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Review

Molecular markers related to patient outcome in patients with IDH-mutant astrocytomas grade 2 to 4: A systematic review

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Abstract ***Background:*** Grading and classification of IDH-mutant astrocytomas has shifted from solely histology towards histology combined with molecular diagnostics. In this systematic review, we give an overview of all currently known clinically relevant molecular markers within IDH-mutant astrocytomas grade 2 to 4.

Methods: A literature search was performed in five electronic databases for English original papers on patient outcome with respect to a molecular marker as determined by DNA/RNA sequencing, micro-arrays, or DNA methylation profiling in IDH-mutant astrocytomas grade 2 to 4. Papers were included if molecular diagnostics were performed on tumour tissue of at least 15 IDH-mutant astrocytoma patients, and if the investigated molecular markers were not limited to the diagnostic markers *MGMT*, *ATRX*, *TERT*, and/or *TP53*.

Results: The literature search identified 4508 unique articles, published between August 2012 and December 2021, of which ultimately 44 articles were included. Numerous molecular markers from these papers were significantly correlated to patient outcome. The associations between patient outcome and non-canonical IDH mutations, PI3K mutations, high expression of *MSH2*, high expression of *RAD18*, homozygous deletion of *CDKN2A/B*, amplification of *PDGFRA*, copy number neutral loss of chromosomal arm 17p, loss of chromosomal arm 19q, the G-CIMP-low DNA methylation cluster, high total CNV, and high tumour mutation burden were confirmed in multiple studies.

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Conclusions: Multiple genetic and epigenetic markers are associated with survival in IDH-mutant astrocytoma patients. Commonly affected are the RB signalling pathway, the RTK-PI3K-mTOR signalling pathway, genomic stability markers, and (epigenetic) gene regulation.

1. Introduction

With the 2016 revision of the World Health Organization (WHO) classification of central nervous system (CNS) tumours, the taxonomy of adult-type diffuse gliomas has shifted from purely histological evaluation towards molecular diagnostics after [1]. Astrocytomas and oligodendrogliomas became characterised by the presence of an IDH mutation, and separated by the absence or presence of combined deletion of chromosomal arms 1p and 19q (1p/19q codeletion) regardless of histological characteristics. In addition to these classification markers, the WHO of 2021 introduced molecular grading of IDH-mutant astrocytomas: IDH-mutant astrocytomas with homozygous deletion of *CDKN2A/B* are now appointed as grade 4 even when lacking necrosis and microvascular proliferation [2]. Although associations with prognosis of several other molecular markers have been described, no other molecular markers than *CDKN2A/B* are currently linked to a specific grade. While the clinical relevance of other molecular markers remains to be determined, these markers may provide insights into the composition of IDH-mutant astrocytomas, aid further tumour grading, and provide opportunities for targeted therapy. Thus, in this systematic review we set out to describe all molecular markers within IDH-mutant astrocytomas grade 2 to 4 which have reported to be of potential clinical significance.

2. Materials and methods

We performed a literature search for original papers written in English on adult human patients with IDH-mutant astrocytomas grade 2 to 4 investigating patient outcome with respect to a molecular marker as determined by DNA sequencing, RNA sequencing, microarrays, or DNA methylation profiling. The literature search was performed on the 7th of January 2022 using the electronic databases Embase, Medline, Web of Science, Cochrane, and the top 200 hits of Google Scholar with the search queries as mentioned in Supplementary Table 1.

