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A screening based approach to find new paths for targeted treatment in chondrosarcoma

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Chapter 9

Discussion and Future perspectives

Discussion

The studies presented in this thesis describe the use of compound and siRNA screens to identify new targeted treatment options for patients with chondrosarcoma. Using available chondrosarcoma cell lines as a model we investigated apoptotic proteins, kinases and metabolic regulators in a non-biased way to identify most promising hits. In addition the role of individual Bcl-2 family members Bcl-2, Bcl-xl and Bcl-w was investigated. Results reveal a role for Bcl-2 family member Bcl-xl, anti-apoptotic and cell cycle regulator Survivin, Cell cycle regulators AURKA, CHK1 and PLK1 and mTOR as important survival proteins in chondrosarcoma. Moreover treatment with Bcl-xl or CHK1 inhibitors could chemo-sensitize a subset of chondrosarcoma cell lines. Furthermore alterations in the Rb1 pathway have been identified as a marker for radioresistance in chondrosarcoma patient samples. In this chapter these findings are discussed in a broader perspective.

Bcl-xl as most important Bcl-2 family member in chondrosarcoma

The apoptosis pathway is an attractive target in the treatment of cancer, and has been studied extensively. Cancer cells can develop several mechanisms to evade apoptosis including upregulation of anti-apoptotic proteins or downregulation/inactivation of pro-apoptotic proteins or pore-forming proteins Bax and Bak [1]. The mechanism that has now been shown for all chondrosarcoma subtypes, except for periosteal chondrosarcoma, is upregulation of anti-apoptotic Bcl-2 family members [2-4]. In **chapter 3** of this thesis we confirmed this also for mesenchymal chondrosarcoma and showed that these cells can be sensitized towards conventional chemotherapy by inhibiting Bcl-2 family members. In **chapter 4** we looked at the individual Bcl-2 family members and showed that particularly Bcl-xl is important and might be most promising in targeted treatment of chondrosarcoma [5]. We showed that Bcl-2 family members Bcl-2 and Bcl-xl were highly expressed in chondrosarcoma tissue samples and showed a correlation of expression with histological grade. Selective inhibition of Bcl-2 did not result in any decrease in viability in a panel of ten chondrosarcoma cell lines, nor a decrease in tumour growth in an orthotopic rat chondrosarcoma model. However selective Bcl-xl inhibition was effective in a subset of chondrosarcoma cell lines and in vivo tumour growth was slowed down. In addition a sensitizing effect was observed with chemotherapy in a subset of chondrosarcoma cell lines, but not in vivo. These data indicate

that Bcl-xl is the dominant/most important anti-apoptotic family member in chondrosarcoma.

No correlation between protein expression levels and response rate of chondrosarcoma cell lines to specific inhibitors was observed in our study, which can complicate selection of eligible patients. The group of Letai et al developed a method called BH3 profiling to determine the dependency of a certain tumour on individual anti-apoptotic proteins. Mitochondria of cancer cells are isolated and exposed to a panel of different pro-apoptotic synthetic BH3 peptides after which the initiation of MOMP and/or apoptosis is measured. How well these mitochondria respond to the different peptides can be measured and can give an indication of the threshold to achieve apoptosis in these cells, as well as identification of the anti-apoptotic proteins they are most dependent on [6]. In addition a modified version of this method can also be used to predict cytotoxic responses of cancers to different treatment strategies [7] and already showed its predictive value in pre-clinical models of different types of hematologic malignancies [8-10]. This strategy is an attractive method to determine chondrosarcoma dependency on Bcl-2 family members and to predict responses to apoptosis inducing treatments. Further research should determine whether this method is also suitable for solid tumours.

Selective targeting of Bcl-2 family members is highly advantageous in terms of toxicities, compared to combined inhibition using for example ABT-737 or its orally equivalent ABT-263 (navitoclax). However targeting of Bcl-xl seems more problematic compared to targeting Bcl-2 or Mcl-1 due to its expression on platelets. This might be solved by careful dosing and timing of drug administration. In addition, combinations with other treatments might lower the effective dose and consequently also toxicity [11].

