DNA methylation profiling for molecular classification of adult diffuse lower-grade gliomas

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Abstract

Background: DNA methylation profiling has facilitated and improved the classification of a wide variety of tumors of the central nervous system. In this study, we investigated the potential utility of DNA methylation profiling to achieve molecular diagnosis in adult primary diffuse lower-grade glioma (dLGG) according to WHO 2016 classification system. We also evaluated whether methylation profiling could provide improved molecular characterization and identify prognostic differences beyond the classical histological WHO grade together with IDH mutation status and 1p/19q codeletion status. All patients diagnosed with dLGG in the period 2007–2016 from the Västra Götaland region in Sweden were assessed for inclusion in the study.

Results: A total of 166 dLGG cases were subjected for genome-wide DNA methylation analysis. Of these, 126 (76%) were assigned a defined diagnostic methylation class with a class prediction score ≥ 0.84 and subclass score ≥ 0.50. The assigned methylation classes were highly associated with their IDH mutation status and 1p/19q codeletion status. IDH-wildtype gliomas were further divided into subgroups with distinct molecular features.

Conclusion: The stratification of the patients by methylation profiling was as effective as the integrated WHO 2016 molecular reclassification at predicting the clinical outcome of the patients. Our study shows that DNA methylation profiling is a reliable and robust approach for the classification of dLGG into molecular defined subgroups, providing accurate detection of molecular markers according to WHO 2016 classification.

Keywords: DNA methylation profiling, Diffuse lower-grade glioma, DNA methylation-based classification, Molecular classification, Prognosis

Background

Diffuse lower-grade gliomas (dLGGs; World Health Organization [WHO] grade II and III) are primary infiltrative neoplasms of the central nervous system (CNS) that exhibits a highly variable clinical behavior and diverse biological features [1, 2]. These tumors recur frequently and will eventually undergo malignant transformation to gliomas of higher grades, worsening the prognosis of the patients [2–4].

For many years, the classification of diffuse gliomas relied purely on histopathological criteria and was subjected to high inter-observer variability, with substantial inconsistency in predicting clinical outcomes [1–5]. Major recent advances in genomic analysis have expanded our understanding of the molecular alterations characterizing dLGG, identifying central
molecular biomarkers with diagnostic and prognostic capabilities. In the revised WHO 2016 classification, the incorporation of molecular biomarkers, such as mutations in the isocitrate dehydrogenase (IDH) genes 1 and 2 and codeletion of the 1p and 19q chromosomal arms (1p/19q codeletion), together with histological features, offers a more objective clinical prognostic stratification of patients with dLGG [6–8].

DNA methylation is the most extensively studied epigenetic mechanism, as it plays a key role in the regulation of gene expression and in the development of cells. Aberrant alterations of the methylome are found in several human diseases, including cancer [9, 10]. In recent years, genome-wide DNA methylation profiling has emerged as a powerful analytical tool for characterization of a wide variety of CNS tumors and has been shown to be a highly robust and reproducible technique for profiling fresh-frozen tumor samples and tumors archived as formalin-fixed paraffin-embedded (FFPE) samples [11, 12]. Furthermore, the use of DNA methylation profiling in the classification of CNS tumors, has been recognized to be a valuable asset to stratify patients into clinically relevant subgroups [13–16] and to facilitate an integrated diagnosis when diagnostic discrepancies are encountered [17–19].

In this study, we investigated the value of using DNA methylation profiling in adult patients with dLGG. Through generation of genome-wide methylation profiles from 166 tumor specimens, we retrospectively assessed the capacity of methylation profiling to achieve WHO 2016 classification directly and whether methylation profiling could add any useful molecular refinement to the WHO 2016 classification. We further evaluated the methylation-based classification in survival analyses for outcome prediction and compared this with the classical WHO grading in addition to IDH and 1p/19q codeletion status. We demonstrate that methylation profiling is a valuable technique for providing reliable diagnostic and prognostic information for patients with dLGG.