After deduplication, papers were screened for inclusion based on title and abstract, and subsequently full-text assessment was performed. We specifically excluded conference abstracts, *in vitro* experiments, animal studies, papers with an IDH-mutant astrocytoma sample size <15, papers measuring a molecular marker in the cerebrospinal fluid or serum, and papers limited to the assessment of *MGMT*, *ATRX*, *TERT*, and/or *TP53*. Screening of titles and abstracts, full-text assessment, and data extraction were performed manually and in duplicate by the two first authors (CMST and WRV). After individual assessment, results were compared and final decisions were made by consensus. Quality assessment was not performed, and therefore no papers were excluded due to poor quality. From the included papers we extracted the number of IDH-mutant astrocytoma patients, the investigated molecular marker, survival data, the WHO tumour grades, if the cohort was publicly available, and whether the cohort was used as a discovery or validation cohort if applicable. Full gene names are summarised in Supplementary Table 2. When the sample size with or without the molecular marker was not mentioned in the text of the articles, the number of patients per group was extracted from the Kaplan–Meier curve if possible. For the survival data, no confidence intervals were calculated nor were median survival times measured by the authors of this review. The review was not registered, nor was a review protocol prepared. All used data has been reported in the manuscript.

3. Results

The literature search identified 9087 articles, of which 4508 unique papers remained after deduplication. After screening of titles and abstracts, 4311 articles were deemed irrelevant for our review and were hence excluded. A full-text assessment of the remaining 197 articles resulted in exclusion of 153 additional articles. The majority of these papers did not look into IDH-mutant astrocytoma patients specifically (n = 132) or described a cohort of IDH-mutant astrocytoma patients smaller than 15 (n = 10). As described in our flowchart (Fig. 1), we ultimately included 44 articles [3–46]. All included articles were published between August 2012 and December 2021. Articles described either personally assembled data sets, freely available public data sets such as The Cancer Genome Atlas (TCGA) and the Chinese Glioma Genome Atlas (CGGA), or a combination of personal and public data sets. In total 26 different data sets were used for the included articles. The investigated molecular markers are summarised both below and in Table 1, subdivided in the following

