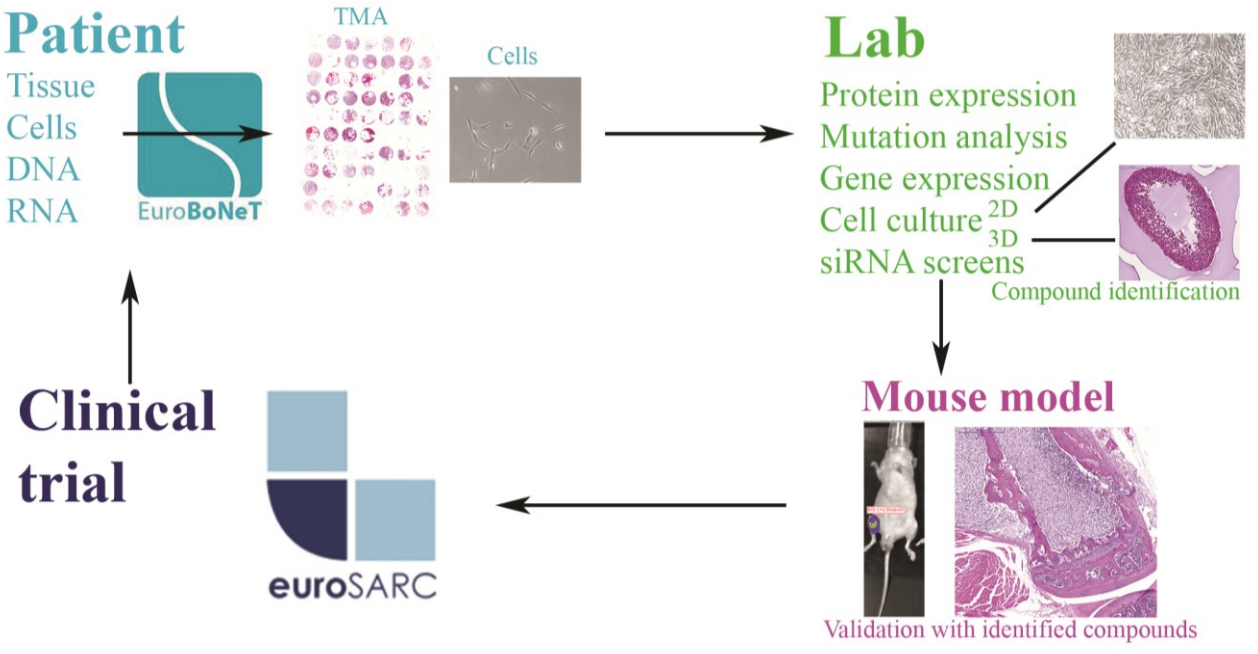

Figure 9.1. Chondrosarcoma from bench to bedside. Tumor material obtained from the patient can be used for several research purposes. Tissue embedded in paraffin can be used for protein expression and tissue microarrays are especially useful for the detection of proteins on multiple tumor tissues at once. Tissue microarrays are increasingly used for the identification of biomarkers. The isolation of fresh cells can be used for 2D and 3D cell culture to study in vitro tumor cell behavior and to possibly generate new cell lines. Frozen tissue can be used for DNA , RNA and protein analyses. Using cell culture models and siRNA screens, targets for treatment can be identified and compound screens can be performed. Compounds proven successful *in vitro,* can then be validated in the orthotopic mouse model, which will also allow for safety testing of combination strategies. Collaborative efforts such as EuroBoNet and euroSARC have enabled the collection of large databases for pre-clinical studies and are now facilitating clinical trials. For a rare malignancy such as chondrosarcoma, large cohort studies such as also performed in this thesis would have been impossible without the existence of such networks.