**Results**

**Cohort characteristics**

Between 2007 and 2016, a total of 210 adult patients underwent surgical resection for primary dLGG at the neurosurgical department at the Sahlgrenska University Hospital (Gothenburg, Sweden). Of all cases, 168 patients were subjected for methylation profiling, were we excluded two patients due to poor tumor quality after quality assessment of the methylation array data. The clinical characteristics of the included 166 patients are listed in Table 1.

| Table 1 Clinical characteristics of the studied population with diffuse lower-grade gliomas (n = 166) |
| Variables | Lower-grade gliomas (%) |
| Grade II, n = 74 | Grade III, n = 92 |
| --- | --- |
| **Gender** | | |
| Female | 26 (35) | 40 (43) |
| Ratio male:female | 1.9 | 1.3 |
| **Age at diagnosis—years** | | |
| Mean±SD | 48±13.5 | 41±13.6 |
| **Age at diagnosis—groups** | | |
| 18–29 | 7 (9) | 16 (17) |
| 30–39 | 17 (23) | 24 (26) |
| 40–49 | 17 (23) | 23 (25) |
| 50–59 | 16 (22) | 21 (23) |
| 60–69 | 15 (20) | 6 (7) |
| ≥ 70 | 2 (3) | 2 (2) |
| **Tumor location** | | |
| Frontal | 40 (54) | 51 (55) |
| Insular | 3 (4) | 3 (3) |
| Occipital | 1 (1) | 2 (2) |
| Parietal | 6 (8) | 11 (12) |
| Temporal | 24 (32) | 24 (26) |
| Basal ganglia | 0 | 1 (1) |
| **Type of surgery** | | |
| Biopsy | 6 (8) | 2 (2) |
| Resection | 68 (92) | 90 (98) |
| **Size of tumor** | | |
| < 4 cm | 17 (23) | 14 (15) |
| 4–6 cm | 31 (42) | 49 (53) |
| > 6 cm | 26 (35) | 29 (32) |
| **Histopathological diagnosis (WHO 2007)** | | |
| Astrocytoma | 45 (61) | 46 (50) |
| Oligoastrocytoma | 15 (20) | 30 (33) |
| Oligodendroglioma | 14 (19) | 16 (17) |
| **Molecular biomarkers** | | |
| IDH-mutant | 14 (19) | 1 (1) |
| IDH-wildtype | 2 (3) | 4 (4) |
| 1p/19q codeletion | 18 (24) | 14 (15) |

SD, Standard deviation

**Molecular characterization and reclassification according to WHO 2016 CNS classification system**

Assessment of the IDH mutation status and 1p/19q codeletion is central in current diagnostics of diffuse gliomas [1]. Therefore, we evaluated the robustness of methylation profiling in detecting these diagnostic biomarkers compared to the clinically used molecular techniques for such purpose, i.e., immunohistochemistry (IHC), next-generation sequencing (NGS), Sanger sequencing, fluorescent *in situ* hybridization (FISH)
Determination of \textit{IDH} mutation status by Sanger sequencing analyses of 165 of the cases detected 120 tumors harboring \textit{IDH} mutations. The \textit{IDH} mutation-associated G-CIMP phenotype, inferred from the DNA methylation array data, showed 100% sensitivity and specificity when compared to Sanger sequencing. After re-examination of the dLGG cases, two cases showed discrepant results compared to IHC. The discrepant cases were re-analyzed by tissue microarray in combination with immunohistochemistry for the \textit{IDH} mutation R132H and \textit{ATRX} mutation analysis, which validated the results from Sanger sequencing/methylation profiling.

Of 59 dLGG cases with clinical determination of 1p/19q codeletion status we excluded three cases from the analysis due to suspicion of normal brain tissue after evaluation of their CNV profiles generated from the methylation array. Of the included 56 cases, 32 cases presented 1p/19q codeletions. From the CNV profiles, we identified 1p/19q codeletions in 32 out of the 57 cases with a sensitivity of 94% and a specificity of 92% (Fig. 1). The samples with discrepant results (n = 4) were re-evaluated by a specialist in clinical neuropathology (TOB). For one of the four discrepant cases, re-examination revealed a misinterpretation of the FISH result at time of diagnosis and the case did not harbor a 1p/19q codeletion, corroborating the results from the methylation analysis. Re-evaluation of the remaining three discrepant cases, including additional clinical, radiological, histological and molecular data, supported the results from methylation analysis, increasing the sensitivity and specificity of the methylation array to 100%.

