



Pickles, J. C., Fairchild, A. R., Stone, T. J., Brownlee, L., Merve, A., Yasin, S. A., Avery, A., Ahmed, S. W., Ogunbiyi, O., Gonzalez Zapata, J., Peary, A. F., Edwards, M., Wilkhu, L., Dryden, C., Ladon, D., Kristiansen, M., Rowe, C., Kurian, K. M., Nicoll, J. A. R., ... Jacques, T. S. (2020). DNA methylation-based profiling for paediatric CNS tumour diagnosis and treatment: a population-based study. *The Lancet Child and Adolescent Health*, 4(2), 121-130.
[https://doi.org/10.1016/S2352-4642\(19\)30342-6](https://doi.org/10.1016/S2352-4642(19)30342-6)

Peer reviewed version

License (if available):
CC BY-NC-ND

Link to published version (if available):
[10.1016/S2352-4642\(19\)30342-6](https://doi.org/10.1016/S2352-4642(19)30342-6)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via Elsevier at [https://www.thelancet.com/journals/lanchi/article/PIIS2352-4642\(19\)30342-6/fulltext#%20](https://www.thelancet.com/journals/lanchi/article/PIIS2352-4642(19)30342-6/fulltext#%20). Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

The impact of molecular profiling on CNS tumour diagnosis and treatment: A paediatric population-based study

Jessica C Pickles PhD^{1,2}, Amy R Fairchild BA^{1,2}, Thomas J Stone PhD^{1,2}, Lorelle Brownlee MBBS², Ashirwad Merve PhD², Shireena A Yasin PhD², Aimee Avery BSc², Saira W Ahmed BSc², Olumide Ogunbiyi BSc², Jamie Gonzalez MSc², Abigail F Peary BSc², Marie Edwards MSc², Lisa Wilkhu MSc³, Carryl Dryden BSc³, Dariusz Ladon PhD³, Mark Kristiansen PhD⁴, Catherine Rowe MSc⁵, Kathreena M Kurian FRCPATH (Neuro)⁶, Prof. James AR Nicoll FRCPATH^{7,8}, Clare Mitchell PhD⁸, Tabitha Bloom⁸, David A Hilton MD⁹, Prof. Safa Al-Sarraj FRCPATH¹⁰, Lawrence Doey BA¹⁰, Paul N Johns FRCPATH¹¹, Leslie R Bridges FRCPATH¹¹, Aruna Chakrabarty FRCPATH¹², Azzam Ismail FRCPATH¹², Nitika Rathi FRCPATH¹³, Khaja Syed MSc¹³, G. Alistair Lammie FRCPATH¹⁴, Clara Limback-Stanic PhD¹⁵, Prof. Colin Smith MD¹⁶, Antonia Torgersen FRCPATH¹⁶, Frances Rae CSci¹⁶, Rebecca M Hill PhD¹⁷, Prof. Steven C Clifford PhD¹⁷, Yura Grabovska MRes¹⁷, Daniel Williamson PhD¹⁷, Matthew Clarke MBBCh¹⁸, Prof. Chris Jones PhD¹⁸, David Capper MD^{19,20}, Martin Sill PhD²¹, Prof. Andreas von Deimling MD^{22,23}, Prof. Stefan M. Pfister MD^{21,24,25}, David T.W. Jones PhD^{21,26}, Prof. Darren Hargrave², Jane Chalker FIBMS³, Prof. Thomas S Jacques FRCPATH^{1,2}

¹Developmental Biology and Cancer Research & Teaching Department, UCL Great Ormond Street Institute of Child Health, London, UK

²Department of Histopathology, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK

³Specialist Integrated Haematology and Malignancy Diagnostic Service-Acquired Genomics, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK

⁴UCL Genomics, UCL Great Ormond Street Institute of Child Health, London, UK

⁵Department of Neuropathology, North Bristol NHS Trust, Bristol, UK

⁶Brain Tumour Research Centre, University of Bristol, UK

⁷Cellular Pathology, University Hospital Southampton NHS Foundation Trust, Southampton, UK

⁸BRAIN UK, Clinical & Experimental Sciences, University of Southampton, Southampton, UK

⁹Cellular and Anatomical Pathology, University Hospitals Plymouth NHS Trust, Plymouth, UK

¹⁰Department of Clinical Neuropathology, Kings College Hospital NHS Trust, London

¹¹Department of Cellular Pathology, St George's University Hospital NHS Foundation Trust, London, UK

¹²St James's University Hospital, The Leeds Teaching Hospitals NHS Trust, Leeds, UK

¹³Department of Neuropathology, The Walton Centre NHS Foundation Trust, Liverpool, UK

¹⁴University Hospital of Wales, Cardiff, UK

¹⁵Department of Histopathology, Charing Cross Hospital, London, UK

¹⁶Western General Hospital, NHS Lothian, Edinburgh, UK

¹⁷Wolfson Childhood Cancer Research Centre, Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne NE1 4LP, UK

¹⁸Division of Molecular Pathology, The Institute of Cancer Research, London, UK

¹⁹Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Department of Neuropathology, Berlin, Germany

²⁰German Cancer Consortium (DKTK), Partner Site Berlin, German Cancer Research Center (DKFZ), Heidelberg, Germany.

²¹Hopp Children's Cancer Center Heidelberg (KITZ), Heidelberg, Germany

²²Department of Neuropathology, University Hospital Heidelberg, Heidelberg, Germany

²³Clinical Cooperation Unit Neuropathology, German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany

²⁴Division of Pediatric Neurooncology, German Cancer Research Center (DKFZ) and German Cancer Consortium (DKTK), Heidelberg, Germany

²⁵Department of Pediatric Oncology, Hematology, Immunology and Pulmonology, Heidelberg University Hospital, Heidelberg, Germany

²⁶Pediatric Glioma Research Group, German Cancer Research Center (DKFZ), Heidelberg, Germany

Author for correspondence:

Prof. Thomas S Jacques

UCL GOS Institute for Child Health,

30 Guilford Street

London WC1N 1EH

UK

Tel: +44 (0)20 7829 8663

Email: t.jacques@ucl.ac.uk

Keywords: paediatric brain tumours; methylation array; molecular pathology; neuropathology; methylation classifier; real world evidence, World Health Organisation

Abbreviations: CNS central nervous system; DKFZ German Cancer Research Centre; ETMR

embryonal tumour with multilayered rosettes; FISH fluorescence *in situ* hybridisation; FFPE formalin-fixed paraffin-embedded; GOSH Great Ormond Street Hospital; HGNET high grade neuroepithelial tumour, IHC immunohistochemistry; NOS not otherwise specified; PFR probe failure rate, PNET primitive neuroectodermal tumour; WHO World Health Organisation.