In our study we showed that single inhibition of Bcl-2 is ineffective in pre-clinical models, but we did not specifically look into Mcl-1 as a possible important anti-apoptotic protein in chondrosarcoma. Previous studies showed RNA as well as protein expression of Mcl-1 in chondrosarcoma cell lines and several pre-clinical studies suggest it can play a role in resistance mechanisms in solid tumours. It would be interesting to study its role in chondrosarcoma, for example using BH3 profiling [11-15].

Survivin; a pro-survival protein with a broad range of functions

As discussed in **chapter 5**, Survivin was identified as an important survival protein in chondrosarcoma cells by performing an apoptosis focussed siRNA screen [16]. Survivin has multiple functions based on its location in the cells. It's cytoplasmic function is mainly preventing apoptosis, while its nuclear function is regulating mitosis as part of the chromosomal passenger complex (CPC) [17]. Survivin was found to be highly expressed in a large panel (>200) of chondrosarcoma tissue samples of all different subtypes. Expression was found in the nucleus as well as in the cytoplasm, indicating that the apoptotic as well as its cell cycle related function is important in chondrosarcoma cells. No expression was observed in normal articular cartilage.

The exact mechanism by which Survivin inhibits apoptosis is not well understood. The current hypothesis is that Survivin has most inhibitory activity when in complex with XIAP (X-linked inhibitor of apoptosis) and HBXIP (hepatitis B X-interacting protein). Survivin is a member of the IAP family, which consists of eight different members. Only the canonical member XIAP can directly inhibit caspase activity, however interaction with other IAPs can improve its stability and increase its inhibitory effect [17]. When in complex with XIAP and HBXIP, Survivin can inhibit caspase 9, 8 and 3/7 activation [18] and increase the activity of other IAP family members acting in the extrinsic apoptosis route [19]. In addition Survivin has been shown to prevent apaf1 release from mitochondria and bind to the IAP inhibitor Smac/Diablo [20].

The function of Survivin in the cell cycle is essential for mitosis. It is a member of the CPC and ensures that chromosomes are properly aligned by targeting the CPC towards the centrosomes in prometaphase. This process is highly dependent on interaction with BUBR1 and AURKB (Aurora kinase B). In addition the CPC coordinates chromosome segregation and cytokinesis.

How Survivin is regulated is not completely understood. The main pathways that have been shown to regulate its expression are EGFR, IGF-1R or HER2/3 tyrosine kinase receptors, which will lead to activation of RAS and/or AKT and downstream signalling activation of mTOR and NF-kb. HIF-1 α and STAT3 can function as a transcriptional activator of Survivin expression. Several reports suggest that wild type P53 can transcriptionally repress Survivin expression, which will lead to (amongst others) an increase

in apoptosis [21]. In addition, post-translational modifications, during different phases of mitosis, are regulating Survivin function and stability.

Since Survivin is regulated by a variety of different pathways and mechanisms it is hard to speculate which one is most important or most active in chondrosarcoma, however based on available literature and the results described in this thesis the hypothesis is that mTOR can play an important role. In addition, since chondrosarcomas are hypoxic tumours, its connection to HIF-1 α would be interesting to investigate further, for example in 3D models that more closely mimic the patient situation.

A positive correlation between Survivin and P53 overexpression (suggestive for mutated *TP53*) was shown in conventional chondrosarcoma (chapter 5). In addition, chondrosarcoma cell lines with mutant *TP53* were more sensitive for Survivin inhibitor YM155, indicating that especially *TP53* mutant chondrosarcomas might be eligible for treatment with Survivin inhibitors. YM155 is an indirect inhibitor of Survivin; it inhibits the transcription of Survivin by disrupting the RNA binding protein ILF3/NF110 [22]. In addition, the binding of zinc transcription factor SP1 to the Survivin promoter is prevented by YM155 [23]. Since the SP1 transcription factor has more binding regions that activate transcription of genes involved in proliferation and cell cycle progression, there will likely be additional effects of YM155 [24]. This is also reflected in the different responses of cell lines towards YM155 treatment.