We next reclassified the dLGG cases by integrating molecular data (\textit{IDH} mutation status and 1p/19q codeletion status) from IHC, NGS, Sanger sequencing, FISH and MLPA into the histopathological diagnosis. As our results showed that DNA methylation profiling provides accurate information of 1p/19q codeletion status (Fig. 1), we also incorporated 1p/19q codeletion status from CNV profiles to those cases where 1p/19q codeletion was not analyzed at time of clinical diagnosis (n = 107). Molecular reclassification of the dLGG cases according to the WHO 2016 criteria is shown in Additional file 1: Supplementary figure S1.

**DNA methylation-based classification**

DNA methylation profiles from the dLGG samples were analyzed by a DNA methylation-based classifier [13]. Of the 166 profiled cases, 79% (131/166) were assigned a defined DNA methylation class by the classifier tool with a class prediction score \( \geq 0.84 \) (Fig. 2a). For 96% (126/131) of those cases, the classifier assigned a specific methylation subclass with a prediction score \( \geq 0.50 \). The classifier was not able to predict a methylation class with a prediction score \( > 0.30 \), herein denoted as “unclassified” cases. This outcome was observed in 6% (10/166) of the cases and will be discussed further below.

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**Table 2** Schematic overview of the diagnostic analyses performed for determining molecular biomarkers according to WHO 2016 classification

| Molecular diagnostic biomarkers | Molecular techniques used in clinical diagnostics | Methylation profiling |
|--------------------------------|-----------------------------------------------|-----------------------|
| Tumor grade                    | Histology                                     | –                     |
| \textit{IDH} mutation status   | Immunohistochemistry                          | G-CIMP\(^c\)          |
| 1p/19q codeletion\(^b\)        | Next-generation sequencing                    |                       |
|                                | Sanger sequencing                             |                       |
|                                | Fluorescent in situ hybridization             | Copy number variation profiles |
|                                | Multiplex ligation-dependent probe amplification |                       |

\(^a\) \textit{IDH}, isocitrate dehydrogenase gene family  
\(^b\) Codelet, complete codeletion of 1p/19q chromosomal arms  
\(^c\) Glioma CpG island methylator phenotype
We identified four distinctive methylation classes of brain tumors in the dLGG cohort with a class prediction score $\geq 0.84$ (Fig. 2b). The majority of the cases were classified as glioma IDH-mutant (84%, 110/131), while 12% (16/131) were classified as glioblastoma IDH-wildtype. In addition, 2% (2/131) of the cases were classified as other tumor types than diffuse glioma, i.e., low-grade glioma MYB/MYBL1 and (anaplastic) pleomorphic xanthoastrocytoma.

The methylation class glioma IDH-mutant comprised 48% (51/106) 1p/19q codeleted oligodendroglioma tumors, 45% (48/106) astrocytoma and 7% (7/106) high-grade astrocytoma (Fig. 2c). Within the glioblastoma IDH-wildtype methylation class, we found five methylation subclasses of which the methylation subclass receptor tyrosine kinase II (RTK II) represented the majority of the cases (47%, 7/15), followed by the 20% (3/15) RTK I and mesenchymal 20% (3/15).