Abstract

Background: Our aim was to assess the added value of incorporating molecular profiling into routine diagnostic tumour practice. There is marked variation in the implementation of genomic data in tumour diagnosis, while optimal integration with conventional diagnostic technology remains uncertain despite several studies reporting improved diagnostic accuracy and selection for targeted treatments and stratification for trials. There is a need for the evaluation of the added value in routine clinical practice and impact on conventional, as well as experimental, treatments.

Methods: We assessed the diagnostic and clinical utility of DNA methylation-based profiling in childhood central nervous system (CNS) tumours using two large national cohorts. The first cohort included routinely diagnosed CNS tumours in the UK (n=306) and the second was enriched for cases that historically have been diagnostically difficult (n=195). In the first cohort, we assessed how the methylation profile altered or refined diagnosis in routine clinical practice and estimated how this would affect standard patient management. For the research cohort, we determined how many cases could be solved using currently standard pathology, how many could only be solved using the methylation profile and how many remained unsolvable.

Findings: Molecular profiling added a unique contribution to clinical diagnosis in 35% (107/306) of cases in routine diagnostic practice, and we estimated that it could change conventional management in 4% (11/306) of patients. In historically difficult to diagnose tumours, 51% (99/195) could be diagnosed using standard methods, with the addition of methylation profiling solving a further 17% (34/195) of cases. The remaining 32% (62/195) cases were unresolved despite specialist pathology and methylation profiling.

Interpretation: Together these data provide estimates of impact that could be expected from routine implementation of genomic profiling into clinical practice, and also indicate limitations where additional techniques will be required. We conclude that DNA methylation arrays are a useful diagnostic adjunct for childhood CNS tumours.

Funding: We are grateful for funding from the Brain Tumour Charity, Children with Cancer UK, Great Ormond Street Hospital Children's Charity (INSTINCT), Olivia Hodson Cancer Fund, Cancer Research UK, and the National Institute of Health Research. All research at Great Ormond Street Hospital (GOSH) NHS Foundation Trust and UCL GOS Institute of Child Health is made possible by the NIHR GOSH Biomedical Research Centre.

Research in Context

Evidence before this study

The development of a CNS tumour classifier based on DNA methylation array data poses huge promise as a diagnostic adjunct in neuropathology. The reference dataset used to build the DKFZ algorithm is based on a large cohort of retrospective data and was validated using prospective internal and external data from multiple centres. We undertook a review of studies published following the description of the algorithm (using Pubmed, 2018-2019, search terms: *methylation AND brain AND (cancer OR tumor OR tumour) AND diagnosis AND array*). The DKFZ group published a practical implementation guideline which presents data from their internal cohort and a separate study focussed on the diagnostic workflow in adult brain tumours and how a methylation assay is performed in an adult context. The WHO does not yet incorporate many of the molecular CNS subtypes recognised by the community and methylation classifier. There is no current evidence presenting uniquely paediatric data and so far, no discussion of the clinical impact, while the 'real-time' added value of methylation profiling has not been addressed.

Added value of this study

We found that DNA methylation analysis added significantly to the diagnosis and treatment of children above and beyond specialist molecular pathology. Methylation analysis altered tumour subtyping in 35% of cases when used in routine practice and was predicted to change conventional treatment for 4% of children. Using an archival cohort, 51% of difficult to diagnose cases were identified without resorting to a DNA methylation profiling, which further subtyped 17% of cases. We identified a group of cases that remain unclassifiable despite molecular neuropathology and methylation profiling, suggesting that novel subtypes remain to be identified.

Implications of all the available evidence

The data suggests that methylation profiling not only provides significant clinical impact in childhood brain tumours by refining diagnosis and changing treatment, but strongly suggests that it should be considered as a routine test in assessing such children.

INTRODUCTION

Incorporation of complex molecular pathology into routine practice can improve diagnostic accuracy and tailored treatments, but poses significant challenges^{1, 2}. Not only are there practical factors (cost, access and accreditation), but it is unclear whether we can extrapolate from curated research cohorts to clinical practice. In particular, genome-wide profiling studies have primarily taken place within research environments or as part of clinical trials^{3, 4}. Their implementation in routine clinical practice is at best variable and at worst, controversial.

Genomic data is of particular importance for paediatric brain tumours. Many have been reclassified based on their molecular profiles⁵⁻⁸. Also, genomic data has impact on cancer treatment for children, many of whom are at a high risk of death or disability depending at least in part on their treatment. The recent description of a methylation classifier for CNS tumours holds promise as an aid to diagnosis^{9, 10}. The methylome, probably reflecting the cell of origin, is maintained during tumour evolution and is a stable marker of tumour type¹¹.

To reflect the differences between adult and paediatric CNS tumours, the classifier model was developed using a large reference cohort containing over 40% of samples from patients under the age of 19 years⁹. However, paediatric cases are under-represented in the original internal and external validation cohorts.

Stringent study settings (such as randomised trials) can bias data by limiting inclusivity at recruitment. It has become clear that there is specific added value in 'real world evidence'^{12, 13}. Therefore, we assessed the impact of routine methylation profiling in clinical practice and estimated its added value in the context of modern standard-of-care pathology using real world data collected during a 2 year period. We determined how often refinement of diagnosis would change treatment. Our experience is an exemplar of how genomic approaches can be assessed once translated into clinical practice.

Methods

Study design and participants

Our data includes two national cohorts: a diagnostic cohort of 306 samples to measure the impact on routine diagnosis and a second archival cohort encompassing 195 diagnostically unresolved cases (supplementary Table 1).