II. Cell lines as model systems to study chondrosarcoma

In order to establish representative models fully representing chondrosarcoma heterogeneity, in **chapter 3** the existing cell line panel for chondrosarcoma was expanded. Growing chondrosarcoma cells in culture is a challenge, and often overgrowth of fibroblasts is observed. At the start of this thesis, four stable chondrosarcoma cell lines were available, all derived from conventional central chondrosarcoma. One more cell line derived from conventional central chondrosarcoma and two derived from dedifferentiated chondrosarcoma were generated, and over time four additional cell lines were developed by others (1). This panel is representing the genetic heterogeneity of chondrosarcoma as three cell lines harbor IDH1 mutations, two IDH2 mutations, and five TP53 mutations. Moreover, all show loss of p16 expression, not always due to CDKN2A mutations/chromosomal aberrations, emphasizing the role of p16 in progression (table 3 chapter 3). The importance of a cell line panel representing the full heterogeneity of chondrosarcoma is subsequently illustrated in both **chapter 7** and **chapter 8**. In **chapter 7**, TP53 mutant cell lines were more sensitive to tyrosine kinase inhibition in combination with doxorubicin than TP53 wildtype cell lines. In **chapter 8** receptor tyrosine kinase profiling revealed heterogeneity in receptor tyrosine kinase activation among the cell lines. For instance, only SW1353 was particularly sensitive to MEK inhibition, which was based on the presence of an NRAS mutation, which was subsequently found in 12% of conventional central chondrosarcomas. These results strongly emphasize the importance of a broad and extensive cell line panel, representing the full heterogeneity of chondrosarcoma.

III. Orthotopic mouse model to study chondrosarcoma

Cell lines can provide information about tumor cell behavior, however, during the development of new therapeutic strategies, information about the tumor microenvironment should also be taken into account. The natural niche for chondrosarcoma is in the bone, and the communication between healthy bone and tumor cells as well as the influence of blood supply and the immune system on tumor development and chemoresistance cannot be investigated using cell lines. A reliable mouse model, representing human chondrosarcoma, could aid in providing answers about *in vivo* tumor behavior and drug response, and bridge the gap between cell lines and clinical trials.
As the natural niche for chondrosarcoma is in the bone, in **chapter 4**, orthotopicchondrosarcoma mouse models were created. A chondrosarcoma grade II cell line with IDH2 and TP53 mutations, and a chondrosarcoma grade III cell line wild type for IDH and TP53 were used. Rather than subcutaneous xenografting of tumor tissue or cell lines, luciferase transformed cell lines were injected in the tibiae. Therefore, tumor growth could be monitored throughout the duration of the experiment. Using doxorubicin, we show that this model is a valuable tool to

monitor *in vivo* tumor growth over time and will prove useful when testing new therapeutic strategies.

IV. Apoptosis signaling in conventional chondrosarcoma and rare chondrosarcorcoma subtypes

In **chapter 5** 2D and 3D cell culture models were used to explore the underlying causes of chemoresistance in conventional chondrosarcoma. We first established that resistance was not due to the extracellular matrix or multidrug resistance pump activity. As the literature showed high expression of the anti-apoptotic proteins Bcl-2 and Bcl-w in high grade conventional chondrosarcoma, the role of Bcl-2 proteins in chemoresistance was further investigated in the cell lines using the BH-3 mimetic ABT-737 (fig 9.2). Intermittent combination therapy with doxorubicin or cisplatin allowed for a dramatic reduction in concentrations used and induced apoptosis in all cell lines, indicating that Bcl-2 anti-apoptotic proteins are important in chemoresistance and that inhibition of Bcl-2 family members sensitizes chondrosarcoma cells for subsequent treatment with conventional chemotherapeutic agents.
In **chapter 6**, the original hypothesis was to correlate the morphological resemblance of clear cell chondrosarcoma, dedifferentiated chondrosarcoma and mesenchymalchondrosarcoma with the different stages of the growth plate by studying proteins involved in growth plate signaling. To this end a tissue microarray study was performed, however, protein expression patterns in the different subtypes were not correlated to differentiation stages in the growth plate. Interestingly, in both dedifferentiated and mesenchymal chondrosarcoma, differences in protein expression could be observed between the cartilaginous components and the anaplastic components, indicating distinctive pathway activations in the respective malignant components. In all subtypes, strong expression of anti-apoptotic Bcl-2 family members was found, suggesting a specific upregulation of anti-apoptotic proteins in chondrosarcoma irrespective of subtype. For dedifferentiated chondrosarcoma, 2 cell lines were available, and combination treatment of ABT-737 with doxorubicin or cisplatin showed a reduction in cell viability, indicating a similar resistance mechanism in place as in conventional chondrosarcoma. The uniformity of inhibition of chondrosarcoma cell proliferation through the combination of Bcl-2 family inhibitors with chemotherapy indicates that this strategy is a strong therapeutic candidate for chondrosarcoma treatment.