Phase I and II clinical trials show that YM155 is well tolerated, but minimal anti-tumour effects have been observed in mono- as well as combination treatment strategies [24, 25]. New Survivin inhibitors have been under development, of which the most promising one is the development of vaccines for cancer immunotherapy. Promising results have been achieved for glioblastoma multiforme and other solid tumours [26-28].

Cell cycle proteins as therapeutic targets

Besides Survivin other cell cycle regulators that might be important in chondrosarcoma have been identified using a combined siRNA screen and compound screen focussed at kinases, as described in **chapter 6** of this thesis. Hits that were identified in both screens were selected for further validation. Using this unbiased approach we identified AURKA (aurora kinase A), PLK1 (Polo like kinase 1) and CHK1 (Checkpoint kinase 1) as important kinases for chondrosarcoma cell survival [29]. These kinases are

all involved in cell cycle regulation, and are essential for proper cell division [30]. Cancer cells show aberrant cell cycle activity, which can be caused by deregulation of cell cycle proteins, or upstream cell signalling pathways [30]. This gives an opportunity to target cell cycle proteins specifically in cancer cells in mono treatment or in combination with chemotherapy [31].

PLK1 and AURKA are both involved in the progression from G2 to S phase during the cell cycle and are important for mitosis and cytokinesis [32]. CHK1 is activated in response to DNA damage by ATM or ATR, and cells will halt the cell cycle in S or G2 to repair the damage. Overexpression of these three cell cycle regulators has been shown in a variety of different tumours and correlates with a worse prognosis [33-35].

A subset of chondrosarcoma patients showed RNA expression of AURKA and CHK1, while PLK1 expression was low compared to normal cartilage. This indicates that PLK1 might not be a good target for chondrosarcoma treatment. AURKA was expressed in chondrosarcoma patient samples, and inhibitors for AURKA are already in clinical trials [30]. Monotherapy with alisertib, the most developed AURKA inhibitor was tested in different solid tumours including sarcoma, and showed a partial response in one out of six patients with dedifferentiated chondrosarcoma [36]. Although first results seemed encouraging [37], a large Phase III trial in lymphoma was discontinued due to lack of clinical response compared to the comparator arm [38]. Combination treatment studies show conflicting results, depending on the dose and tumour type, regarding adverse effects, maximum tolerated dose and efficacy [39-42]. Larger phase III randomized control studies should determine whether combination therapy is superior over standard treatment. High CHK1 RNA expression was correlated towards a worse overall survival, and inhibition of CHK1 sensitized chondrosarcoma cell lines towards conventional chemotherapy treatment. This was also previously shown in other sarcoma types [43-45]. CHK1 is part of the DNA damage pathway, and combination treatment with DNA damaging agents will lead to synthetic lethality. This means that a combination of the two treatments will lead to cell death, while treatment with only one of the two will not cause any or only minimal effect. First generation inhibitors of CHK1 were discontinued due to toxicity, however second generation inhibitors show less toxicity and are currently tested in combination with chemotherapy [34, 46, 47].

Defects in cell cycle regulators and DNA damage pathways are a general hallmark of cancer. The three cell cycle related kinases that we found in this

study are deregulated in a large portion of cancers. In chondrosarcoma specifically CHK1 might be the most interesting and most potent target to investigate further, since this was correlated to overall survival and synthetic lethality with chemotherapy was shown. A correlation was observed between *TP53* mutation status and sensitivity, which is also reported in the literature [48-50]. This indicates that the higher grade chondrosarcomas, harbouring a mutation in *TP53* (20%) [51] might benefit most from inhibitors targeting the cell cycle, as monotherapy or in combination strategies.