Diagnostic Cohort: We included CNS tumours spanning a 24-month period (Sept 2016- Sept 2018) in patients under 19 years of age, with a DNA methylation array performed in real-time at Great Ormond Street Hospital (GOSH) and where a consultant neuropathologist: i) requested the array and ii) included the data in a clinical diagnostic report. The decision to perform an array was at the discretion of the neuropathologist but most primary high-grade tumours were profiled (see results). Local and referred cases were included, with a small number (n=19) received from centres from outside of the UK. Cases undertaken for research purposes were excluded.

Archival cohort: We searched for UK cases of high-grade CNS paediatric tumours (grades III and IV) reported between 1990 and 2018, that either failed to achieve a confident diagnosis at the time of initial diagnosis or fell into historical groups now recognized to represent a mixture of tumour groups (e.g. CNS PNETs/supratentorial PNETs). We excluded cases with an initial histological diagnosis of medulloblastoma, glioblastoma, ependymoma, AT/RT, Ewing's sarcoma, teratoma and sarcoma as these were beyond the study's scope.

Ethical approval: clinical cohort approval was given by Great Ormond Street NHS Foundation Trust as service evaluation (Registration No. 2301). The archival cohort was analysed under ethical approval granted by BRAIN UK tissue bank (REC: 14/SC/0098, References 16/007 and 17/007)

Procedures

Methylation profiling: DNA was extracted from 50 µm formalin-fixed paraffin-embedded (FFPE) tissue taken either as 5x10 µm rolls or macrodissected sections to enrich for tumour content. DNA was extracted using the Promega Maxwell 16 FFPE Tissue LEV DNA Purification Kit and quantified using a Nanodrop, before bisulphite conversion of up to 500 ng DNA, using the Zymo EZ DNA Methylation-Gold kit. Bisulphite converted FFPE DNA was

treated with the Illumina Infinium FFPE DNA Restore kit. DNA was assayed using Illumina Infinium MethylationEPIC BeadChip arrays processed by UCL Genomics, according to the Infinium HD FFPE Methylation Assay automated protocol (Illumina).

Methylation classification: methylation data was imported into R v3.5.3 using *minfi*¹⁴ (version 1.22.1) and normalised with the included function *preprocessIllumina*. Missing CpG beta values were imputed using the *impute.knn* function implemented in the *impute* package¹⁵. A DNA methylation classification model (MNP v11b2, current at the time of implementation) was used (www.moleculareuropathology.org/mnp)⁹. This resulted in an output indicating the best match of tumour diagnosis and a corresponding calibrated score⁹, ranging from 0-1.

Reporting methylation outputs: during the period of clinical implementation, if the classifier's predicted output gave a calibrated score ≥ 0.9 , the methylation class was used in the clinical report in keeping with previous publications⁹. When scores were < 0.9 , the classifier was deemed to be of uncertain significance and was not included in the clinical report¹⁰. All outputs were considered alongside standard neuropathology^{16, 17} and when relevant, further testing was performed to confirm the result.

Copy number (CN) plots were generated from methylation data using *Conumee*¹⁸. During the study timeframe study, CN plots were not routinely reported clinically. We retrospectively reviewed the plots to identify diagnostically relevant changes in specific tumour types. Gains and losses were called when probe intensity was ± 0.15 on log2 scale and amplifications were called when > 0.6 ^{19, 20}.

The rationale for undertaking methylation analysis in clinical cases was categorised as: (i) tumours that were difficult to diagnose, (ii) rare tumour types requiring further confirmation, (iii) cases where the purpose of the array was to determine the subtype of a tumour and (iv) cases with limited available diagnostic material.

The diagnostic impact of the predicted methylation class in clinical cases was reviewed by a single observer (JCP) independently of the reporting pathologist, and categorised as (i) classification that confirmed the final diagnosis but did not add additional information, (ii) classification that confirmed and refined the final diagnosis, providing additional molecular subtyping not available by histopathology alone, (iii) classification that amended the initial diagnosis, leading to a change in final diagnosis, (iv) classifications of uncertain significance and (v) classifications considered potentially misleading.

Methylation data from the clinical cohort that modified the final diagnosis were assessed by a senior paediatric neuro-oncologist (DH) to determine if the data would indicate a change in therapeutic strategy according to relevant national guidance for childhood tumours in the UK at the time of diagnosis. We considered methylation data to have had a clinical impact when the patient's treatment would have differed if the data had not been available. The same oncologist identified cases where the methylation data could in the future help to triage patients for forthcoming trials; such as targeted therapies or improved risk stratification. We analysed this latter data separately as it is more subjective.

Review of archival cohort: an immunohistochemical panel was performed on the cases from the archival cohort, including GFAP, synaptophysin, Ki-67, NeuN, OLIG2, INI-1, SMARCA4, LIN28A, mutant H3K27M, H3K27 trimethylation, CD56, CD99, EMA, p65/RELA and L1-CAM²¹. FFPE sections were cut at 3 µm and automated staining performed on a Leica BOND-MAX. The histopathology was reviewed by a paediatric neuropathologist (TSJ) to determine if a confident diagnosis could be offered on the basis of the neuropathological features. DNA methylation arrays and the classifier algorithm were performed as described above using MNP v2 and v4. Where appropriate, cytogenetic testing (fluorescence in-situ hybridisation, FISH) and confirmation of suspected mutations (sequencing, RT-PCR) was performed.

Mapping samples against reference cohorts: sample methylation data were read into R using Minfi and preprocessed using the included preprocessIllumina function. Beta values were extracted and matched against those in the MNP reference cohort dataset (v4)⁹. These combined data were used to generate t-SNE plots containing both reference and GOSH cohorts for manual inspection of clustering in cases where GOSH samples could not be robustly classified by the MNP algorithm. Research samples whose co-ordinates were within or directly adjacent to methylation reference subgroups were considered to belong to the respective tumour subgroup.

Statistical analysis

Confidence intervals of 95% were calculated in R using the prop.test function from the stats package. Statistical testing was undertaken in SPSS (IBM version 24), significance set to ≤ 0.01 . Pairwise correlations of continuous data were tested using Spearman's rank test, while pairwise comparisons of population variance were tested using the Kruskal-Wallis test.

