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General Introduction

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1. DIFFUSE GLIOMAS

Diffuse gliomas are the most common type of malignant brain tumors in adults, with an incidence of ~5 per 100 000 adults in the United States each year (1, 2). In the past decade, diffuse gliomas were classified into astrocytoma, oligodendroglioma and oligoastrocytoma based on histological features described by the World Health Organization (WHO) in 2007 (3). Diffuse gliomas are graded from II to IV. Grade II/III gliomas are classified as low grade gliomas (LGG) (3). Grade IV diffuse gliomas are the most aggressive form of gliomas, also known as glioblastoma multiforme (GBM). GBMs are further stratified into primary (those that arise de novo, and comprise ~90% of all GBMs) and secondary GBMs (those that progress from gliomas of lower grades).

Treatment decisions are dependent on the subtype of gliomas. Standard treatments for diffuse glioma patients include surgical resection followed by either radiotherapy (RT), chemotherapy (usually temozolomide or TMZ) or a combination thereof (4-6). However, current treatment strategy has limited improvement on the overall survival of patients (~6 month for RT and 3 months for TMZ in GBM) and almost all patients eventually die from disease progression (7-9).

A major problem of the WHO 2007 classification system based on histological appearance has been the significant intra- and inter-observer variation (10, 11). The technological advances in sequencing technology have led to the identification of almost all cancer genes in gliomas. Interestingly some of the genetic changes segregate in defined histological subtypes but correlate better with patient survival than the histological classification of gliomas (12-15). For example, *isocitrate dehydrogenase 1/2* (*IDH1/2*) mutations occur in 60-80% of Grade II/III gliomas. However, tumors with wildtype *IDH* have a significantly worse prognosis compared with *IDH*-mutated, even within tumors of identical grade (Fig. 1). Primary GBMs (pGBM), are mainly *IDH*-wildtype and they have a median overall survival of 9.9 months (16). Secondary GBMs (sGBM) account for 10% of GBMs and they often harbor mutations in *IDH* and have a better prognosis compared with the *IDH* wild-type GBMs, with a median overall survival of 24 months (16).

1p/19q co-deletion has been associated with oligodendroglial histological features and this patient group was more responsive to chemotherapy than those with intact 1p/19q (6, 17, 18). Molecular alterations in the *alpha thalassemia/mental retardation syndrome X-linked* (*ATRX*) or *tp53* gene have been identified in a more astrocytic-like subset of Grade II/III gliomas and are almost always mutually exclusive with 1p/19q co-deleted gliomas.