Metabolic vulnerabilities in chondrosarcoma; mTOR as central player

Another vulnerability investigated in chondrosarcoma was targeting metabolism. This might be a therapeutic opportunity since approximately 50% of the chondrosarcomas shows a mutation in *IDH1* or *IDH2*, which will lead to the production of oncometabolite D2HG. *IDH1* and *IDH2* have an important function in the citric acid cycle and mutations in these genes might possibly lead to a dysregulated metabolic state and cause vulnerability. In **chapter 7** the execution of a custom-made metabolic compound screen performed in three different chondrosarcoma cell lines is described. This was followed by validation of the most promising compounds on a metabolic level using the Seahorse analyser. In concordance with a recent publication of Zhang et al., we reported that cholesterol inhibitors are effective in chondrosarcoma cells, especially in the *IDH1* mutant line, and lower mitochondrial respiration levels were observed [52]. Dual mTORC1 and C2 inhibitor sapanisertib was found as the most potent inhibitor of oxidative and glycolytic metabolism, and was further validated in a panel of cell lines and in an orthotopic mouse model. Cell lines were sensitive for treatment with sapanisertib, although a plateau was reached at 10-30% viability. Mouse tumours treated with sapanisertib were growing slower compared to control tumours, however resistance was acquired after +/- 8 weeks of treatment [53].

mTOR can form two different complexes; mTORC1 and mTORC2. They both have different functions. mTORC1 is involved in many cellular processes, including mRNA translation, autophagy, amino acid signalling and lipid, glucose and nucleotide metabolism. Its activation is regulated by growth factors and intracellular and environmental stress including energy, oxygen, amino acid and DNA damage levels. The function of mTORC2 is less studied, but it is involved in cytoskeleton regulation, metabolism and cell survival.

mTORC2 is activated by Pi3K regulated mechanisms, but possibly also by metabolic signals [54-56].

Compared to Rapamycin, which is only inhibiting mTORC1, treatment with sapanisertib led to larger decreases in oxidative phosphorylation and glycolysis indicating that mTORC1 and mTORC2 both are regulating metabolism in chondrosarcoma cells. This was not dependent on *IDH1*. Previous reports suggest that D2HG, the oncometabolite produced by cells harbouring a mutation in *IDH1* or *IDH2*, inhibits mTOR signalling [57]. We did not observe any difference in metabolic response towards mTOR inhibitors between *IDH1* mutant cells in which D2HG production was inhibited, compared to control conditions. In addition all tested chondrosarcoma cell lines had a high basic level of P-S6 and P-AKT expression indicating that mTOR signalling is highly active in these cells.

One of the metabolic pathways that is regulated by mTOR is glutamine metabolism. Most cancer cells depend on glutamine for their survival [58]. Our lab previously showed that this is also the case for a subset of chondrosarcoma cells, which was again independent of *IDH* mutation status [59]. Inhibiting glutamine in chondrosarcoma cells resulted in a dose dependent decrease in viability. In addition, chondrosarcoma tissue samples showed an increased expression of GLS, the enzyme converting glutamine to glutamate, which was correlated with histological grade, but not with *IDH* mutation status [59]. Both mTORC1 and mTORC2 promote glutaminolysis and inhibiting mTOR in chondrosarcoma might be partially effective because of its effect on glutamine. Resistance to mTOR inhibition might be caused by adapting mechanisms in AKT, which will lead again to an increase in glutaminolysis [60]. Previous research in lung squamous cell carcinoma xenografts showed that combining dual mTOR inhibition with glutamine inhibition overcomes resistance to mTOR inhibitors [61]. Since we also observed resistance in our *in vivo* experiments it would be interesting to further explore this combination also in chondrosarcoma.