Role of the funding source

The funder had no role in the study design, data collection, analysis and interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Implementation of methylation profiling in routine clinical practice

To determine the impact of molecular testing on diagnostic yield, we reviewed the GOSH experience of methylation profiling in childhood CNS tumours. For the period under consideration, standard-of-care involved morphology, immunohistochemistry for standard diagnostic markers, FISH and single gene analysis (Sanger sequencing and qPCR)¹⁷. During the two-year period studied, we diagnosed 484 brain tumours in patients under 19 years of age. The estimated brain tumour incidence in this age group is 633 cases per year²² in the UK. Therefore, this cohort is approximately 40% of the nation's childhood CNS tumours (242/633, total national population of 66 million²³). We undertook diagnostic DNA methylation arrays in 306 cases or 63% of our total cases; the equivalent of 24% of the population's childhood brain tumours (Figure 1A).

Clinical cases (n=306) were those that were referred by a neuropathologist for diagnosis (Figure S1). Most high-grade tumours were profiled (Figure 1B). We categorised cases according to the primary indication for profiling (Figure S2): 162 cases were requested specifically to establish a tumour subtype (53%) e.g. medulloblastoma; 115 cases were undertaken as they were diagnostically difficult cases (38%); 19 were undertaken where the diagnosis was certain but the tumour type was considered unusual in the age group (6%); and ten were undertaken to improve diagnostic certainty when the biopsy was small (3%).

Diagnostic yield of real-world methylation profiling

The principal outcome from the arrays was classification using an algorithm from the German Cancer Research Centre (DKFZ)⁹. In keeping with previous publications^{9, 10}, a calibrated score greater than 0.9 indicates successful classification (see below for analysis of scores <0.9). From the entire diagnostic cohort (irrespective of DNA quality), 49% (149/306) cases gave a calibrated score of 0.9 or greater. Diagnostic impact was assessed by reviewing the reports to determine what effect the array had on the real-time diagnosis (Figure 2)^{9, 24}. Overall, we report a total recall rate of 48% (146/306), and a precision rate at n (calibrated scores >0.9) of 98% (146/149).

Cases where the array confirmed but did not refine the final diagnosis accounted for 26% (39/149) of classifying cases or 13% (39/306) of all cases (Figure 2). Diagnoses in this group

mostly belonged to CNS tumours with molecular subgroups that were identifiable by existing diagnostic tests e.g. WNT-activated medulloblastoma.

Array predictions that both confirmed and refined the final diagnosis by providing additional molecular subtyping data, not otherwise available by existing histopathological or molecular evaluation, accounted for 64% (99/149) of the classifying cases or 32% (99/306) of all cases (Figure 2). Many of these tumours were those where an array was then used as the primary method of subtyping, e.g. some subtypes of medulloblastoma and high-grade glioma.

There were five array predictions that led to an amended final diagnosis (Supplementary table 2A), including two newly recognized tumours, high grade neuroepithelial tumour (HGNET) with *MN1* alteration and HGNET with *BCOR* alteration²⁵.

In three cases, the array made a prediction that was considered potentially significant, but that could not be confirmed, and we regarded these results as of uncertain significance (Supplementary table 2B).

In no case was the array entirely misleading if taken in the context of other radiological and pathological data. We only considered 3 array predictions to be potentially misleading if interpreted in isolation (Supplementary table 2C) but the clinical management was not likely to have changed. The first case was an embryonal tumour that classified as a pineoblastoma however, on review there was no radiological involvement of the pineal gland. It is possible that this is a limitation of the radiology rather than the array, or it may represent an ectopic origin for pineoblastoma¹⁰. In the remaining two cases, the array predicted control brain tissue but the result was disregarded as there was morphological evidence of tumour. The corresponding sections were reviewed and the prediction was explainable because of low tumour content.

Factors that affected diagnostic yield

To explore the factors that affected the score, we reviewed potential confounding factors including technical factors, clinical indication, patient age and diagnosis.

All cases were processed irrespective of the DNA quality. Therefore, to analyse the impact of technical factors, we compared the percentage of failed probes to the calibrated score. As expected, we found a negative correlation between probe failure rate (PFR) and calibrated

score ($\rho=-0.39$, $p<0.0001$ figure S3). Next we binned cases into four groups based on PFR (Figure 3A). No cases with a PFR over 5% and few over 2% gave a calibrated score over 0.9. However, there remained significant number of non-classifying cases with low PFRs suggesting DNA quality only accounts for a proportion of the unclassified cases.

We hypothesised that the classifier results may vary in different clinical contexts. To address this, we analysed the calibrated score based on the patient's age, site of preparation, final diagnosis and the clinical indication for the array. We found no correlation between age and calibrated score ($p=0.75$) suggesting that the classifier worked equally well across all ages of children and young adults (Figure S3). However, the calibrated score varied according to the indication for undertaking an array ($p<0.0001$, Figure 3B). The highest scores were from cases undertaken for sub-typing or confirmation of confident diagnoses. Intermediate scores were observed when the arrays were undertaken to solve diagnostically challenging cases whilst the lowest scores came from cases with small biopsies. This suggests that the expected success of methylation analysis depends on the clinical question being addressed.

To determine if the performance of the classifier differed in different tumour types, we considered how the calibrated scores varied according to the final diagnosis ($p<0.0001$, Figure 3C). The tumour type with the highest success rate was medulloblastoma in which 59 of the 79 cases profiled (75%) produced a confident subtype. Ependymoma had more modest results, where 15 of the 29 cases (52%) classified. Notably most *RELA*-altered ependymomas (7 out of 9 cases) successfully classified by array.

Fifty-nine patients were diagnosed with a high-grade astrocytoma (grades III and IV) and of these, 41% ($n=24$) were classifiable (Figure 3C). However, the rate was higher in specific subtypes e.g. diffuse midline gliomas with a histone H3K27 mutation had confident scores in 7 out of 10 cases and G34 mutated glioblastomas had confident scores in all 4 cases. Methylation data increased grade to glioblastoma in 10 high-grade astrocytomas.

In low-grade glioma or glioneuronal tumours, a calibrated score ≥ 0.9 was assigned in only 33% of cases (28/85), compared to our cohort's average of 49% (Figure 3C). This suggests that this tumour group is particularly challenging to classify by this technique.

Copy number data

Inferred copy number (CN) data generated from methylation arrays were not included in the clinical reports during the time of this study. However, to assess the potential of CN data, we

retrospectively compared the CN results to existing diagnostic data (i.e. FISH) in medulloblastoma and embryonal tumours with multilayered rosettes (ETMR).

