Preclinical validation of Notch therapeutics for cancer treatment

Summary

Cancer is one of the major causes of mortality worldwide. Despite all the advances in molecular targeted therapies and cancer diagnosis, the burden of cancer is increasing worldwide. Many tumors initially respond to treatment but ultimately acquire resistance to radiotherapy and chemotherapy and recur. More than half of all cancer patients receive radiation therapy as part of a curative or palliative treatment often in combination with surgery or chemotherapy. Signaling pathways that control normal cell renewal in adult tissues are often deregulated in cancer and contribute to treatment failure. In this thesis, we studied the role of one such pathway, NOTCH signaling, at the basic and therapeutic level. We investigated the regulatory mechanism of NOTCH4 receptor activation compared to NOTCH1. We demonstrated the contribution of NOTCH activity in treatment resistance and finally we applied a theranostic approach (combining a precise and advanced imaging modalities and the treatment) to conduct a translatable therapeutic targeting of the pathway.

In **Chapter 1**, a general introduction into NOTCH signaling pathway and its inappropriate regulation in different cancer types has been outlined. The therapeutic targeting of the NOTCH pathway in cancer cells and the tumor microenvironment as well as the use of advanced techniques and devices in NOTCH preclinical research with the aim to improve therapy outcome has been introduced. A literature review is followed in **Chapter 2**, in which the role of NOTCH in mediating resistance to radiotherapy and the different intrinsic and extrinsic mechanisms involved has been discussed. This review also extensively outlines different strategies to target NOTCH in cancer cells and provides an overview of both pre-clinical and clinical studies on how targeting NOTCH could become a novel approach for the improvement of tumor treatment by performing appropriate treatment scheduling and patient selection who will most likely benefit from treatment with NOTCH-targeting therapeutics. Additionally, it argues the importance of NOTCH signatures for patient selection. Critically, this review also acknowledges the failure of clinical trials using Notch inhibitors by explaining our incomplete understanding of the unique and redundant functions of the Notch receptors and our inability to select the correct patients and lack of knowledge on the correct timing of interventions.

In **chapter 3**, we present a first comprehensive analysis of the requirements for ligand-dependent and -independent NOTCH4 receptor activation in mammalian cells and describe its differences in comparison to NOTCH1. We find that NOTCH4 receptors can be activated by ligands but to a much lesser extent compared with NOTCH1. Unlike NOTCH1, we showed that NOTCH4 is inefficiently S1 processed thereby less presented on the cell surface and not

susceptible to ligand-independent activation either by heterodimerization domain (HD) activating mutations or by using EDTA as Ca2+ chelators. Progressive deletion of NOTCH4 extracellular domain resulted in an increased transcriptional activity and removing C-HD resulted in a fully active NOTCH4 intracellular domain. Our domain swapping analysis indicated that there is a strong repressive element within the N-HD of NOTCH4 keeping it inactive compared to NOTCH1 suggesting the importance of the NOTCH4 HD domain in restraining its activity. These data demonstrate important structural and functional differences between NOTCH4 compared to NOTCH1.

In **chapter 4**, the contribution of NOTCH activity in radiation resistance of non-small cell lung cancer (NSCLC) has been addressed. We find that *in vitro*, NOTCH activity did not affect the proliferation or intrinsic radiosensitivity of NSCLC cells. However, xenografts expressing a constitutively active ligand-independent form of NOTCH1 (N1ΔE) grew significantly faster, were more hypoxic and showed a radioresistant phenotype. In contrast, xenografts with blocked NOTCH activity (DNMAML) grew slower than wild type tumors. These data indicate an important role of the tumor microenvironment in determining the outcome of NOTCH activity in this context and imply that blocking NOTCH activity in NSCLC might be a promising intervention to improve outcome after radiotherapy.

In **chapter 5**, we establish a preclinical platform in an orthotopic glioblastoma (GBM) model that enables us to accurately delineate tumors, do a planning contrast enhanced micro-CT and follow tumor growth using bioluminescence imaging (BLI). We find a strong correlation between CT volume and BLI-integrated intensity (Pearson coefficient (r) = .85, p = .0002) as well as microCT-delineated tumor size with tumor size obtained via histologic analysis (Pearson coefficient (r) = .88, p = .005). These data imply that BLI intensity can be used to derive tumor volume but that the use of both contrast-enhanced micro-CT and BLI provides complementary tumor growth information, which is particularly useful for modern small animal irradiation devices that make use of micro-CT and BLI for treatment planning, targeting, and monitoring.

Chapter 6 outlines the feasibility of image-guided radiotherapy employing image-guided small animal micro-irradiators using small animal treatment planning software (*SmART*-plan). We establish a radiation-dose response curve in our established orthotopic GBM model and identify an appropriate treatment radiation dose that allows room for therapeutic synergies in combination treatments for example with targeted inhibitors. We demonstrate a precise uniform radiation in all *in vivo* treatment groups at all doses tested

(4,8 and 12 Gy) and dose volume histograms (DVHs) shows accurate dose coverage in the planning target volume which results in tumor growth delay. The image-guided multiple cross-firing beams on a rotating gantry delivers a prescribed dose to an isocenter directed at the center of the target (tumor) and results in greater dose homogeneity and less dose at the organ at risk (OAR). To evaluate the efficacy of combination treatment with chemotherapy, radiotherapy and NOTCH inhibitors in GBM *in vitro*, we also developed a robust 3D spheroid model of glioma cells. We find that 3D spheroid cultures showed a dose-dependent growth delay upon single and combination treatments. These data implies that the both these *in vitro* and *in vivo* techniques can be combined for clinically relevant testing of novel drugs combined with radiation.

We extended the application of our established preclinical workflow from chapter 5 and 6 in **chapter7**, in which the efficacy of a clinically relevant NOTCH inhibitor in combination with radiation and alkylating chemotherapy using temozolomide (TMZ) has been evaluated. We demonstrate that while NOTCH blockade alone does not affect proliferation rate, its combination with radiation and/or TMZ attenuates the proliferation rate of glioma cells *in vitro*. Similarly, we show that NOTCH inhibition as mono-therapy is not effective, while its combination with standard of care treatment (radiation+TMZ) results in a significant and pronounced spheroid growth delay. Moreover, we find that while NOTCH blockade alone has a modest inhibitory effect *in vivo*, its combination with either radiation or TMZ remarkably and significantly enhances its therapeutic effect and improves outcome by >60 days. Moreover, combining TMZ, radiation and Notch inhibitor results in a strong tumor growth delay and in 1 out of 4 mice tumor cure is observed. These findings indicate that Notch inhibition combined with standard of care therapeutic approaches can provide improved survival benefit for GBM and encourage further translational and clinical studies.

In **chapter8**, a general discussion again summarizing the findings within this thesis, reports other studies in which a role for NOTCH in treatment resistant by regulating cancer cell growth and stemness has been demonstrated as well as outlines current challenges for the reliable translation of the new interventions into clinical research.