In future research it would be relevant to focus on identifying a metabolic signature for cancer cells to select patients eligible for treatment with certain metabolic inhibitors. This has been done by Momcilovic et al. in lung and head and neck squamous cell carcinoma and osteosarcoma. Expression of a specific set of proteins combined with PET imaging and histology predicted response towards dual mTOR inhibition and glutamine inhibitors in xenograft models [61]. In addition an 11 gene signature together with expression of GAC, a specific isoform of GLS1, was predictive of glutamine

sensitivity in breast cancer. They also showed that upon GLS1 knock down TCA and glutathione intermediates were downregulated and ROS production was increased in breast cancer cell lines. These observations indicate that the combination of glutamine inhibitors together with redox modifiers in patients that show a high prediction score might be worthwhile to investigate [62].

It is important to look into this metabolic signature on a tissue specific, and if possible patient specific level. For example in chondrosarcoma no specific metabolic profile has been found for *IDH* mutated chondrosarcomas, while gliomas harbouring an *IDH* mutation do show for example an increased sensitivity for glutaminase inhibitors [63].

Defects in CDKN2A/RB1 as a marker of radio resistance?

Chondrosarcomas are relative radioresistant tumours; conventional radiotherapy (linear accelerator based) is only given to patients with inoperable tumours, or metastatic disease [64, 65]. A recent retrospective study showed that a small group of patients might still benefit from conventional radiotherapy [65]. In **chapter 8** a study is described to identify possible markers for radioresistance or -sensitivity in chondrosarcoma. Our cell line panel showed a heterogeneous response towards γ -radiation, which was confirmed by a recent publication by Girard et al. [66]. By using an ex vivo culture system we identified that alterations in the RB1 pathway might correlate with radiotherapy resistance in chondrosarcoma [67]. Chondrosarcoma tumour explants were cultured, treated with radiotherapy and afterwards analysed for the induction of double strand breaks using a previously published γ -H2AX assay [68, 69]. Subsequently, tumour material was sequenced and mutations frequently observed in known oncogenes and tumour suppressor genes were analysed. Mutations in *IDH1* and *IDH2* were detected in approximately 50% of chondrosarcomas, but were not associated with the amount of γ -H2AX foci. This was in concordance with the fact that we did not observe a difference in radio sensitivity between *IDH1* mutant cells, in which production of D2HG was inhibited using AGI-5198, and non-treated cells. Conversely alterations in *CDKN2A* and *RB1* were associated with less γ -H2AX foci after radiation, which indicates radio resistance in these patients. In line with our results a previous study described that restoring P16 in chondrosarcoma cell lines increased radio sensitivity [70]. Other studies report an increase in radio sensitivity in tumours that display defects in the RB1 pathway [71-74], indicating a context specific effect. Alterations in one of the components of the RB1 pathway have been

described in the majority of high grade conventional chondrosarcomas, indicating that alterations in this pathway are correlating with a more malignant phenotype [75-78].

The amount of γ -H2AX foci has been shown to be a good marker for the number of double strand breaks induced by radiation and correlations with patient response and amount of foci have been observed in several studies [68, 79-81]. However there are also studies that do not find a correlation between γ -H2AX foci and radiosensitivity [82-84], indicating that this might also be tissue and context specific. The fact that we observe less γ -H2AX foci in RB1 pathway mutated tumours might be explained by an effect of the mutation on cell cycle distribution, which will lead to differences in the amount of repair and the pathway that will be used to repair the breaks. Our study describes the observation that we find more alterations in RB1 pathway genes in tumours that show less γ -H2AX foci after radiation compared to tumours that do not show alterations in RB1 pathway genes. Research more focussed on downstream repair pathways might lead to the identification of the mechanism behind this observation. In addition our panel of chondrosarcoma tissues is very small and heterogenous, and further research in a larger cohort is needed to confirm our findings. Besides its well-known role in the cell cycle RB1 is also involved in a multitude of other cellular processes, including metabolic processes [85]. Cells with inactivated RB1 showed upregulated glutaminase activity and glutamine incorporation into GSH, indicating that tumours with alterations in the RB1 pathway are more susceptible for treatment with glutamine inhibitors [86-88]. We recently published that glutamine can be a potentially therapeutic target for patients with high grade chondrosarcoma [59], although the exact mechanism remained unclear. The most glutamine sensitive cell line, SW1335, also showed the highest GSH levels, and the highest radioresistance, as measured by γ -H2AX foci counting. This cell line harbours a mutation in CDKN2A, influencing the RB1 pathway. It would be interesting to investigate whether alterations in the RB1 pathway might predict for sensitivity for glutamine inhibitors in chondrosarcoma. In addition inhibition of AURKA has been identified as synthetic lethal in cells with defective RB1 [89], suggesting this might be another therapeutic vulnerability for patients with high grade RB1 defective chondrosarcoma. Whether alterations in the RB1 pathway can also predict for sensitivity to proton or high let radiation treatment will be a subject for further investigation.