MYC and *MYCN* amplification are poor prognostic factors in medulloblastoma²⁶. Both FISH and CN plots were available for 62 out of 79 reported medulloblastomas, with a total of 12 patients reported to have either *MYC* (n=6) or *MYCN* (n=7) amplification by FISH (one patient had the unusual combination of both) (Figure 4 and S4). The CN plots had a specificity of 100% for both *MYC* and *MYCN* amplification compared to FISH. The sensitivity of the array was only 67% for *MYC* and 57% for *MYCN* compared to FISH. It is likely that the low sensitivity is due to the difficulty in detecting focal amplifications by bulk DNA techniques.

We assessed amplification of the microRNA cluster on chromosome 19 (*C19MC*) that defines *C19MC*-altered ETMRs. In all four ETMRs, *C19MC* amplification was detected by both FISH and by array CN plot analysis (data not shown).

***MGMT* promoter methylation assessment in paediatric cases**

MGMT promoter methylation is considered of prognostic relevance in adult gliomas and associated with sensitivity to alkylating chemotherapy (temozolomide)²⁷ but its value in childhood cancers remains unconfirmed^{28, 29, 30, 31}. We found that *MGMT* promoter methylation was rare in children and mostly restricted to small subsets of tumour types (see supplementary material).

Impact on therapy

We reviewed all cases where the methylation array modified the diagnosis to determine if the additional data would have implications for treatment according to standard protocols used in the UK. In 11 patients, the diagnostic modification would have mandated a change in management based on current protocols (supplementary Table 4). To estimate the potential impact of these changes, we expressed this as a percentage of all arrays performed (irrespective of technical failures), finding that 4% (CI +/-2%) of cases (11/306) would have an impact on standard management.

We also estimated which might predict treatment changes that could be offered in the next few years in trials. When considering these patients, a future change in therapy was possible for 6% of cases (18/306); bringing the total estimated upper limit on future therapeutic impact to 10% (CI +/-3%) of cases tested.

Impact of suboptimal classification scores

At the time of the diagnosis, only scores ≥ 0.9 were reported in our clinical practice. However lower scores have been suggested to be useful when used with caution¹⁰ and are reported at other centres²⁴. As these outputs were not used for real-time diagnosis, we categorised them according to how the classifier's prediction related to the final diagnosis (Figure 5A). The lower the calibrated score, the greater the risk of a misleading result (Fig. 5B and C). We conclude that cases with low calibrated scores should be considered with caution, and used alongside other testing.

The impact of methylation profiling in diagnostically challenging cases

The data in the first cohort estimates the impact of methylation profiling in routine clinical practice. The data suggests it is successful in well-defined entities but shows variable results where the diagnosis was uncertain. To explore this, we determined the impact on tumours that have defied diagnosis. We hypothesised that a proportion of such tumours might be diagnosable by current pathological criteria without advanced molecular technology, while others may be solved by methylation profiling and finally, some may remain unresolved despite contemporary histology and molecular analysis.

We collated a national cohort of archival high-grade brain tumours that either failed to achieve a confident diagnosis at the time of the initial diagnosis or fell into groups that are now recognised to represent a mixed group of molecular entities (e.g. CNS PNETs/supratentorial PNETs). Cases with sufficient tissue (n=195) were subjected to a standardised immunohistochemistry panel and the histological features were reviewed by a neuropathologist (TSJ).

Based on the histopathology, we made a confident diagnosis (i.e. cases where the immunohistochemistry gave an unambiguous results e.g. INI1 loss in AT/RT) in 51% of the research cohort (99/195), suggesting that many cases can be resolved without further molecular testing (Figure 6A and B). In 17% (34/195) we were able to achieve a confident diagnosis using methylation profiling in combination with histology (Figure 6A and C). The remaining 32% of cases (62/195) could not be confidently resolved using the array or histopathology. 15 of these cases were explained by technical problems (12 cases had high PFRs and three had low tumour content). A single case (an infantile hemispheric glioma (IHG))

was resolved by the latest version of the methylation classifier (mnp.v4). We suggest that the remaining 46 unsolvable cases are a combination of novel entities, tumour predisposition syndromes⁹, and cases where the technology is not optimal for their identification (Figure 6A).

To test this, we plotted the unsolved samples against the original DKFZ reference cohort (Figure 6D). On t-SNE plot, we assessed the proximity of unsolvable cases to known methylation classes. We estimate that only 35% (16/46) of the unsolvable cases are examples of known entities, already part of the existing methylation classifier, and the remainder may be novel entities.

We noted a handful of unsolved cases clustered near but separately from the reference cases (Figure 6D). These cases may be rare variants of known CNS tumours, for example ETMR-like tumours with *DICER1* mutations³² or potentially novel, undescribed CNS tumour entities.

Discussion

Using two national cohorts of paediatric CNS tumours, we have evaluated the impact and limitations of incorporating DNA methylation arrays into routine diagnostic practice. We found that methylation profiling alters the diagnosis in 35% (CI +/-5%) of cases and affects treatment in 4% (CI +/-2%) of patients.

We have been conservative in measuring the added value of these techniques beyond standard-of-care testing¹⁷ and considered changes in patient management according to current protocols. We assessed all arrays including those that failed, not preselecting the best quality samples, as we wished to determine how the arrays performed based on real clinical samples. We suggest that these are acceptable approaches for measuring the realistic clinical impact of molecular profiling on clinical practice, avoiding unrealistic expectations from clinicians.

The data nonetheless supports routine use of methylation profiling for childhood CNS tumours. Although the numbers may appear low, considering the debilitating long-term impact of treatment on the developing brain and the poor prognosis of many childhood brain tumours, changing treatment in as few as 3-4% of children is likely to be highly significant. This would equate to 23 children annually in the UK²², 274 in Europe and 120 in the United States³³. Based on this data, and contrary to adult practice²⁴, we use methylation profiling in all CNS parenchymal tumours, where there is sufficient tissue and tumour content.