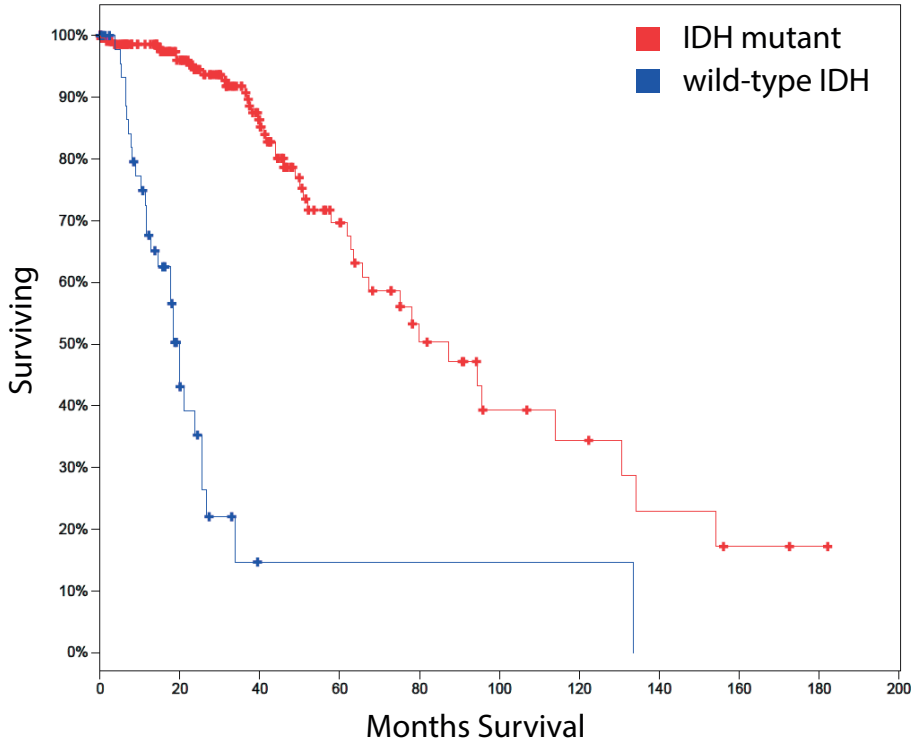

Figure 1. Diffuse glioma patients with mutations in IDH have a better prognosis than the ones with wild-type IDH (TCGA database_283 LGG samples).

The improvement in prognostic classification of gliomas by the molecular markers has led to an update of the WHO classification in 2016. In this update, gliomas are firstly divided into astrocytoma, oligoastrocytoma, oligodendroglioma and GBM based on histology. They are further classified using molecular markers including 1p/19q co-deletion, mutations in *IDH1/2* and *alpha thalassemia/mental retardation syndrome X-linked* (*ATRX*)/*TP53* (Fig. 2) (16). For example, the combination of mutations in both *IDH* and *TP53* defines a molecular astrocytic group of gliomas and the combination of *IDH* mutations and 1p/19q co-deletion defines a molecular oligodendritic group of gliomas. Within histologically identified GBMs, *IDH* mutational status separates pGBM from sGBM.

Besides the molecular markers incorporated in the WHO 2016 classification for central nervous system (CNS) tumors, other molecular markers have been identified that are significantly associated with classification and clinical outcome (15). For example, increased telomerase activity has been discovered in several malignancies including melanomas, liposarcomas and hepatocellular carcinomas (19-21). In diffuse gliomas,

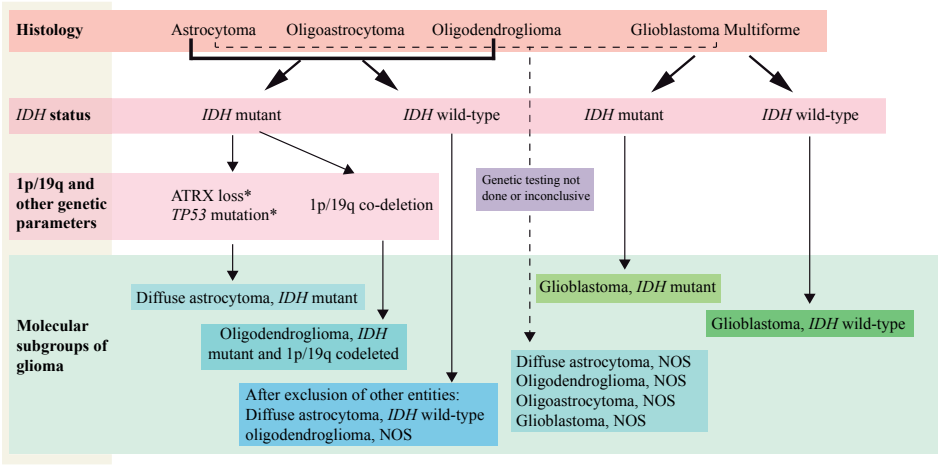

Figure 2. 2016 WHO classification for diffused gliomas using histological and genetic features (16).

mutations in two different genes result in an increased telomere length: mutations in the *ATRX* gene and in the *telomerase reverse transcriptase* (*TERT*) promoter. Mutations in the *TERT* promoter region are present in almost all GBMs and oligodendroglial tumors (28, 29).