Future perspectives

In this thesis the use of two focussed siRNA screens to identify new therapeutic targets for chondrosarcoma patients is described. Since the time the siRNA screens were initiated, several technical improvements have been become available, the most important one of which is the discovery of the CRISPR technique. Using CRISPR technology genes can be knocked out very specifically, with reduced aspecific effects compared to siRNAs. In addition to the method used, other improvements are the use of isogenic cell pairs, rather than screening a panel of different cell lines, to study the role of a specific gene. This rules out cell line specific effects that are not related to the effects specific for the cancer type. Furthermore, pooled screening is also an option to detect genes that show synthetic lethality with the gene of interest. Using pooled screening, all different genes are studied at once, cultured for a prolonged period of time and the start and the end population of cells is sequenced to detect which genes are important in the specific cell line.

Another important step is creating 3D models that more closely resemble the tumour in the patient. Cell lines are easy for large screens, but not always good models to do translational research. In our study for example we found that chondrosarcoma cell lines are sensitive to certain types of chemotherapy, while patients do show resistance. In addition we do show a high expression of PLK1 in the cell lines, while expression was absent in the chondrosarcoma primary tumour tissues. This illustrates that cell lines might not be the best models to validate results. In my opinion cell lines are a good model for performing genetic and compound screens, but for validation studies, it would be better to use a 3D model, next to the cell lines, that more closely resembles the patient situation.

In the research described in this thesis, one of the most important aims is to find therapeutic options for patients with inoperable and metastatic disease. In addition, the mechanism behind resistance towards conventional chemotherapy remains to be identified, although there have been some clues from research in cell line models. In this thesis we describe a role for Bcl-xl and CHK1 as potential important proteins in chemoresistance. Since previous studies show that cell lines and 3D cultures show differential sensitivities towards chemotherapy, it will be an important next step to validate these findings in relevant chondrosarcoma 3D models.

In addition, several other potential targets have been identified, including mTOR and Survivin. A clinical trial testing mTOR inhibitors in combination

with cyclophosphamide in chondrosarcoma patients is currently ongoing. In the chondrosarcoma xenograft model after an initial delay in tumour growth, resistance towards mTORC1 and mTORC2 inhibitor sapanisertib was observed within a few weeks. Combination treatment using mTOR inhibitors with glutamine inhibitors might be an interesting strategy to overcome this resistance, and has been shown previously in a lung cancer xenograft model [61].

In conclusion, we found Bcl-2 family member Bcl-xl, cell cycle regulator CHK1 and metabolic regulator mTOR as important mediators of chondrosarcoma survival and chemoresistance. The clinical value of these findings should be further exploited in future studies. In addition, personalized approaches like BH3 and metabolic profiling to select eligible patients for specific treatments might hold great value for future exploration, since no general marker predicting sensitivity to specific inhibitors is available at the moment.

Future translational studies to identify new therapeutic targets for chondrosarcoma patients should focus on CRISPR screens and compound screens in either a large panel of chondrosarcoma cell lines, or isogenic cell pairs to study a specific genetic defect. The validation of these findings should include a 3D based model that most closely resembles the patient situation, and genomic and expression analysis in patient material with the ultimate goal to bring the findings to the clinic.

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