Classification rates vary depending on the clinical cohort to which the classifier is applied. The DKFZ validation cohorts reported variable classification rates across 5 external centres (no tumour match in 5-42% of cases, average 22%)⁹. One adult study reported 44% of tested CNS tumours were unclassifiable using a calibrated threshold of 0.84²⁴. In our paediatric cohort, we observed a significant proportion of unclassified cases (51%), possibly explained by the frequent complex diagnostic cases seen in childhood neuropathology.

Our data suggest that as well as technical factors, clinical ones such as the clinical question and the tumour type are important predictors of the classifier's performance. This is important as the *a priori* likelihood of a meaningful prediction depends on the question being asked. For example, if the purpose is to subtype medulloblastoma, we would expect a high success rate. If in contrast, an array is used to solve a difficult case that has defied conventional diagnostic approaches, it is less likely to succeed. In the second cohort, we included cases that had

historically defied diagnosis or had diagnoses that were historical (e.g. supratentorial PNET). We were able to offer a confident diagnosis in 51% of cases using histological techniques alone without an array. In part, this reflects an increased recognition of specific entities (e.g. diffuse midline glioma, H3K27 mutant) and the availability of more specific markers (e.g. antibodies against mutant H3K27). However, it emphasises that new diagnostic techniques should not be directly compared to historical diagnoses.

The second cohort also identified tumours that we were unable to diagnose with pathology or methylation profiling. Although it is not certain what factors account for these 'un-diagnosable' cases, we consider that they are either novel entities or recognised tumour types where current technology cannot diagnose them. The latter may be due to technical factors (such as low tumour content) or intrinsic limitations of the diagnostic methods. However, based on the clustering of the cases (Figure 6), we estimate that only 35% of these 'un-diagnosable' cases are recognised entities. This gives an upper limit to the number of cases that are potentially novel (65%) and these warrant further investigation.

Access to specialist pathology varies globally (both molecular and histological expertise) and that the impact of methylation arrays on diagnosis might reflect this. We speculate the impact of methylation profiling might be greater in non-specialist centres, although the frequency of complex cases may be reduced. Additionally, as novel immunohistochemical markers are identified, the impact of profiling may change. For example, H3K27me3 was recently introduced to discriminate ependymoma PFA from PFB³⁴. Had this been implemented at the time, the overall diagnostic impact of the arrays would have changed from 35% to 34% (107/306 and 103/306) with no change in treatment impact.

Subgrouping of some tumours may currently have minimal clinical impact (e.g. within pilocytic astrocytoma), however evidence for risk stratification will only become clear once sufficient outcome data is linked to molecular subtyping. Identification of targetable driver mutations (e.g. BRAF and NTRK) is largely dependent on sequencing data that methylation profiling cannot explicitly provide; in our experience classification can help triage genetic testing and in the future we expect this to increase as more experimental treatments are made available.

Finally, we compared inferred copy number data to standard FISH testing. We conclude that for some indications, methylation profiling could replace FISH (e.g. *C19MC* in ETMR). It may also be helpful to identify diagnostically relevant chromosomal changes (e.g. *CDKN2A/B* loss in PXA or monosomy chr6 in medulloblastoma, data not presented) or to indicate fusion events¹⁰. However, we caution against absolute replacement for traditional testing because of

the effects of tumour heterogeneity or low tumour content. The danger of missing prognostic information by CN alone is highlighted by the limited sensitivity for *MYC* and *MYCN* amplification in medulloblastoma.

The limitation of our study arises from the nature of the cohorts. While the clinical cohort captures very many of the UK's children's brain tumours, there may be sampling biases e.g. possible enrichment for difficult to diagnose tumours or those requiring molecular data for full subtyping. The research cohort is limited to high-grade tumours, and we have not explored a retrospective study of low-grade tumours. It will be useful to continue to review the impact when all tumours are routinely profiled.

We have used methylation profiling of childhood brain tumours to develop an approach to assess the impact of genomic technology in clinical practice. This real-world evidence approach complements those of traditional controlled prospective studies. We suggest that this is a general approach that allows new genomic technology to be. Also established techniques could be assessed using these criteria; how much of the established immunohistochemistry would show similar clinical impact if tested in the same way?

Acknowledgements

We are grateful to the referring centres and pathologists (see supplementary Table 3). Archival tissue samples were obtained from the NHS Trusts as part of the UK Brain Archive Information Network (BRAIN UK) which is funded by Brain Tumour Research and the Medical Research Council. We are grateful to Lydia Llewellyn (University Hospital of Wales Cardiff) for help in obtaining tissue. We are grateful for funding from the Brain Tumour Charity, Children with Cancer UK, Great Ormond Street Hospital Children's Charity, Olivia Hodson Cancer Fund, Cancer Research UK and the National Institute of Health Research. This work as supported by the INSTINCT network funded by The Brain Tumour Charity, Great Ormond Street Children's Charity and Children with Cancer UK, and the EVEREST centre funded by The Brain Tumour Charity. All research at Great Ormond Street Hospital NHS Foundation Trust and UCL Great Ormond Street Institute of Child Health is made possible by the NIHR Great Ormond Street Hospital Biomedical Research Centre. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. We thank Prof. Neil Sebire and Prof. Juan Pedro Martinez-Barbera for their comments on the manuscript.

Contributors

JCP and TSJ designed the study, analysed data, and wrote the manuscript. AM, SAY, TSJ

reported clinical cases. ARF, CR, KMK, JARN, CL, TB, DAH, SS, LD, PNJ, LRB, AC, AI, NR, KS, AGL, CLS, CS, AT, FR, RMH, SCC, YG, DW, MC, CJ provided archival cases, data and/or clinical annotation. TJS, DH, LB, AA, SWA, OO, JG, AFP, ME, LW, CD, DL, MK, JC carried out diagnostic testing and/or data analysis. DC, MS, AvD, SMP, DTWJ developed and/or maintain the classifier algorithm. All authors reviewed and accepted the paper before submission.

Declaration of interests

DC, MS, AvD, DTWJ, SMP have a patent "DNA methylation based method for classifying tumor species of the brain" (EP3067432A1) pending. The other authors declared no direct conflicts of interest but report the following interests outside the work: DC and AvD have patents relating to IDH1 R132H antibody and BRAF V600E antibody. TSJ and AM are directors and shareholders of Repath Ltd. TSJ is a director and shareholder of Neuropath Ltd. DH reports non-financial support from Dabrafenib and Trametinib (Novartis), grants, personal fees and non-financial support from Selumetinib (AstraZeneca), grants, personal fees and non-financial support from Larotrectinib (Loco/Bayer), personal fees and non-financial support from Entrectinib (Roche).

