General discussion and future perspective

Diffuse gliomas are the most common type of malignant primary brain tumors. New treatment options are urgently needed as the prognosis for the majority of glioma patients remains dismal. Novel therapies may be developed if we have a better understanding on the oncogenic pathways involved. In this thesis, we focused on the molecular function and clinical implication of mutations in *IDH1* and *EGFR*, which are involved in the oncogenesis of two distinct subtypes of diffuse gliomas.

ONCOGENIC BIOLOGY OF *IDH* MUTATIONS

Tumors depend on their acquired genetic changes for growth and therefore these changes are good targets for treatment. Several large-scale sequencing studies on diffuse gliomas have identified common genetic events that drive oncogenesis in the various glioma subtypes. One of the most common genetic changes in diffuse low grade gliomas (LGG) involves the *IDH1* gene. Mutations in *IDH1* (or similar mutations in *IDH2*) belong to the earliest genetic changes in LGGs and they are almost always clonal (i.e. present in all tumor cells). Mutations in *IDH1* or *IDH2* alter the normal function of these proteins and result in an enzyme with a novel activity whereby D2HG is produced. Patients with *IDH*-mutated gliomas have a better prognosis and benefit more from chemotherapy/radiation therapy than patients with *IDH* wildtype gliomas [1, 2].

Since mutations in *IDH* genes are amongst the most common identified in LGGs, and because of their clonality and mutation-specific enzymatic activity, they are considered a good target for therapy. Indeed, inhibitors targeting the mutant-specific activity have been developed and these are currently being tested for clinical activity. Although promising clinical responses have been reported in acute myeloid leukemia (AML) patients, the clinical benefit for glioma patients has thus-far been limited [3-5], though the field is still awaiting reports on the clinical trials. The response to IDH inhibitors in AML patients is also remarkable considering the fact that mutations in AML are most-often subclonal, indicating that the tumor was not dependent on the mutation for initial growth. Additional research into the molecular mechanisms affected by mutant IDH1 and IDH2 therefore is required. However, this research is hampered because there are only few preclinical model systems of *IDH*-mutated gliomas.

In this thesis, we have described the generation of two model systems for *IDH*-mutated gliomas. In **Chapter 2** we report on an *in vivo* transgenic zebrafish model system for *IDH1* mutations with CNS-specific expression at the early stage of the embryonic development. Although the D2HG level was significantly increased, our zebrafish remained healthy and no tumors were formed in our models, also not in a *Tp53* mutant

background. Although other transgenic model systems for *Idh* mutations (mice and drosophila) developed phenotypes (brain hemorrhage or wing expansion defects), thusfar no gliomas were identified in any of the *in vivo* animal model systems [6, 7]. This absence suggests that *IDH* mutations alone are insufficient to initiate glioma formation. However, expression of mutant IDH1 in the subventricular zone did result in precancerous subventricular nodules, which suggests involvement of mutant IDH at the early stages of tumor development [8]. The absence of full blown tumors is in contrast to AML where hematopoietic expression of *IDH* mutations alone is sufficient to initiate leukemia in several mouse models and mutant IDH is involved in maintaining the malignancies [9-12].

There are several possibilities as to why no gliomas are formed in the *in-vivo* model systems. Perhaps additional genetic changes should be incorporated including (a combination of) mutations in *TP53*, *ATRX* and/or other, less-common, mutations. Alternatively, the model systems created to-date did not target the correct cell of origin for gliomas. In this case, alternative promoters should be used to drive expression of mutant IDH.

In **Chapter 3** we report on establishing short term cultures of LGGs and show that these cultures retain mutant tumor cells and other driver mutations (albeit at a lower VAF compared to the VAF in the original tumor) and therefore may offer an *in vitro* assay to study downstream pathway alterations and to determine the efficacy of (new) therapeutics. As only very few *IDH*-mutated primary tumor lines have been established to date, and those that have contain many more genetic changes and may even no longer be dependent on the mutation for growth, our assay is a welcome addition to study LGGs. Apart from primary patient-derived lines [13], other options to create *in vitro* model systems include the creation of tumor models using human-induced pluripotent stem cells (HiPSCs) by transforming neuro progenitor cells (NPCs) [14].

The importance of further examining the molecular pathways affected by *IDH1* mutations is shown in **Chapter 4** where we identify a novel pathway that is inhibited by mutant IDH1. Our results demonstrate that MUL1 is a novel binding partner of IDH1 and its function in activating NF-kB is inhibited in *IDH1*-mutated cells, ultimately leading to less sensitivity to TNFα-induced apoptosis. The data described in this chapter can help understand gliomagenesis and identify novel targets for treatment in IDH mutant gliomas.