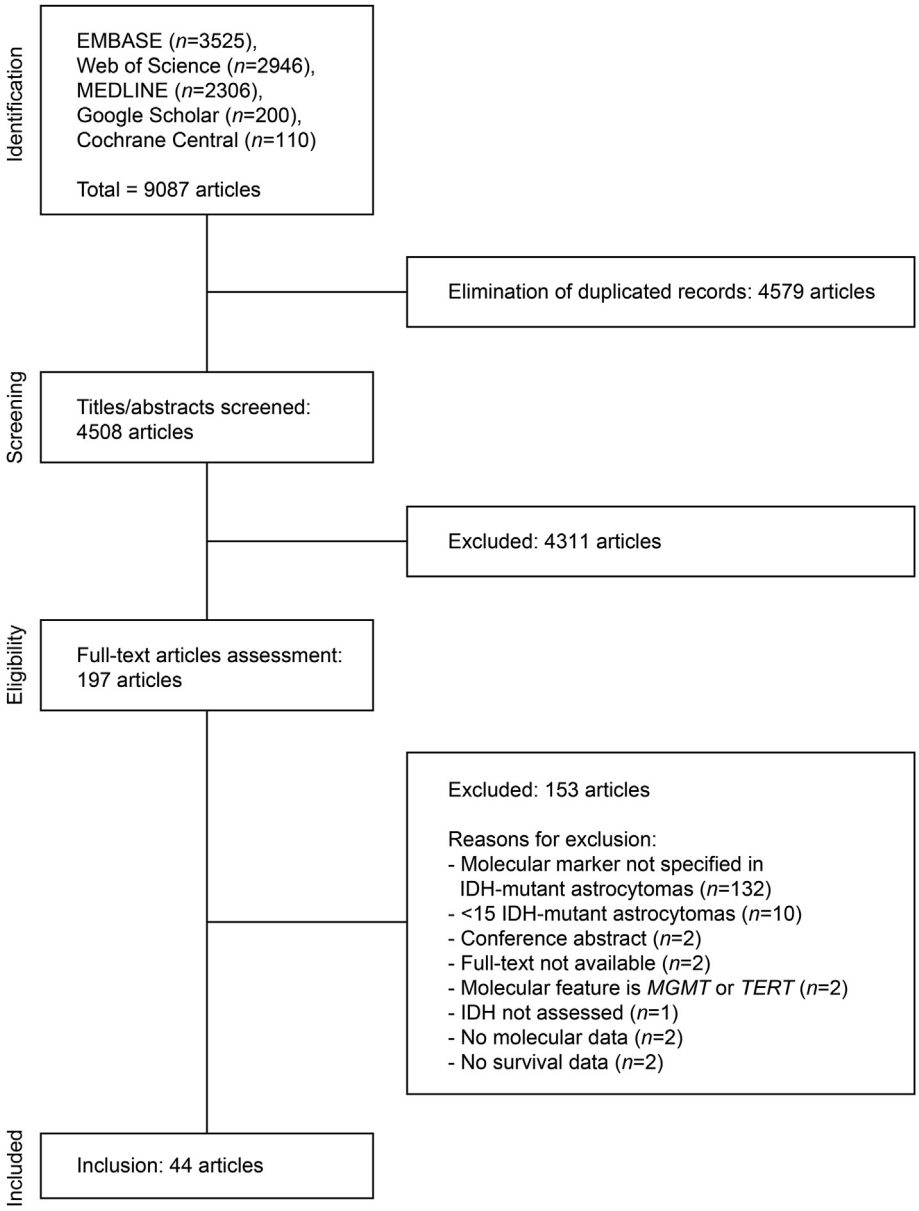

Fig. 1. Flowchart describing the systematic approach for the inclusion of articles.

categories: single gene mutations, RNA expression of single genes, copy number alterations (CNAs) of single genes, larger CNAs, genome-wide changes, and a combination of mutation signatures, copy number signatures, signalling pathways and RNA expression signatures.

3.1. Gene mutations

Mutations in *KMT2D* [21] were associated with prolonged overall survival in one cohort. The presence of so-called 'non-R132H IDH mutations' [33], as opposed to the canonical $IDH1^{R132H}$ mutation, was associated with improved overall survival in two out of three investigated cohorts. Mutations in PI3K genes in general [18], and mutations in *PIK3R1* [6] alone, were significant markers of poor prognosis in multiple cohorts, although this clinical effect was not replicated in every cohort [6,34]. Mutations in *PIK3CA* [6] alone and mutations in *PTEN* [39] were not associated with overall survival.

3.2. Gene expression

High expression of multiple individual genes were negatively associated with prognosis in single cohorts of IDH-mutant astrocytoma grade 2 to 4, i.e. *HOTAIRM1* [3], *HOXD11* [36], *MCM6* [9], and *MPC2* [20]. High expression of *PROX1* [31] was associated with poor overall survival in two independent cohorts. However, it was evaluated as a grouped value in one cohort and as a continuous value in another cohort, thereby the same

statistical analysis to assess the expression of *PROX1* was strictly speaking not validated in an independent cohort. A negative association was found between overall survival and increased expression of *MSH2* and *RAD18* when expression was used as a continuous variable in both the CGGA and TCGA cohorts [24]. However, when expression was dichotomised, high expression of *RAD18* was correlated with a worse outcome only in the TCGA cohort, and high expression of *MSH2* did not show any correlation with overall survival in both cohorts. Upregulation of other genes, such as *FREM2* [46], *TXNDC12* [38], and the eIF3-complex [11] were only associated with poor prognosis in one cohort per gene, but were not significant in other cohorts. High expression of *ADAR3* [44], *PER1* [16] and *WDFY3-AS2* [42] were associated with improved overall survival in grade 2 and 3 IDH-mutant astrocytomas, and for *PER1* and *WDFY3-AS2* also in grade 4 IDH-mutant astrocytomas. The expression of *TGM2* [3] and *MPC1* [20] were not associated with overall survival.