V. Survival pathways in conventional chondrosarcoma

In **chapters 7** and **8** increased kinase signaling was hypothesized to contribute to increased chondrosarcoma survival and as such to chemoresistance. As the Src pathway was previously found to be active in chondrosarcoma (2), its role in

chemoresistance was investigated in **chapter 7** . Moreover, since TP53 mutations are described to play a role in chemoresistance as well (3;4), a possible correlation between response and functional p53 was investigated. Cell lines with mutant TP53 were found to be especially sensitive to the combination of doxorubicin with dasatinib (Src inhibition, fig 9.2). However, dasatinib as a single agent has been shown to overcome chemoresistance in other malignancies despite TP53 mutations (5;6). Thus, also in chondrosarcoma resistance mechanisms could be operable that can be overcome by dasatinib. Interestingly, Src inhibition was additionally found to uniformly inhibit migration across all cell lines, suggesting that chemoresistance and migration act through different pathways.
In **chapter 8,** the chondrosarcoma cell line panel was used toinvestigate activation of receptor tyrosine kinases which further confirmed the heterogeneity of chondrosarcoma cell lines. Moreover, mutation analysis revealed an NRAS mutation in one cell line (SW1353) and in 12% of conventional chondrosarcomas. Downstream pathway analysis showed that despite heterogeneity in RTK activation, all cell lines showed activation of the PI3K/mTOR pathway (fig 9.2). Going back to human chondrosarcoma tissues using tissue microarrays, using pS6 immunohistochemistry, mTOR pathway activation was confirmed in 69% of conventional and 44% of dedifferentiated chondrosarcomas. Using both cell lines and xenograft mouse models, dual PI3K/mTOR inhibitors were found to successfully inhibit chondrosarcoma tumor growth.
In 2D cell culture, a combined strategy of dual PI3K/mTOR inhibitors with MEK inhibitors was found to be most successful, especially in NRAS mutated cells. However, in this study as well as in the literature, in RAS mutated cells such an approach does not lead to the induction of apoptosis.

VI. Leads for new therapeutic strategies to overcome chemoresistance in chondrosarcoma

Chemoresistance mechanisms in chondrosarcoma prove to be complicated. This thesis shows the activation of apoptosis pathways as well as survival pathways. In all cell lines inhibition of Bcl-2 family members with BH3 mimetics in combination with conventional chemotherapeutic agents leads to a dramatic reduction in cell viability. Src inhibition with dasatinib was found to be especially successful in TP53 mutant cell lines when combined with doxorubicin. In addition, circumventing the heterogeneity of upstream RTK activation, PI3K/mTOR activation was found to be a common downstream activation mechanism across cell lines. Moreover, preliminary data show that mTOR inhibition in combination with doxorubicin overcomes chemoresistance (van Oosterwijk et al, unpublished results), providing a third option for treatment in chondrosarcoma. Thus, using the bench to bedside pipeline, the importance of Bcl-2 family members, Src family kinases, and the PI3K/mTOR pathway in chemoresistance was demonstrated. Moreover, in vitro studies suggest a role for combination strategies, in which

inhibition of one of these three pathways renders the tumor cells sensitive to conventional chemotherapeutic agents, overcoming chemoresistance.