Apart from the prognostic biomarkers listed above, there are only few biomarkers that predict response to treatment. Of those, MGMT-promoter methylation is the most robust and is predictive of response to TMZ. TMZ is a commonly used chemotherapy agent which catalyzes alkylation of thymine and guanine, leading to DNA damages and initiation of apoptosis (22). O6-methylguanine-DNA methyltransferase (MGMT) mediates DNA damage repair system by removing alkyl groups and thus prevents apoptosis (23). Therefore patients with MGMT loss showed relatively better responses to TMZ treatment (24). In diffuse gliomas, MGMT promoter methylation has been identified in about 80 % LGGs and 40 % GBMs (25).

2. ISOCITRATE DEHYDROGENASE

Isocitrate dehydrogenase (IDH) catalyzes conversion of isocitrate to α-ketoglutarate (αKG) using $NAD(P)^+$ as a co-factor (Fig. 3, left panel) (26). IDH has three isozymes, IDH1, IDH2 and IDH3. Both IDH1 and IDH2 function as homodimers using NADP + as co-factors. They differ with respect to their subcellular localization with IDH1 being localized in the cytoplasm and peroxisomes and IDH2 in mitochondria. IDH3 func-

tions as a heterotetramer in the mitochondria using NAD^+ as its oxidizing agent and is involved in the citric acid cycle for ATP production (27).

In 2008, one of the first genomic sequencing projects in GBM identified mutations in *IDH1* in 12 % of these tumors (28). As mentioned, *IDH1* mutations correlated with better survival in GBM patients. Subsequent whole genome sequencing efforts have identified *IDH1/2* mutations in multiple malignancies including LGGs, acute myeloid leukemia (AML), chondrosarcomas and cholangiocarcinomas (29-32). *IDH* mutations are a hallmark of LGGs. In gliomas, most of the identified mutations in *IDH* are in *IDH1* and over 90 % of reported *IDH1* mutations are a missense mutation, where arginine at position 132 is replaced by a histidine ($IDH1^{R132H}$). Some patients in whom no mutations in *IDH1* were identified harbored mutations in the *IDH2* gene. Mutations in *IDH2* affect the amino acid R172, an amino acid that is analogous to R132 in IDH1 (29).

The $IDH1^{R132H}$ mutations are mostly heterozygous and the generated mutant enzyme is likely to dimerize with the wildtype counterpart (Fig. 3, right panel). Mutant IDH1 uses αKG as a substrate to produce D-2hydroxyglutarate or D2HG (33). D2HG shares structural similarity with αKG and the accumulation of D2HG inhibits, via competitive inhibition, a number of αKG-dependent enzymes. As a cellular key component, αKG is involved in a wide range of pathways including regulating epigenetic modifications.