In order to investigate the diagnostic value of methylation profiling, we compared the WHO 2016 molecular reclassification with methylation-based classification. Methylation profiling provided similar molecular characterization of dLGG cases as the integrated molecular diagnosis (Fig. 3). dLGG cases reclassified as oligodendrogliomas, harboring IDH mutations and 1p/19q codeletions, fall into the same methylation subgroup independently of the histopathological grade. IDH-mutant astrocytic gliomas were defined into two different methylation subgroups (astrocytoma or high-grade astrocytoma) that did not fully correspond to the assigned WHO grade. IDH-wildtype astrocytomas were the most heterogeneous molecular group as the grade II and grade III tumors were stratified into distinct methylation subgroups of glioblastoma or different molecular entities (i.e., low-grade glioma MYB/MYBL1 or (anaplastic) pleomorphic xanthoastrocytoma) that are not included in the category of "diffuse astrocytic and oligodendroglial tumors" in the WHO 2016 classification system. The IDH-wildtype astrocytic gliomas classified as glioblastoma IDH-wildtype (n = 15) harbored other features characteristic of glioblastoma [20]. These included EGFR amplifications (87%, 13/15), whole chromosome 7 gain (53%, 8/15) or whole chromosome 10 loss (73%, 11/15). The IDH-wildtype astrocytic glioma classified as low-grade glioma MYB/MYBL1 by methylation profiling, was negative for IDH1/IDH2 and BRAF mutations by
sequencing analysis and was 1p/19q non-codeleted. The diagnosis was changed to angiocentric glioma grade I (WHO 2016) after re-evaluation by the neuropathologist. Likewise, the IDH-wildtype astrocytoma classified as (anaplastic) pleomorphic xanthoastrocytoma harbored a BRAF V600E mutation and was IDH1-wildtype detected by Sanger sequencing. In addition, CNV analysis showed focal deletions of CDKN2A/B and no other chromosomal aberrations consistent with IDH-wildtype glioblastomas were found. This case was reevaluated and the diagnosis changed to anaplastic pleomorphic xanthoastrocytoma (WHO 2016).

**DNA methylation profiling for prediction of overall survival**

We further investigated the potential of methylation profiling in predicting prognosis of the dLGG patients compared with the WHO 2016 molecular reclassification. Patients with tumors classified as control tissues by the classifier tool and entities with \( n = 1 \) were excluded from this survival analysis. Methylation-based classification provided similar prognostication compared to molecular reclassification using IDH mutation status and 1p/19q codeletion status according to WHO 2016 (Fig. 4a, b). Among the glioma IDH-mutant group, 1p/19q codeleted oligodendrogloma had a better prognosis (median survival not reached) than did patients with an astrocytoma subclass (median survival 115 months), who in turn had better prognosis than patients with high-grade astrocytoma subclass (median survival 60 months).

**MGMT promoter methylation and CDKN2A/B homozygous deletion as prognostic biomarkers in dLGG**

The MGMT promoter methylation status is a predictive biomarker that can be used for treatment allocation in patients with glioblastoma [21, 22]. It has also been demonstrated to be an independent prognostic biomarker for high-risk low-grade glioma patients who received radiotherapy (RT) with adjuvant temozolomide (TMZ) treatment [23]. In our cohort, the MGMT promotor was methylated in 92% (97/106) and unmethylated in 8% (9/106) of the tumors classified as glioma IDH-mutant (Table 3). Of the 97 patients with a methylated promotor, 74% (72/97) received chemotherapy with TMZ and/or procarbazine, lomustine and vincristine. In the unmethylated group, six patients received such treatment. In the IDH-wildtype glioma group (i.e., glioblastoma, low-grade glioma MYB/MYBL1 and (anaplastic) pleomorphic xanthoastrocytoma), MGMT promotor was methylated in 29% (5/17) and unmethylated in 71% (12/17) of the cases. Four patients in the methylated group were treated with alkylating agents compared to nine patients in the unmethylated group.

Homozygous deletion of the CDKN2A/B gene has been identified as a biomarker for improved grading of IDH-mutant diffuse astrocytic gliomas [24]. We therefore studied the prognostic value of CDKN2A/B in our classified tumor cohort (\( n = 123 \)). Homozygous deletion of the CDKN2A/B gene was detected in a larger proportion of
the glioblastoma IDH-wildtype tumors (9/15) compared to glioma IDH-mutant tumors (9/106) (Fisher’s exact test p value < 0.001). Survival analysis did not show significant differences according to CDKN2A/B status within the defined molecular subgroups, possibly due to the limited number of cases.