3.3. Gene copy number alterations

The homozygous deletion of *CDKN2A/B* was found to be associated with poor prognosis in multiple studies [6,7,21,29,32,34,44]. Homozygous deletion of *RB1* was associated with poor overall survival in grade 2 to 4 IDH-mutant astrocytoma in one study [32], while in two other cohorts no significance association with overall survival and progression-free survival was seen [7,34]. *FOCAD* loss [8] was negatively associated with overall survival in one cohort, and *PDGFRA* [32,34] amplification in two separate cohorts. *CDK4* [34], *MYCN* [32] and *MET* [34] amplification were associated with poor prognosis in a single cohort, but this association could not be replicated in other cohorts [7,28,32,34]. None of the other single gene deletions or amplifications were associated with overall survival [26,28,32,43].

3.4. Large copy number alterations

We defined large CNAs as copy number changes the size of single chromosomal bands up to entire chromosomes. A positive association was demonstrated between overall survival and copy number neutral loss of heterozygosity (CNLOH) of chromosomal arm 17p [22] in patients with grade 2 to 4 IDH-mutant astrocytomas. Negative correlations were found between overall survival and loss of chromosomal arms 9p [6], 10q [39], 11p [6], 19q [4], and 22q [6]. However, correlations between overall survival and losses of chromosomal arms 9p, 11p, and 22q, were not found in other tested cohorts [6], and the correlation for chromosomal arm 10q was only reported in one patient cohort [39]. The negative correlation for loss of 19q was demonstrated in two cohorts of grade 2 IDH-mutant astrocytomas but not in two cohorts of mixed grade 2 and 3 IDH-mutant astrocytomas [4,6]. Gains of chromosome 7 [39] and chromosomal arm 8q [27] were found to be associated with a worse overall survival, but could not be validated in other cohorts [6,39]. No significant association with patient outcome were found for other losses or gains of chromosomal arms or chromosomal bands [6,39].

3.5. Genome-wide changes

Genome-wide DNA methylation profiles with a higher methylation state such as the Heidelberg cluster 'IDH-mutant astrocytomas lower-grade' (A_IDH), and the so-called 'high methylation cluster' were correlated with a prolonged overall survival in a single patient cohort [32]. Conversely, DNA methylation profiles with a lower methylation state such as glioma-CpG island methylator phenotype (G-CIMP)-low [10,40] in two cohorts, and risk of progression to G-CIMP-low [17] in one cohort are correlated with a worse overall survival in grade 2 to 4 IDH-mutant astrocytoma patients. Other genome-wide aberrances such as a high total copy number variation (CNV) [25,30,32,34], or high tumour mutational burden [5] had an unambiguously negative outcome on survival in multiple patient cohorts as well.

3.6. Pathways and signatures

The individual genes and miRNAs comprising the investigated pathways and signatures are mentioned in Table 2 [6]. The chromosomal instability signature described a set of gene mutations which were associated with poor overall and progression-free survival in a single cohort [30]. A combination signature of *CDKN2A/B* homozygous deletion and *CDK4* amplification showed that harbouring either of these CNAs was negatively associated with overall survival and progression-free survival in the grade 2 and 3 IDH-mutant astrocytomas of the TCGA [25]. However, this association was not observed in the grade 4 IDH-mutant astrocytoma cohort [25].

Five of the investigated pathways were taken from one article using two databases (a personal database and TCGA) in which altered signalling pathways were defined as mutations or CNVs in at least two genes of the pathway [6]. Of these, the RB pathway was negatively correlated with overall survival in both databases, whereas the receptor tyrosine kinase (RTK)-PI3K-mTOR pathway only displayed this negative correlation within the personal database but not the TCGA database [6]. The histone methyltransferase (HMT) pathway, the NOTCH pathway, and the switch/sucrose non-fermentable (SWI/SWF) pathway were not correlated to survival in either database [6].