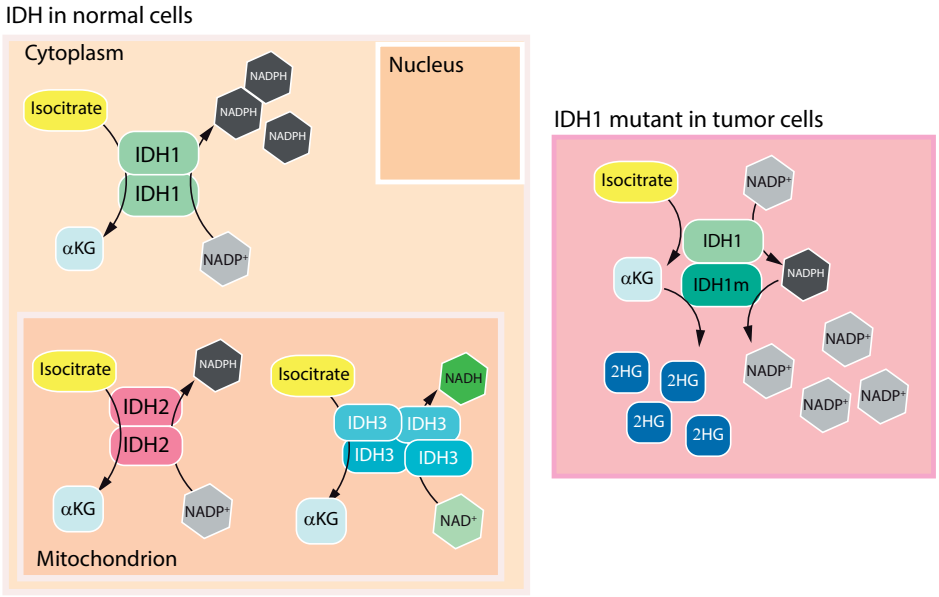

Figure 3. IDH in normal and tumor cells

Examples include Tet methylcytosine dioxygenase 2 (TET2) and Lysine Demethylase 4A (KDM4A)/JMJD2A, enzymes involved in the demethylation of DNA and histones (34-36). The increased level of D2HG also affects the hydroxylation of HIF-1α by inhibiting the Egl nine homolog 1 (EGLN1) prolyl hydroxylase (37), which leads to an upregulation of HIF1α-inducible genes including *vascular endothelial growth factor* (*VEGF*) (38, 39). It should be noted that, due to the different affinities for D2HG, the αKG-dependent enzymes are affected at various levels (35, 36). As a result of the competitive inhibition of αKG-dependent oxygenases by D2HG, *IDH1*-mutant cells ultimately remain in an undifferentiated state (40).

Since IDH1 is thought to play a role in oncogenesis and D2HG production is the key activity of mutant IDH1, several groups have tried to identify mutant IDH1 inhibitors. The first report came from *Popovici-Muller et al.* describing a series compounds with almost 90 % inhibition of D2HG production. The most promising compound also proved active in a U87 glioblastoma xenograft mouse model (41, 42). IDH1 and IDH2 inhibitors are currently being tested in clinical trials (43). A recent clinical trial for relapsed or refractory AML patients showed that treatment with IDH1 inhibitor, ivosidenib was correlated with persistent remission (44). Results of the trials for gliomas have not been reported yet.

However, targeting *IDH* mutations by decreasing D2HG production has been rising concerns and *in vitro* and *in vivo* studies have shown quite conflicting results. For example, some research groups showed decreased proliferation, less colony formation and increased differentiation after the inhibitor treatment to glioma, AML and chondrosarcoma cell lines and their corresponding xenograft models (45-47). On the other hand, in the sarcoma cell line H1060 that harbors an endogenous $IDH1^{R132C}$ mutation, inhibiting D2HG production did not affect the oncogenic properties such as proliferation or migration (48). Additionally, *Molenaar et al.* suggested that D2HG sensitizes tumor cells to ionizing radiation (IR) and inhibiting D2HG production resulted in decreased sensitivity to IR (49). Taken all studies together, other therapeutic targets or treatment strategies for *IDH*-mutated tumors remain to be characterized.

Understanding the molecular mechanism of gliomagenesis driven by *IDH* mutations is a key to seeking potential therapeutic targets. However, understanding the molecular mechanisms of mutant IDH1 has been hampered by the lack of good *in vitro* and *in vivo* model systems. It has been suggested that glioma cells with *IDH1* mutations cannot be propagated in a standard laboratory setting (50, 51), though few successful cultures have been reported (52).

To date, only few animal models have been described for *IDH* mutations in gliomas. One of the first attempts using a brain-specific *IDH1*-mutant knock-in mouse model was embryonically lethal due to D2HG-induced defects in collagen maturation (53). No glial tumors were formed in this model system.