Evaluation of dLGG cases with low classification scores and unclassified cases

To fully substantiate the impact of methylation-based classification for the WHO 2016 classification system, we evaluated the cases with low class/subclass prediction scores as these classification results can still provide important diagnostic information [25]. Three percent (5/166) of the tumors were assigned a methylation class (prediction score ≥ 0.84) but were not further subclassified (prediction score < 0.50), and for 21% (35/166), the class prediction score ranged between 0.30 and 0.83 (Fig. 2a and 5a) or were unclassified. The median methylation class score notably varied across the methylation classes (Fig. 5a). Methylation-based classification suggested, in the majority of the cases, diagnoses supported by CNV signatures and prognostic outcomes of the predicted methylation class/subclass (Additional file 1: Figure S2A–B). For example, the molecular reclassification of IDH-mutant, 1p/19q codeleted oligodendrogliomas (n = 3) was in accordance with the given methylation subgroup. IDH-wildtype astrocytic gliomas with a glioblastoma classification (n = 9), presented typical chromosomal changes (EGFR amplification, chromosome 7 gain or chromosome 10 loss) and prognosis of glioblastoma with the exception of the case classified as glioblastoma IDH-wildtype, midline (Additional file 2: table S1). For other IDH-wildtype astrocytic gliomas, particularly of grade II, the suggested methylation class was not associated with dLGG. For one of these cases, the methylation class plexus tumor, subclass pediatric B was predicted with a score of 0.30 by the classifier and the CNV profile from the methylation array was not indicative of a plexus tumor [26]. Other tumors were classified with the brain control tissue methylation classes (hemispheric cortex, hypothalamus, white matter or inflammatory tumor microenvironment) with prediction scores varying between 0.31 and 0.81. Tumor CNV profiles were identified in 44% (4/9) of these tumors.

For better interpretation of the cases with low classification prediction scores and unclassified cases, we accounted for tumor purity as low neoplastic cell content could influence the classification of the tumors [13]. As the proportion of neoplastic cells was not assessed prior to running the samples in the methylation array, we used the R package InfiniumPurify [27, 28] to estimate tumor purity. Tumor purity ranged from 21 to 97% (Fig. 5b) in the dLGG cohort. Among the cases with low prediction scores, 30% (9/30) showed a tumor purity of 70% or lower (Additional file 1: Figure S2B). The median tumor purity score was quite high among the methylation classes except for the control tissue class with an estimated

| Table 3 | Diagnostics by DNA methylation profiling of diffuse lower-grade gliomas with assigned methylation classes and subclasses (n = 126) |
|---------|----------------------------------------------------------------------------------------------------------------------------------|
| Methylation class | Molecular biomarkers according to methylation profiling |
|              | IDHa wt (n = 20) | IDH mut (n = 106) | Codelb (n = 51) | MGMTc unmeth (n = 24) | MGMT meth (n = 102) |
| IDH glioma (n = 106) | | | | | |
| 1p/19q codeleted oligodendroglioma | 0 | 51 | 51 | 0 | 51 |
| Astrocytoma | 0 | 48 | 0 | 7 | 41 |
| High-grade astrocytoma | 0 | 7 | 0 | 2 | 5 |
| Glioblastoma IDH wt (n = 15) | | | | | |
| Mesenchymal | 3 | 0 | 0 | 3 | 0 |
| Midline | 1 | 0 | 0 | 0 | 1 |
| MYCN | 1 | 0 | 0 | 1 | 0 |
| RTK I | 3 | 0 | 0 | 2 | 1 |
| RTK II | 7 | 0 | 0 | 5 | 2 |
| Pleomorphic xanthoastrocytoma | 1 | 0 | 0 | 0 | 1 |
| Low-grade glioma MYB/MYBL1 | 1 | 0 | 0 | 1 | 0 |
| Control tissue | 3 | 0 | 0 | 3 | 0 |

* IDH, isocitrate dehydrogenase gene family, wildtype (wt) and mutated (mut)  
  ** Codel., complete codeletion of 1p/19q chromosomal arms  
  *** MGMT, O6-methylguanine-DNA methyltransferase promotor, methylated (meth) or unmethylated (unmeth)
median tumor purity score below 70% (Fig. 5b). In some cases, the low methylation class predicted score could be explained by the low neoplastic cell content but not all (Additional file 1: Figure S2C). Using a tumor purity cut-off of ≥70% as suggested by Capper et al. [25], 152 cases remain and 10% (15/152) of these have a low class prediction score. However, the low number of neoplastic cells could still provide a valid classification in some cases.