Figure Legends

Figure 1. Routine implementation of DNA methylation arrays into a specialist paediatric neuropathology centre. (A) Annual proportion of UK's cases reported with and without a DNA methylation array (EPIC, 850K) at our neuropathology centre. Annual UK incidence (n=633) based on CRUK data²². Values plotted are on an annual basis, i.e. Total number of cases in the cohort are halved. Values at the top of each plotted bar are the sum of cases by age. (Total cases reported = 484, total cases with an array = 306). (B) Proportions of arrays performed in all cases reported during two years with a final diagnosis of medulloblastoma, ependymoma, low- and high-grade gliomas.

Figure 2. DNA methylation classification has additive diagnostic value in 35% ±5% of CNS paediatric cases (n=107). Graphical representation of the overall impact of arrays on routine diagnostic practice for paediatric CNS tumours. We considered an array to have additive value when the output refined (99) or amended (5) the diagnosis or added other relevant diagnostic data, of uncertain significance (3).

Figure 3. Diagnostic yield of DNA methylation arrays is highest in well characterised CNS tumours. Calibrated scores for cases were broken down (A) by binned probe failure rate

(PFR), (B) by indication for array and (C) by broad diagnosis. The red dotted lines in B and C represent the 0.9 calibrated threshold, values equal and above this value were accepted for diagnostic reporting. The numbers are sample numbers; the heavy black line is the median, the boxes are the interquartile range and the whiskers are the range excluding outliers. Outliers are plotted as circles (if 1.5-3.0x box length from the box edge) and asterisks (if more than 3.0x box length from the box edge).

Figure 4. Accuracy of inferring amplification data from DNA methylation array data.

Amplification data inferred from CN plot The Brain Tumour Charity was compared to the results of reported cytogenetic data (FISH), for 62 medulloblastoma patients. (A) Sensitivity and specificity calculations for *MYCN* and (B) *MYC*. For example, CN plots and example cytogenetic imaging, see supplementary figure S4.

Figure 5. Impact of suboptimal scoring predictions on the final reported diagnosis. (A)

One in every 3 suboptimal scoring predictions (range 0 to <0.9) will be misleading (or 35%, 55/157). (B) Precision of classifier predictions broken down by calibrated score range. Calculated by the sum of outputs that matched the final diagnosis and were plausible but unconfirmed, divided by the total number of cases. For scores 0.7 - 0.9, 1 in every 14 predictions is estimated to be misleading; compared to scores above 0.9 where only 1 in every 50 predictions was misleading (C) Median calibrated score by diagnostic impact.

Figure 6. Modern pathology in combination with DNA methylation profiling can assign a final diagnosis to 68% of difficult to diagnose cases. (A)

Review of 195 archival cases using current neuropathology practices and how the diagnosis was reached. Insert represents 62 undiagnosable cases which were further analysed. (B) Breakdown of cases diagnosed by local standard pathology methods without requiring array data (n=99) and (C) cases reviewed that required pathology and array data for a molecular diagnosis (n=34). (D) t-SNE plot of 47 remaining unsolvable cases passing QC which failed to classify (black) overlaid on the DKFZ reference cohort of known CNS tumour subgroups (coloured points). Samples that clustered within or adjacent to known groups were considered to match to the corresponding molecular subgroup. Abbreviations: HGG: high grade glioma, LGG: low grade glioma, PXA: pleomorphic xanthoastrocytoma, GNT: glioneuronal tumour, MB: medulloblastoma, ETMR: embryonal tumour with multilayered rosettes, ATRT: atypical teratoid/rhabdoid tumour, NB: neuroblastoma, HGNET: high grade neuroepithelial tumour, NOS: not otherwise specified; GBM G34: glioblastoma IDH wildtype H3.3 G34 mutant; GBM RTK I: glioblastoma IDH wildtype subclass RTK I; GBM RTK II: glioblastoma IDH wildtype subclass RTK II; GBM RTK III: glioblastoma IDH wildtype subclass RTK III; GBM MYCN: glioblastoma IDH wildtype

subclass MYCN; GBM MES: glioblastoma IDH wildtype subclass mesenchymal; GBM MID: glioblastoma IDH wildtype subclass midline; MB, G3: Medulloblastoma, subclass group 3; MB, G4: Medulloblastoma, subclass group 4; ETMR: embryonal tumour with multilayered rosettes.