In 2013, *Leenders et al.* reported on glioma xenografts using a patient derived high-grade oligodendroglioma cell line with $IDH1^{R132H}$ mutation (54). In a *Drosophila* model, UAS-Idh-R195H, an $IDH1^{R132H}$ mutation homologue, was induced, which resulted in activation of *tp53* expression and subsequently led to neuronal degeneration and defects in wing expansion (55). Nevertheless, none of the published *in vivo* model systems have generated gliomas by introducing CNS-specific expression of *IDH* mutations alone (53, 55-57).

3. EPIDERMAL GROWTH FACTOR RECEPTOR

Epidermal growth factor receptor or EGFR, is a transmembrane receptor tyrosine kinase protein. The protein is activated following binding of ligands which include epidermal growth factor (EGF), transforming growth factor α (TGFα) and amphiregulin (58, 59). Activated EGFR triggers several signaling cascades including MAPK, AKT and STAT pathways. Activation of these pathways ultimately promote cell differentiation and proliferation (60, 61). Abnormal EGFR activities have been identified in several cancer types including gliomas and pulmonary adenocarcinoma (62, 63). *EGFR* amplification and mutations have been reported in about 57% pGBM patients (64). High copy DNA amplification in gliomas is often seen as double-minutes (extrachromosomal copies of the gene). A subset of GBMs with EGFR amplification harbor additional mutations such as *EGFR variant III* (*EGFRvIII*, an intragenic deletion of exons 2-7). This mutation results in constitutive activation of the receptor (65). Targeted therapies for EGFR including monoclonal antibodies or tyrosine kinase inhibitors have been tested in multiple phase II clinical trials for pGBM patients but did not show significant improvement on overall survival (66, 67).

4. SCOPE OF THIS THESIS

To improve the clinical outcome of glioma patients, there is considerable need to discover novel treatment options for patients. This firstly requires better understanding of the molecular mechanism of the driver mutations in each subgroup of gliomas.

In **Chapter 2 and 3**, we report on the creation of *in vitro* and *in vivo* model systems for understanding the function of driver mutation *IDH1* in LGG. This includes (a) a transgenic zebrafish model with CNS-specific expression of mutations in *IDH1* and (b) short-term primary glioma culture systems using gliomas with *IDH1* mutations. Both model systems showed increased levels of D2HG due to mutations in *IDH1* and can be used as drug screen model systems targeting mutations in *IDH1*. Our *in vivo* model suggests that expression of *IDH1* mutation alone at the early embryonic stage during zebrafish development is insufficient to promote gliomagenesis and even combining *IDH1* mutation with *Tp53* mutation did not increase the tumorigenesis incidences.

In **Chapters 4** and **5**, we further studied the molecular pathways affected by driver mutations in LGG and GBM. For both *IDH1* and *EGFR* mutations, we identified novel binding partners. We discovered that NF-kB, is a novel pathway affected by D2HG produced by mutant IDH1 enzyme, which ultimately may explain why *IDH*-mutated glioma cells keep on proliferating. To study tumor-specific effects of *EGFR* mutations in GBM, we made different *EGFR* clones harboring mutations that are either common to GBM or lung cancer. Our results suggest that each mutation has different binding partners and subsequent activation of downstream pathways. These results argue for the development of mutation specific inhibitors.

Surgical resection of recurrent GBMs is performed only in a minority of patients and treatment strategies using targeted-therapy are heavily dependent on the molecular data of primary tumors. In **Chapter 6** we showed that most of *EGFR* amplification in the primary tumor was retained at tumor recurrence therefore indicates that molecular data obtained in the primary tumor can be used to predict the *EGFR* status of the recurrent tumor. However, half of the *EGFRvIII* expression in the initial tumor is not retained in the recurrent tumor. A final chapter describes gene-expression analysis of samples included in the EORTC22033-26033 clinical trial. We showed that previously defined intrinsic glioma subtypes, subtypes based on unsupervised expression analysis of gene expression data, are prognostic for progression-free survival in EORTC22033-26033 clinical trial samples.

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