Discussion
Genome-wide DNA methylation characterization of CNS tumors has facilitated the classification of tumors into molecularly defined subgroups with distinct clinical prognoses and outcomes [13–16]. Several institutional centers have started to evaluate the impact of DNA methylation analysis toward its implementation into clinical practice [13, 17, 18, 29, 30]. The value of methylation profiling in the diagnosis and prognosis of primary dLGG has however not been investigated. We therefore generated methylation data from 166 dLGG tumors, assessed the impact of methylation profiling on clinical molecular diagnostics compared to the WHO 2016 classification system and evaluated its prognostic utility for clinical outcomes.

Molecular classification with DNA methylation analysis was achieved for 76% of the dLGG cases using the MNP classifier [13]. Previous studies have shown classification rates of 49–95% [13, 17, 18, 30]. These differences can be explained by the different cohorts and selection of patients that have been included in the different studies. For example, in the study by Jaunmuktane et al. [17] the authors reported a match in 56% of their adult brain tumor cases with a calibrated score ≥0.84. The higher classification rate in our cohort

![DNA methylation-based classification and tumor purity evaluation. a Left: distribution of the adult diffuse lower-grade glioma (dLGG) cases (n = 166) by methylation class prediction score. The vertical dotted line shows the threshold value of ≥0.84 for assignment to an established methylation class. Right: median methylation class score values by methylation classes. Black dots represent individual tumor cases in each methylation class. b Left: distribution of dLGG cases (n = 166) by tumor purity score estimated with InfiniumPurify [27, 28]. Right: median tumor purity score values by methylation classes (n = 166). IDH glioma: glioma IDH-mutant. GBM: glioblastoma IDH-wildtype. CT: control tissue. UNC: unclassified (non-classifiable cases with a class prediction score threshold ≥0.30). Other methylation classes: low-grade glioma, MYB/MYBL1, (anaplastic) pleomorphic xanthoastrocytoma, low-grade glioma, ganglioglioma and plexus tumor, pediatric B...](image)
using the same cutoff value, could be explained by the population-based setup of our study, rather than examining a mixed cohort of patients with, e.g., diagnostically challenging cases or cases referred for second opinion.

The molecular classes identified in our cohort were strongly related to the IDH mutation-associated G-CIMP status verified by Sanger sequencing analysis. The clinical analysis of IDH mutation status by IHC yielded two discrepant results compared to Sanger sequencing/methylation profiling, warranting some cautions in the interpretation of IHC analysis alone. Likewise, 1p/19q codeletions were detected with high accuracy by analyzing the generated CNV profiles, which were confirmed by the clinical analysis (FISH and/or MLPA). This demonstrates that methylation analysis provides reliable detection of IDH mutations and 1p/19q codeletions and can further resolve inconclusive chromosomal aberrations that could lead to misinterpretation.

The diagnosis and prognostication provided by the methylation analysis showed good concordance with the currently used WHO 2016 classification system, demonstrating that it is a valid method for routine classification. Furthermore, it provides more comprehensive data, allowing detection of rare tumor entities that are easily to be misclassified using conventional techniques, in addition to further identify the more indolent IDH-wildtype gliomas from molecular glioblastoma without the need for additional analyses.

Promotor methylation of the MGMT gene is a strong predictor of therapy response and survival in patients with glioblastoma [21, 22] and has been suggested as a prognostic biomarker for high-risk dLGG treated with RT and TMZ [23]. We cannot draw any conclusions regarding the predictive value of MGMT in a cohort of dLGG. Thus, further studies with larger cohorts are required to validate the value of MGMT promotor methylation in dLGG.

Homozygous deletion of the chromosomal region harboring the CDKN2A and CDKN2B genes was recently proposed as a prognostic biomarker for diffuse gliomas [24]. We assessed its prognostic value in our cohort, but the cohort contained too few dLGG cases with homozygous CDKN2A/B deletions to provide a valid comparison between the groups. Shirahata et al. [24] identified 18% deletions in 211 IDH-mutant astrocytic gliomas, where all deletions were detected in grade III astrocytomas and glioblastomas. In our study, we detected 8% deletions in 106 IDH-mutant gliomas using the same cutoff value. Our IDH-mutant glioma cohort comprised approximately 50% astrocytic tumors with only a minority subclassified as high-grade gliomas, which explains the low number of deletions in the cohort.