Almost all of the signatures based on RNA expression were uniformly negatively correlated with overall survival in patients with IDH-mutant astrocytomas, i.e. the 25-gene 1p/19q risk signature [12], the 2-

gene DNA damage response (DDR) signature [24], the 4-gene signature [35], the 4-miRNA risk classifier [14], the 6-gene risk signature [13], the 70 genes of chromosomal instability (CIN70) expression signature [30], the hypoxia-related survival (HRS) score [15], and the Set1, Set2, and Set3 high-risk genes [5]. Of these, the 4-gene signature [35], the 4-miRNA risk classifier [14], and the HRS score [15], were only assessed in a single patient cohort per signature. The 6-gene expression signature [41] showed a negative correlation with overall survival in patients with grade 2 and 3 IDH-mutant astrocytomas, but mixed results (negative correlation versus no correlation) in patients with grade 4 IDH-mutant astrocytomas. Similarly, mixed results were also observed in the 5-gene risk signature [45], the 7-gene signature [19], and the tumour microenvironment (TME) signature [37]. The lymphocyte activation-associated gene signature [23] and the three *FREM2* associated pathway activation levels (PAL1, PAL2, and PAL3) [46] were not associated with patient outcome.

4. Discussion

Grading of IDH-mutant astrocytomas is still primarily performed by histology alone, though many molecular markers are associated with patient outcome. In this systematic review we have described all currently known clinically relevant markers in IDH-mutant astrocytomas grade 2 to 4, identified with DNA/RNA sequencing or DNA methylation profiling. These molecular markers include individual gene mutations, altered expression of individual genes, copy number alterations of individual genes, large copy number alterations, genome-wide changes, altered gene pathways, and (gene) risk signatures. Though many molecular markers were significantly associated with patient outcome, few markers were validated in separate patient cohorts. Non-canonical IDH mutations, PI3K mutations, high expression of *MSH2*, high expression of *RAD18*, homozygous deletion of *CDKN2A/B*, amplification of *PDGFRA*, copy number neutral loss of chromosomal arm 17p, loss of chromosomal arm 19q, the G-CIMP-low DNA methylation cluster, high total CNV, and high tumour mutation burden, were confirmed clinically relevant molecular markers, either in a separate cohort in the same manuscript or in a cohort of a different manuscript. We have highlighted the biological significance of these validated markers, and their proposed role in the formation and maintenance of IDH-mutant astrocytomas below. Pathways and signatures will not be discussed in detail since it is unclear to what extent the individual genes, included in these pathways and signatures, are related to survival. Moreover, the individual genes and proteins with confirmed clinical relevance, i.e. non-R132H IDH, *CDKN2A/B*, *PDGFRA*, PI3K, *RAD18*, and *MSH2*, are also summarised in their respective pathways in Fig. 2.

4.1. CDKN2A/B and RTK-PI3K-mTOR signalling pathway

The proteins that are encoded by *CDKN2A* ($p16^{INK4A}$ and $p14^{ARF}$) and *CDKN2B* ($p15^{INK4B}$) act as tumour suppressors by regulating the cell cycle and apoptosis. The absence of $p14^{ARF}$, removes the inhibition on MDM2-mediated degradation of the p53 tumour suppressor, ultimately inhibiting apoptosis [47]. In addition, homozygous deletion of both *CDKN2A* and *CDKN2B* removes the inhibition of CDK4 and CDK6 by $p16^{INK4A}$ and $p15^{INK4B}$, resulting in the phosphorylation of the RB protein. Phosphorylation of the RB protein renders it unable to repress the E2F transcription factors, thereby inducing the transcription of E2F target genes that are essential for irreversibly driving the cell cycle into S phase, and for initiation of DNA synthesis [48].

Both amplification of *PDGFRA* and activating mutations in PI3K genes, exert their downstream effect by overactivation of the RTK-PI3K-mTOR signalling pathway [49–51]. Under physiological conditions, activated RTKs, such as PDGFRA, recruit PI3K to the plasma membrane where it catalyses the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol 3,4,5-trisphosphate (PIP3) [50,51]. PIP3 recruits AKT to the plasma membrane where AKT is fully activated through phosphorylation. Active AKT phosphorylates a large array of target proteins which are involved in cell survival, cell growth, cell proliferation, cell migration and angiogenesis [52]. Hyperactivation of the RTK-PI3K-mTOR signalling pathway can, for instance, induce cell cycle progression by removing the inhibition on the CDK2 protein, and subsequently phosphorylate the RB protein [53–55]. It may also block tumour cell apoptosis by inhibition of pro-apoptotic proteins such as caspase 9, or by activation of anti-apoptotic proteins such as MDM2 [56,57].