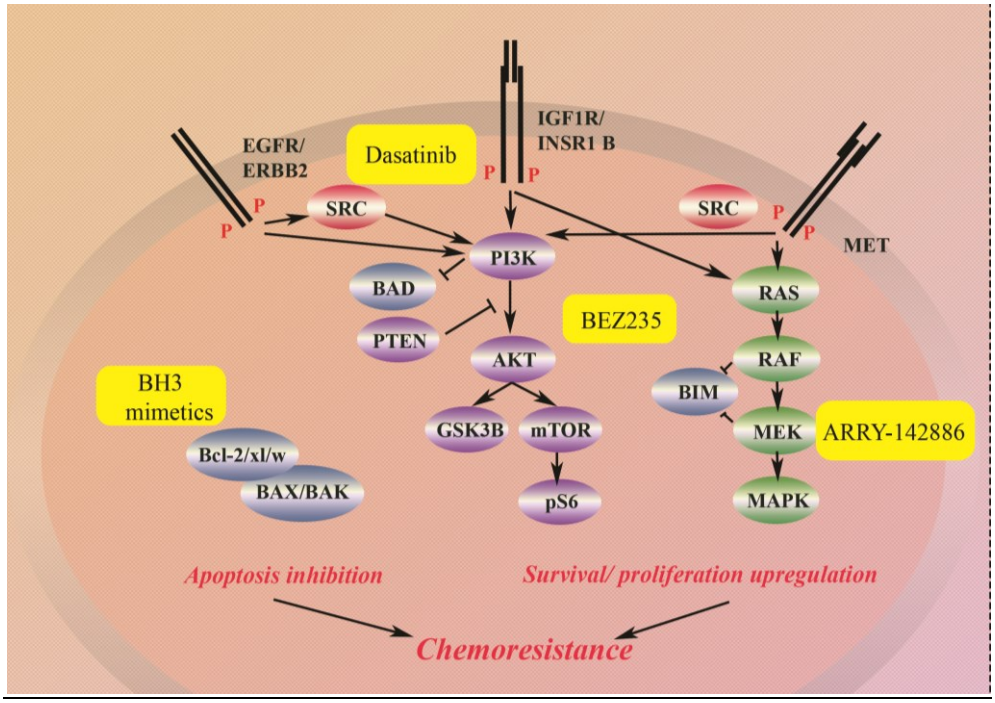

Figure 9.2. Interplay of survival and apoptosis pathways in chondrosarcoma. Activation of EGFR, IGF1R, and MET receptor tyrosine kinases (RTKs) were found using RTK profiling. Further investigation revealed activity of downstream PI3K/mTOR and MAPK pathways. Kinome profiling had previously revealed Src, PI3K, and AKT activity, and immunohistochemistry confirmed high mTOR signaling in human chondrasarcom tissues. The MAPK pathway was found to be activated in a subset of chondrosarcomas due to activating NRAS mutations, therefore downstream targeting with a MEK inhibitor is warranted. Active PI3K/mTOR, Src, and MAPK signaling pathways can lead to increased cell survival, but also promote resistance to apoptosis. Inhibition of apoptosis and upregulation of survival and proliferation can lead to chemoresistance, and targeting these pathways with inhibitors (shown in yellow) was found to successfully inhibit proliferation and overcome chondrosarcoma chemoresistance mechanisms. RAF and MEK can inhibit the transcription of the activating BH3 protein BIM, and PI3K can inhibit the sensitizing BH3 protein BAD. Lack of presence of these BH3 proteins leaves anti-apoptotic Bcl-2 proteins free to sequester the pro-apoptotic proteins BAX and BAK, in which case apoptosis is inhibited. As upregulation of anti-apoptotic Bcl-2 proteins was found in combination with activation of these kinase pathways, a therapeutic approach targeting these pathways simultaneously is advised.