Tumor specimens are heterogeneous mixtures of healthy normal cells and neoplastic cells. Hence, estimates of tumor purity should be made considering when interpreting methylation-based classification of tumors [13]. In our study, we did not set a cutoff value for tumor purity but examined how methylation profiling would work in all dLGG cases regardless of the percentage of neoplastic cells in the tumors. Some of the unclassified cases showed a low percentage of neoplastic cells. However, the majority of the unclassified cases showed a tumor purity above 70%, suggesting that these cases may represent rare tumor entities that are not included in the MNP classifier.

A limitation of this study is the lack of molecular data for all patients at time of diagnosis. The patients in our cohort were diagnosed with dLGG in the era where molecular markers were not required to establish a diagnosis. In addition, we did not conduct prospective analyses, which are needed to elucidate the impact of methylation profiling on patients with changed treatment approach when integrating diagnostic information provided by methylation analysis.

Conclusions
In conclusion, our results substantiate the value of DNA methylation profiling to diagnose and distinguish different dLGG entities, as well as to predict prognosis of the patients. The identification of diagnostic and prognostic biomarkers in a robust high-throughput fashion is feasible through methylation arrays. Relevant glioma diagnostic and prognostic biomarkers such as IDH mutation status, copy number aberrations including 1p/19q codeletions, MGMT promotor methylation status and differentiation of IDH-wildtype tumors into glioblastoma or indolent tumors, can all simultaneously be obtained from one analysis. This may facilitate the current routine molecular diagnostic approaches by replacing multiple analyses for the different biomarkers with one assay.

Methods
Patients and samples
A clinical consecutive cohort of 210 adult patients, diagnosed with primary dLGG (WHO 2007 grade II-III) during 2007–2016 in the Västra Götaland region in Sweden, was evaluated for inclusion. Patients with suspected radiological appearance of a glioblastoma (e.g., ring-like contrast enhancement and necrosis) from which only a diagnostic biopsy was taken, but where biopsy results were a grade II or III glioma, were not included due to the risk of sampling bias [31, 32]. This was the selection criterion for a larger dLGG study, where methylation-based re-analysis was one of the research aims. We also excluded patients with non-available FFPE tumor tissue.
for further analyses. FFPE tumor tissue and clinical data (patient characteristics and molecular data) were collected retrospectively for a total of 168 subjects. Molecular data included IDH mutation status determined for 21 dLGG cases (15 IDH-mutant and 6 IDH-wildtype cases) by IHC and/or NGS and 1p/19q codeletion status for 60 dLGG cases (56 1p/19q codeleted cases) by FISH and/or MLPA determined at time of diagnosis.

**Genome-wide DNA methylation analysis**

*DNA extraction and quantification*

DNA from FFPE tumors was extracted with QIAamp® DNA FFPE kit (Qiagen) following the protocol provided by the manufacturer with an extra digestion step with proteinase K overnight. The extracted DNA was quantified using the Qubit® dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific).

**Bisulfite conversion of DNA, restoration and array processing**

Between 500 and 1000 ng of extracted DNA was bisulfite-converted with the EZ DNA methylation kit (Zymo) and restored with the Infinium HD FFPE Restore Kit (Illumina), according to the instructions supplied by the manufacturer. The Infinium MethylationEPIC BeadChip array (Illumina) was used to generate genome-wide DNA methylation profiles for the tumor samples (UCL Genomics, UK).

**Data analysis**

Raw methylation data (IDAT files) generated from the methylation arrays were normalized, analyzed and assessed for quality controls as previously described [33] using the statistical software R with Rstudio (version 4.0.2). The G-CIMP phenotype, which is strongly associated with IDH mutations in gliomas [25, 34, 35], was characterized by unsupervised hierarchical clustering to identify dLGG samples with IDH mutations. Hierarchical clustering was performed with the 1500 most deviating CpG sites with The Cancer Genome Atlas glioma samples with known G-CIMP status based on methylation data as described by Noushmehr et al.[35]. The O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status was predicted with the R package MGMT-STP27 [37] using the suggested cutoff at 0.358 [36, 37]. Percentage of neoplastic cells was determined with the R package InfiniumPurify [27, 28] with normal methylation data included in the package. For tumor classification, IDAT files were uploaded into a publicly available DNA methylation-based classifier (MNP, version 114b, https://www.molecularneuropathology.org/mnp) [13]. Methylation-based tumor classifications and subclassifications with prediction class scores, indicating probability estimations of the assigned classes, were automatically generated as reports by the classifier tool. The reports were then evaluated according to the recommendations as presented by Capper et al. [25] using the threshold value ≥ 0.84 preferred for clinical settings.