4.2. DNA damage repair and genomic instability

Genomic instability refers to the increased tendency of cells to gain genomic alterations, such as mutations and CNAs, hereby driving tumourigenesis, intratumoural heterogeneity, malignant progression, and therapy resistance [58–60]. *RAD18* and *MSH2* are DNA damage repair genes, which are essential for the maintenance of genomic stability [59,61]. *RAD18* is involved in the activation of the translesion synthesis pathway, and promotes homologous recombination [61], whereas *MSH2* encodes a DNA mismatch repair protein that

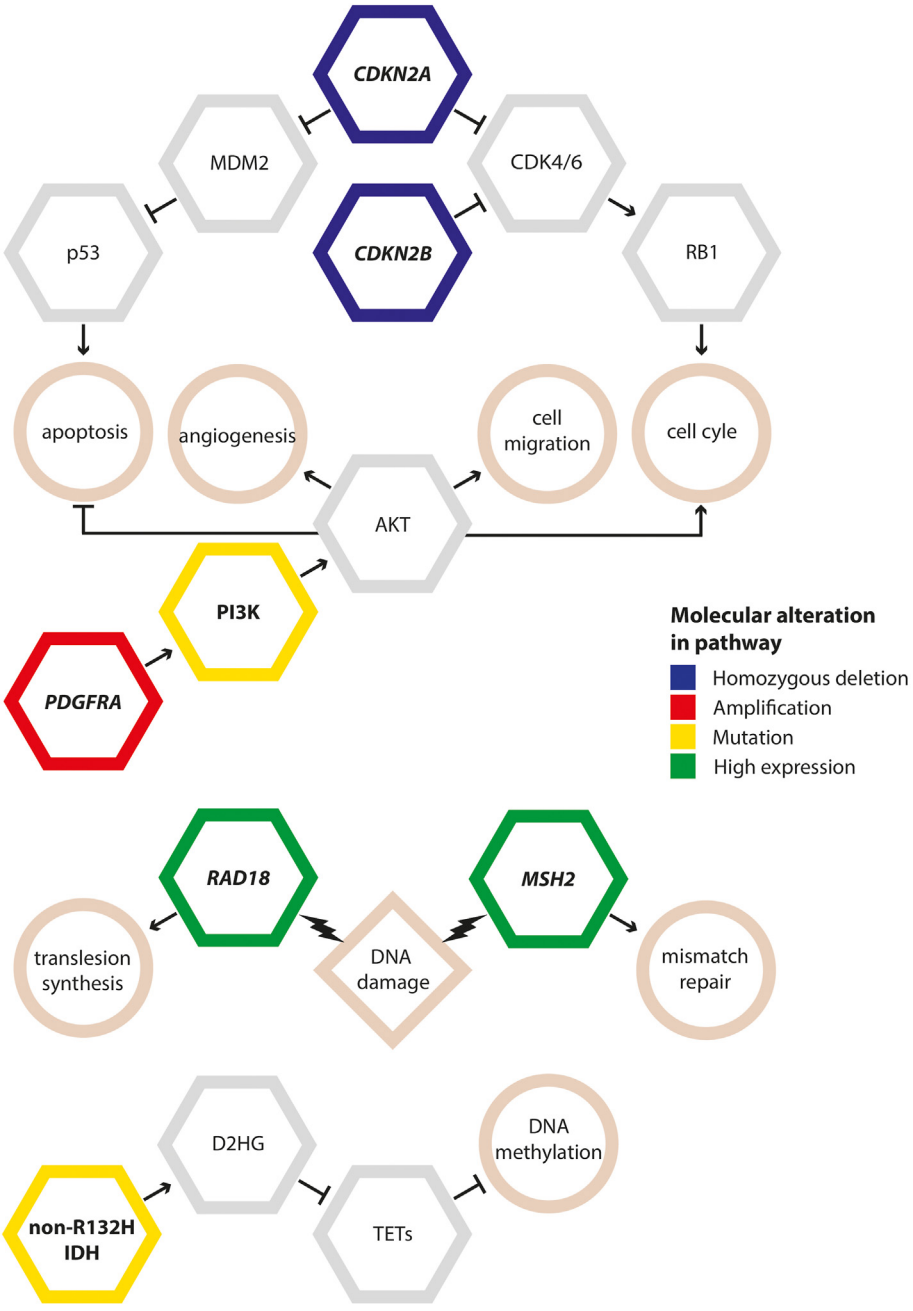

Fig. 2. Graphical overview of molecular markers in their respective pathways. Individual genes and proteins which were associated with survival in at least two independent data sets are coloured in this figure. The pathways concerning *CDKN2A/B*, *PDGFRA*, PI3K, *RAD18*, and *MSH2* portray the physiological processes of these molecular markers. The IDH pathway occurs only due to the specific gain of function mutations. The hexagons represent genes or proteins, the circles represent downstream pathways, and the diamond represents the upstream initiator.

recognises DNA mismatches and initialises DNA repair [59]. Counterintuitively, increased expression of the genomic protectors *RAD18* and *MSH2* were negatively associated with overall survival. A possible explanation could be treatment inefficacy at these higher levels of gene expression; both high expression of *RAD18* and *MSH2* have been correlated to temozolomide resistance *in vitro* [62,63]. However, a retrospective multicenter study on MSH2 protein expression in high-grade gliomas did not find any association with overall survival when correcting for IDH-mutation status [64].

Furthermore, high tumour mutational burden and high total CNV are both indicators of genomic instability. It is unclear if specific affected genes are

responsible for the survival differences, or if these differences are caused by the genomic instability itself. In addition, several CNAs of whole chromosomes and chromosomal arms were shown to be associated with survival. Unfortunately, it is difficult to speculate which genes on these large stretches of DNA may be causal for these survival differences.