Generation of proper model systems is important as it will help understand clinical responses to IDH inhibitors. For example, IDH inhibitors initially showed prominent

efficacy in inhibiting tumorigenic properties of different cancer cell lines with *IDH* mutations [15-17] *in vitro* and *in vivo*, though some of the more recent studies failed to confirm this observation [16, 18]. Other therapies for *IDH*-mutated tumors can also be further explored in these model systems. For example, *Sulkowski et al*. showed that the *IDH* mutation-induced D2HG-dependent deficiency in DNA homologous recombination, results in sensitivity to PARP inhibitor treatment [19]. Alternatively, IDH-mutant cells are highly dependent on the level of NAD + and inhibiting the NAD + salvage pathway resulted in cytotoxicity of IDH-mutant cells [18].

It should also be noted that inhibiting D2HG production of *IDH* mutation may pose a risk to patients. For example, *IDH*-mutated gliomas are more sensitive to chemotherapy and radiotherapy due to impaired DNA repair system[17, 20]. As such, inhibiting mutant IDH activity may actually antagonize chemotherapy efficacy. These data demonstrate that further research to better understand the biology of *IDH* mutations in gliomas is required.

The overall results of the randomized phase III European Organization for Research and Treatment (EORTC) 22033-26033 clinical trial did not show differences in clinical efficacy between radiotherapy (RT) vs temozolomide (TMZ) [21]. In **Chapter 7**, we sub-grouped LGGs from the EORTC22033-26033 clinical trial into previously defined intrinsic glioma subtypes (IGS) using gene expression profiling. We have confirmed the prognostic value of IGS. LGG assigned to IGS-9 (most were *IDH*-mutated with 1p19q codeletion) benefited more from RT than from TMZ whereas this benefit was not observed in IGS-17 (most were *IDH*-mutated with intact 1p19q). However, we did not identify predictive markers for response to treatment and it is of note that the follow-up time was limited.

ONCOGENIC BIOLOGY OF MUTATIONS IN *EGFR*

Activating mutations in *EGFR* have been identified in various cancer types. Interestingly, different mutations in this gene are found in different types of cancer. For example, *EGFRvIII* is frequently identified in GBMs whereas over 40 % of non-small cell lung cancer (NSCLC) patients with EGFR mutations carry the *EGFR L858R* mutation. Both *EGFRvIII* and *EGFR L858R* result in a constitutively active form of EGFR, which activates signaling pathways involved in cell proliferation, differentiation and survival. It should be noted that common *EGFR* mutations in NSCLC are mainly within the tyrosine kinase domain, whereas common *EGFR* mutations in GBMs are mainly in the extracellular domain of the receptor. Importantly, EGFR has been considered as a

good therapeutic target for *EGFR*-mutated GBMs as preclinical models demonstrated sustained dependency on the mutation [22, 23]. However, and in marked contrast to *EGFR*-mutated pulmonary adenocarcinoma, inhibiting EGFR phosphorylation by tyrosine kinase inhibitors (TKIs) did not decrease tumor growth nor improve survival, despite the fact the EGFR phosphorylation was effectively inhibited, also in patients (at least for gefitinib) [24-28]. This suggests that an additional oncogenic function of EGFR is required for tumor growth in gliomas. In this thesis, we first examined whether EGFR remains a target for therapy in recurrent GBMs. In **Chapter 6**, we show that *EGFR* amplification in the majority (85 %) of the primary GBMs is retained at recurrence. However, only about 50 % of *EGFRvIII*-positive primary GBMs retained *EGFRvIII* expression at recurrence. Therefore, care should be taken in using EGFRvIII as target as its status may change [29]. In **Chapter 5**, we have also further examined EGFR and its signaling pathway to understand the differences in treatment response between gliomas and pulmonary adenocarcinomas. We have identified mutant-specific binding partners for different *EGFR* mutations (*EGFRvIII* and *EGFR L858R*), which each activated distinct downstream pathways.

In the future, further research into the molecular pathways affected by EGFR is required to understand the lack of treatment response to EGFR TKIs of glioma patients. The proteins and pathways identified in **Chapter 5** may serve as a starting point to provide new insights for treatment development. Apart from the role of EGFR in signal transduction, several studies have reported a role of EGFR in the nucleus, where it directly binds to DNA and induces transcription of various genes [30-32]. Indeed, several of the mutation-specific EGFR-binding partners have a presumed role in the nucleus. This role may be mutation specific as EGFRvIII reportedly has a higher presence in the nucleus than EGFR L858R [33]. Therefore, future research should also include nuclear EGFR as potential treatment target in gliomas.

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