**Copy number variation analysis**

Copy number variation (CNV) profiles for each individual case were generated from raw methylation data inferred from the methylation array using the R package conumee [38]. 1p/19q codeletion, homozygous deletion of CDKN2A/B, amplification of the epidermal growth factor receptor (EGFR) as well as gain of chromosome 7 and loss of chromosome 10, were visually assessed from the CNV profiles. For determining CDKN2A/B deletion we used the cutoff previously suggested by Shirahata et al. [24].

**Mutation analysis of IDH1/2 and BRAF by Sanger sequencing**

PCR amplification of IDH1, IDH2 and BRAF was performed with 50 ng of extracted DNA using IDH1, IDH2 [39] or BRAF primers. PCR products were purified with ExoSAP-IT PCR Product Cleanup Reagent (Affymetrix) before Sanger sequencing (Eurofins—GATC Biotech).

**Molecular reclassification**

Molecular reclassification of the dLGG cases following the WHO 2016 criteria was based on histopathological grade and IDH mutation status, 1p/19q codeletion status and, in some instances alpha thalassemia/mental retardation syndrome X-linked (ATRX) status, generated at time of diagnosis and retrospectively in our facilities.

**Statistical analysis**

Statistical analyses were performed using the IBM SPSS® Statistics software version 25. We conducted a Kaplan–Meier survival analysis and overall survival curves (from date of surgery to date of death or date of last follow-up) were compared with a log-rank test.

**Abbreviations**

ATRX: Alpha thalassemia/mental retardation syndrome X-linked; CNS: Central nervous system; CNV: Copy number variation; dLGG: Diffuse lower-grade glioma; EGFR: Epidermal growth factor receptor; FFPE: Formalin-fixed paraffin-embedded; FISH: Fluorescent in situ hybridization; G-CIMP: Glioma CpG island methylator phenotype; IDH: Isocitrate dehydrogenase gene family; IHC: Immunohistochemistry; MGMT: O6-methylguanine-DNA methyltransferase promoter; MLPA: Multiplex ligation-probe amplification; NGS: Next-generation sequencing; RTK I/II: Receptor tyrosine kinase I or II; TMZ: Temozolomide; WHO: World Health Organization.
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-021-01085-7.

Additional file 1: Figure S1. This figure presents the molecular reclassification of the adult diffuse lower-grade glioma cohort according to WHO 2016. Supplementary figure S2. This figure presents diagnostic and prognostic evaluations of diffuse lower-grade glioma cases with low methylation class/subclass prediction scores and evaluation of tumor purity.

Additional file 2: Table S1. This table presents the clinical and molecular characteristics of diffuse lower-grade glioma cases with low methylation class/subclass prediction scores.

Acknowledgements

We thank Helene Spøgren for her assistance with the FISH analyses and UCL Genomics for EPIC array processing.

Authors' contributions

ASJ and HC designed the study and coordinated it. SFV performed all experiments except for the histopathological, clinical and radiological evaluations, which were performed by TOB and AC. SFV performed the data analysis with assistance from ASJ and HC. SFV prepared the figures and tables and wrote the manuscript with input from ASJ and HC. AS provided input to the project. All authors read and approved the final manuscript.

Funding

Open access funding provided by University of Gothenburg. This work was supported by the Swedish Cancer Society, the Swedish Research Council (VR 2017-00944), the Swedish Society for Medical Research and the Swedish state under the agreement between the Swedish government and the county councils, the ALF agreement.

Availability of data and materials

Not applicable.

Declarations

Ethical statement

This project was approved by the regional ethics committee in the Västra Götaland region in Sweden (Dnr 1067-16).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 5 January 2021 Accepted: 20 April 2021
Published online: 03 May 2021

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