4.3. IDH mutations and genome-wide DNA methylation

Wild type IDH1 and IDH2 catalyse the conversion of isocitrate to alpha-ketoglutarate (α-KG), in turn both mutant IDH1 and IDH2 convert α-KG to the oncometabolite D-2-hydroxyglutarate (D-2-HG). This conversion effectively results in the competitive inhibition of a large family of α-KG-dependent enzymes which play crucial roles in tissue homoeostasis and (epigenetic) gene regulation including DNA methylation [65]. IDH-mutant glioma are known to display genome-wide DNA-hypermethylation which is most likely due to the inhibition of the α-KG-dependent TET family 5mC hydroxylases [10,66,67]. DNA methyltransferases catalyse DNA methylation by adding a methyl group at the 5′ carbon of the cytosine ring, resulting in 5-methylcytosine (5mC). TET hydroxylases catalyse DNA demethylation by oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), which is subsequently replaced with unmethylated cytosine through various downstream mechanisms [68–70]. Gliomas with non-canonical IDH mutations, the so-called 'non-R132H IDH mutations', are presumed to have further elevated D-2-HG production compared to tumours with an *IDH1*^{R132H} mutation, which would result in even higher genome-wide DNA methylation levels, which are associated with improved outcome [10,66,71].

Previous studies have shown that G-CIMP-low IDH-mutant astrocytomas are more frequently hypomethylated at CCCTC-binding factor (CTCF) binding sites than G-CIMP-high tumours [10,72]. CTCF proteins simultaneously bind to specific hypomethylated DNA sequences and to other CTCF proteins, thereby forming CTCF homodimers. As a result, the bound DNA on either side of the homodimer are brought together to form a chromatin loop [73]. These chromatin loops can prevent enhancers to come into physical proximity of the transcription start site of their gene of interest [72,73]. Since CTCF only binds to hypomethylated binding sites, G-CIMP-low tumours, and likely other lower genome-wide DNA methylation subgroups as well, are able to form more chromatin loops than G-CIMP-high tumours which might alter the overall gene expression of the tumour.