VII. Future prospects

The orthotopic mouse model (**chapter 4**), can be used to explore the *in vivo* efficacy of the combination strategies that were shown here as *in vitro* data suggests these compounds are successful candidates for clinical trials. Future studies might explore the combination of dual PI3K/mTOR inhibitors with MEK inhibition in combination with BH-3 mimetics. This thesis shows the therapeutic potential of PI3K/mTOR inhibitors with MEK inhibitors (**chapter 8**) and of BH3 mimetics (**chapter 5&6**), and recently such an approach was shown to induce apoptosis in RAS mutated cells (7).
To further unravel the role of apoptosis and survival mechanisms in chemoresistance, in the final year a synthetic lethal siRNA screen with doxorubicin and cisplatin has been optimized and performed. The siRNA screen has been performed using the dharmacon libraries for ~80 apoptosis genes and a ~800 kinases. The results of this screen will identify the key players in the pathways described in this thesis and will increase our understanding of the complicated nature of chemoresistance of chondrosarcoma. Moreover, in the future, a similar approach can be used to investigate the resistance to radiotherapy, which will provide answers as to whether this resistance is mediated by similar pathways or that a distinct resistance mechanism is in place. In the coming years, the results from the apoptosis and kinase screen will need to be validated using shRNA, and protein expression of identified genes will be examined on human chondrosarcoma tissue using tissue microarrays. Using 2D cell culture and the orthotopic mouse model, inhibitors will be evaluated for their efficacy targeting the key players identified. This project was designed to corroborate and expand on earlier findings, and the ultimate goal will be proceed to clinical trials. The completion of the bench to bedside pipeline as shown here will hopefully aid in the rapid clinical implementation of effective targeted therapy for chondrosarcoma.

References

(1) Monderer D, Luseau A, Bellec A, David E, Ponsolle S, Saiagh S, Bercegeay S, Piloquet P, Denis MG, Lode L, Redini F, Biger M, Heymann D, Heymann MF, Le BR, Gouin F, Blanchard F. New chondrosarcoma cell lines and mouse models to study the link between chondrogenesis and chemoresistance. Lab Invest 2013.

(2) Schrage YM, Briaire-de Bruijn IH, de Miranda NFCC, van Oosterwijk JG, Taminiau AHM, van Wezel T, Hogendoorn PCW, Bovée JVMG. Kinome profiling of chondrosarcoma reveals Src-pathway activity and dasatinib as option for treatment. Cancer Res 2009;69(15):6216-22.

(3) Donzelli S, Fontemaggi G, Fazi F, Di AS, Padula F, Biagioni F, Muti P, Strano S, Blandino G. MicroRNA-128-2 targets the transcriptional repressor E2F5 enhancing mutant p53 gain of function. Cell Death Differ 2012; 19(6):1038-48.

(4) Huang Y, Jeong JS, Okamura J, Sook-Kim M, Zhu H, Guerrero-Preston R, Ratovitski EA. Global tumor protein p53/p63 interactome: making a case for cisplatin chemoresistance. Cell Cycle 2012;11(12):2367-79.

(5) Bosco R, Rabusin M, Voltan R, Celeghini C, Corallini F, Capitani S, Secchiero P. Anti-leukemic activity of dasatinib in both p53(wild-type) and p53(mutated) B malignant cells. Invest New Drugs 2012;30(1):417-22.

(6) Amrein L, Hernandez TA, Ferrario C, Johnston J, Gibson SB, Panasci L, Aloyz R. Dasatinib sensitizes primary chronic lymphocytic leukaemia lymphocytes to chlorambucil and fludarabine in vitro. Br J Haematol 2008;143(5):698-706.

(7) Tan N, Wong M, Nannini MA, Hong R, Lee LB, Price S, Williams K, Savy PP, Yue P, Sampath D, Settleman J, Fairbrother WJ, Belmont LD. Bcl-2/Bcl-xL Inhibition Increases the Efficacy of Mek Inhibition Alone and in Combination with PI3 Kinase Inhibition in Lung and Pancreatic Tumor Models. Mol Cancer Ther 2013