References

1. Mack SC, Northcott PA. Genomic Analysis of Childhood Brain Tumors: Methods for Genome-Wide Discovery and Precision Medicine Become Mainstream. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2017;35(21):2346-54.
2. Bertier G, Carrot-Zhang J, Ragoussis V, Joly Y. Integrating precision cancer medicine into healthcare-policy, practice, and research challenges. *Genome medicine*. 2016;8(1):108.
3. Forrest SJ, Georger B, Janeway KA. Precision medicine in pediatric oncology. *Current opinion in pediatrics*. 2018;30(1):17-24.
4. Ippen FM, Colman H, van den Bent MJ, Brastianos PK. Precision Medicine for Primary Central Nervous System Tumors: Are We There Yet? *American Society of Clinical Oncology educational book American Society of Clinical Oncology Annual Meeting*. 2018;38:158-67.
5. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta neuropathologica*. 2016;131(6):803-20.
6. Schwalbe EC, Lindsey JC, Nakjang S, Crosier S, Smith AJ, Hicks D, et al. Novel molecular subgroups for clinical classification and outcome prediction in childhood medulloblastoma: a cohort study. *The Lancet Oncology*. 2017;18(7):958-71.
7. Korshunov A, Casalini B, Chavez L, Hielscher T, Sill M, Ryzhova M, et al. Integrated molecular characterization of IDH-mutant glioblastomas. *Neuropathology and applied neurobiology*. 2018.
8. Sturm D, Orr BA, Toprak UH, Hovestadt V, Jones DTW, Capper D, et al. New Brain Tumor Entities Emerge from Molecular Classification of CNS-PNETs. *Cell*. 2016;164(5):1060-72.
9. Capper D, Jones DTW, Sill M, Hovestadt V, Schrimpf D, Sturm D, et al. DNA methylation-based classification of central nervous system tumours. *Nature*. 2018;555(7697):469-74.
10. Capper D, Stichel D, Sahm F, Jones DTW, Schrimpf D, Sill M, et al. Practical implementation of DNA methylation and copy-number-based CNS tumor diagnostics: the Heidelberg experience. *Acta neuropathologica*. 2018;136(2):181-210.
11. Fernandez AF, Assenov Y, Martin-Subero JI, Balint B, Siebert R, Taniguchi H, et al. A DNA methylation fingerprint of 1628 human samples. *Genome research*. 2012;22(2):407-19.
12. Katkade VB, Sanders KN, Zou KH. Real world data: an opportunity to supplement existing evidence for the use of long-established medicines in health care decision making. *Journal of multidisciplinary healthcare*. 2018;11:295-304.
13. FDA. Real-World Evidence 2019 05/09/2019; 2019(07/10/2019). Available from: <https://www.fda.gov/science-research/science-and-research-special-topics/real-world-evidence>.
14. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics (Oxford, England)*. 2014;30(10):1363-9.
15. Hastie T TR, Narasimhan B, Chu G. Impute: impute: Imputation for microarray data. R package version 1.56.0. 2018.
16. Louis DN OH, Wiestler OD, Cavenee WK, Ellison DW, Figarella-Branger D, Perry A, Reifenberger G, Von Deimling A. WHO Classification of Tumours of the Central Nervous System Lyon IARC Press; 2016.
17. Pathologists TRCo. Dataset for tumours of the central nervous system, including the pituitary gland (4th edition). Standards and datasets for reporting cancers. London: The Royal College of Pathologists; 2016. p. 1-41.
18. Hovestadt V ZM. Conumee: Enhanced copy-number variation analysis using Illumina DNA methylation arrays. R package version 1.10.1.

19. Stone TJ, Keeley A, Virasami A, Harkness W, Tisdall M, Izquierdo Delgado E, et al. Comprehensive molecular characterisation of epilepsy-associated glioneuronal tumours. *Acta neuropathologica*. 2018;135(1):115-29.
20. Stichel D, Ebrahimi A, Reuss D, Schrimpf D, Ono T, Shirahata M, et al. Distribution of EGFR amplification, combined chromosome 7 gain and chromosome 10 loss, and TERT promoter mutation in brain tumors and their potential for the reclassification of IDHwt astrocytoma to glioblastoma. *Acta neuropathologica*. 2018;136(5):793-803.
21. Pickles JC, Hawkins C, Pietsch T, Jacques TS. CNS embryonal tumours: WHO 2016 and beyond. *Neuropathology and applied neurobiology*. 2018;44(2):151-62.
22. Children's cancers incidence statistics, Cancer Research UK 2019 01/01/2019; 2019. Available from: <https://www.cancerresearchuk.org/health-professional/cancer-statistics/childrens-cancers/incidence>.
23. Overview of the UK population, Office for National Statistics 2018 01/01/2019. Available from: <https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/articles/overviewoftheukpopulation/november2018>.
24. Jaunmuktane Z, Capper D, Jones DTW, Schrimpf D, Sill M, Dutt M, et al. Methylation array profiling of adult brain tumours: diagnostic outcomes in a large, single centre. *Acta neuropathologica communications*. 2019;7(1):24.
25. Sturm D, Orr BA, Toprak UH, Hovestadt V, Jones DT, Capper D, et al. New Brain Tumor Entities Emerge from Molecular Classification of CNS-PNETs. *Cell*. 2016;164(5):1060-72.
26. Pfister S, Remke M, Benner A, Mendrzyk F, Toedt G, Felsberg J, et al. Outcome prediction in pediatric medulloblastoma based on DNA copy-number aberrations of chromosomes 6q and 17q and the MYC and MYCN loci. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2009;27(10):1627-36.
27. Mansouri A, Hachem LD, Mansouri S, Nassiri F, Laperriere NJ, Xia D, et al. MGMT promoter methylation status testing to guide therapy for glioblastoma: refining the approach based on emerging evidence and current challenges. *Neuro-oncology*. 2018.
28. Buccoliero AM, Castiglione F, Rossi Degl'Innocenti D, Paglierani M, Maio V, Gheri CF, et al. O6-Methylguanine-DNA-methyltransferase in recurring anaplastic ependymomas: PCR and immunohistochemistry. *Journal of chemotherapy (Florence, Italy)*. 2008;20(2):263-8.
29. Nagasawa DT, Trang A, Choy W, Spasic M, Yew A, Zarinkhou G, et al. Genetic expression profiles of adult and pediatric ependymomas: molecular pathways, prognostic indicators, and therapeutic targets. *Clinical neurology and neurosurgery*. 2013;115(4):388-99.
30. Korshunov A, Ryzhova M, Hovestadt V, Bender S, Sturm D, Capper D, et al. Integrated analysis of pediatric glioblastoma reveals a subset of biologically favorable tumors with associated molecular prognostic markers. *Acta neuropathologica*. 2015;129(5):669-78.
31. Eisenstat DD, Pollack IF, Demers A, Sapp MV, Lambert P, Weisfeld-Adams JD, et al. Impact of tumor location and pathological discordance on survival of children with midline high-grade gliomas treated on Children's Cancer Group high-grade glioma study CCG-945. *Journal of neuro-oncology*. 2015;121(3):573-81.
32. Uro-Coste E, Masliah-Planchon J, Siegfried A, Blanluet M, Lambo S, Kool M, et al. ETMR-like infantile cerebellar embryonal tumors in the extended morphologic spectrum of DICER1-related tumors. *Acta neuropathologica*. 2019;137(1):175-7.
33. Global, regional, and national burden of brain and other CNS cancer, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Neurology*. 2019;18(4):376-93.
34. Panwalkar P, Clark J, Ramaswamy V, Hawes D, Yang F, Dunham C, et al. Immunohistochemical analysis of H3K27me3 demonstrates global reduction in group-A childhood posterior fossa ependymoma and is a powerful predictor of outcome. *Acta neuropathologica*. 2017;134(5):705-14.