Exempt from the aforementioned molecular markers, most other molecular markers associated with patient outcome were either only found in a single patient cohort with IDH-mutant astrocytomas, or validation cohorts were unable to replicate the results from the discovery cohort. We were therefore unable to confirm the clinical relevance of these molecular markers. Even for the molecular markers confirmed in two independent data sets the level of evidence might be insufficient for the marker to be readily used for patient prognostication, e.g. loss of 19q was negatively associated with overall survival in two cohorts whereas no correlation was found in two other cohorts. Novel independent data sets are necessary to evaluate the clinical significance of molecular markers especially for those with conflicting data.

On a separate note, for the RNA expression of individual genes, patient cohorts are often dichotomised on the median value of the markers in high and low subgroups. It is unclear whether these divisions translate into any biological differences, and exploratory analyses into (biologically) relevant cut-off points per expression marker would be advisable.

Furthermore, all reported articles were based on either retrospective patient cohorts or post-hoc analyses of prospective patient cohorts. Most of these articles predated the 2021 WHO classification of CNS tumours, and relevance of the identified markers therefore requires confirmation in this setting. The prognostic relevance of the molecular markers in IDH-mutant astrocytomas should ideally be verified in clinical trials in patients which are prospectively selected for the presence or absence of a molecular marker to prevent biases such as differences in post-operative treatment. Since follow-up in IDH-mutant astrocytoma patients can extend to 20 years, a more pragmatic approach is retrospective patient accrual via international consortia to increase the sample size of patients with the presence of a rare molecular marker with in theory readily available follow-up. However, in current large publicly available retrospective data sets on IDH-mutant astrocytomas the clinical annotation is often incomplete or absent, and there is often a publication bias for molecular markers with a significant association with overall survival. This emphasises the need for international consensus on uniform data collection, and standardised publication of all survival results.

Despite the detailed molecular characterisation of IDH-mutant astrocytomas, no effective targeted therapy has yet emerged from these efforts. However, this systematic review has highlighted molecular markers for which inhibitors are available, but have not yet been tested in this specific target population. The identified markers may also lead to the development of novel agents against targets for which no drug is currently available.

In summary, multiple genetic and epigenetic changes are associated with survival in IDH-mutant astrocytoma patients, and commonly affected are the RB signalling pathway, the RTK-PI3K-mTOR signalling pathway, genomic stability markers, and (epigenetic) gene regulation. However, only a select set of molecular markers

was validated in independent patient cohorts. Since validation studies without an association with overall survival are often underreported, the clinical relevance of unconfirmed molecular markers, i.e. identified in a single patient cohort, requires careful consideration. With the increased importance of molecular diagnostics for the grading of gliomas, the need for clinically and biologically relevant validated markers becomes apparent. To this end, international collaborations should be initialised to establish large patient cohorts with consensus on uniform data collection and standardised publication protocols.

Author contributions

CMST and WRV were responsible for the manual data collection. All authors were responsible for the study design, data analysis, data interpretation, writing, and approval of the final version of the manuscript.

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Conflict of interest statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: MJvdB reports grants from Dutch Cancer Foundation, grants from Brain Tumor Charity, grants from Strijd van Salland, grants from MSD formerly Schering Plough, during the conduct of the study; personal fees from Carthera, personal fees from Nerviano, personal fees from Bayer, personal fees from Celgene, personal fees from Agios, personal fees from Abbvie, personal fees from Karyopharm, personal fees from Boston Pharmaceuticals, personal fees from Genenta, outside the submitted work. All other authors declare no competing interests.

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Appendix A. Supplementary data

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