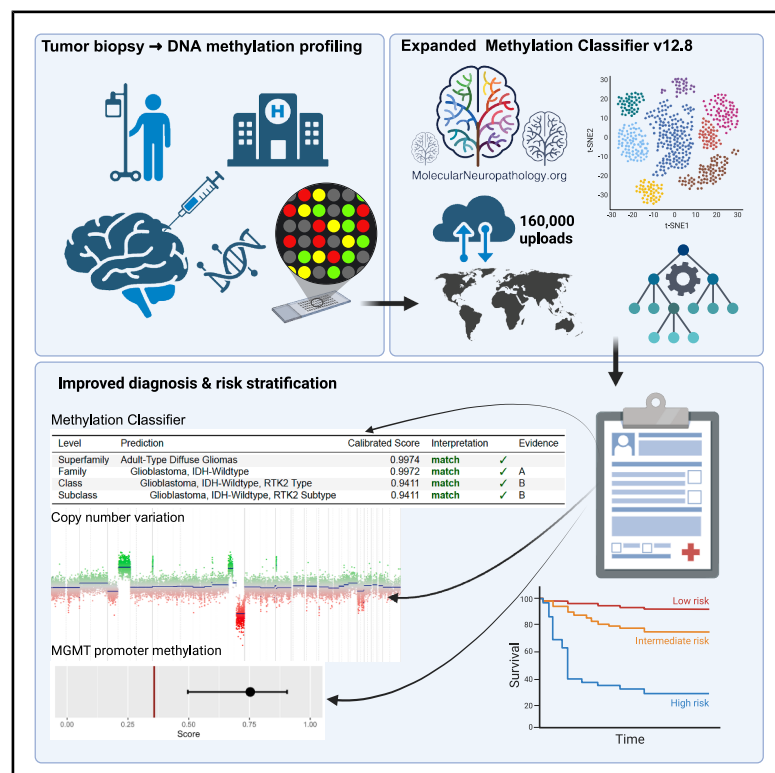# Advancing CNS tumor diagnostics with expanded DNA methylation-based classification

Graphical abstract

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In brief

Sill and colleagues present an expanded DNA methylation classifier for CNS tumors. Building upon the 2021 WHO classification, this resource introduces a hierarchical taxonomy of 184 subclasses. It enhances classification accuracy for rare entities and provides integrated genomic data from a single, streamlined assay for modern neuro-oncology.

Highlights

- Classifier aligns with and extends the 2021 WHO CNS tumor classification

- New hierarchical structure organizes 184 subclasses to inform future classifications

- Integrated assay provides classification and CNV data to streamline molecular workup

- Large-scale data enables robust identification of ultra-rare CNS tumor entities

CellPress

Article

Advancing CNS tumor diagnostics with expanded DNA methylation-based classification

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SUMMARY

DNA methylation-based classification is now central to contemporary neuro-oncology, as highlighted by the World Health Organization (WHO) classification of central nervous system (CNS) tumors. We present the Heidelberg CNS Tumor Methylation Classifier version 12.8 (v12.8), trained on 7,495 methylation profiles, which expands recognized entities from 91 classes in version 11 (v11) to 184 subclasses. This expansion is a result of newly identified tumor types discovered through our large online repository and global collaborations, underscoring CNS tumor heterogeneity. The random forest-based classifier achieves 95% subclass-level accuracy, with its well-calibrated probabilistic scores providing a reliable measure of confidence for each classification. Its hierarchical output structure enables interpretation across subclass, class, family, and superfamily levels, thereby supporting clinical decisions at multiple granularities. Comparative analyses demonstrate that v12.8 surpasses previous versions and conventional WHO-based approaches. These advances highlight the improved precision and practical utility of the updated classifier in personalized neuro-oncology.

INTRODUCTION

DNA methylation-based classification has become a central pillar of state-of-the-art diagnostics in neuro-oncology. Most prominently, the fifth edition of the World Health Organization

(WHO) classification of central nervous system (CNS) tumors[1] lists DNA methylation profiling as a desirable or even essential method for accurately diagnosing several tumor types. In addition, methylation profiling is now recommended by multiple guideline authorities and medical societies, such as the National

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Cancer Cell
Article

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DNA methylation encodes a unique combination of information—the heritable marks of cell-of-origin and changes incurred during tumor initiation and progression. This makes it a stable and reliable resource for tumor typing. Here, we present the diverse landscape of CNS tumors represented by the Heidelberg methylation classifier v12.8 and its utility in clinical routine diagnostics. DNA methylation-based classification of CNS tumors was pioneered with the public release of the Heidelberg CNS tumor classifier v11, which was trained on a reference set of 2,801 samples comprising 91 classes primarily based on the existing WHO tumor types.[6] The classifier, and all subsequent updates, were made available to the scientific community for the past 9 years (2016–2025) on the molecularneuropathology.org platform. At the time of data freeze in October 2024, over 160,000 profiles worldwide were analyzed on the platform. In addition to analyses and database management for the community, the platform included an end user license agreement (EULA) that offered users to share data for further development. This facilitated the accumulation of diverse DNA methylation profiles from across the globe. As the data repository expanded, a considerable number of uploaded samples failed to align with any of the 91 classes in v11, thus prompting exploratory analyses that led to identification of previously undefined or misclassified

tumor types. We mainly employed unsupervised approaches to identify novel clusters using methylation data with further validation relying on ancillary methods like DNA/RNA sequencing, immunohistochemistry, etc. Taken together, these findings laid the groundwork for creating an updated reference set for v12.8. Multiple novel methylation-defined or -supported entities from v12.8 are now recognized by the WHO 2021 guidelines, such as the diffuse glioneuronal tumor with oligodendroglioma-like features and nuclear clusters (DGONC).[7] The classifier has been utilized and validated in independent cohorts across diverse regions and setups, demonstrating its universal robustness and potential clinical utility.

The value of methylation classification primarily lies in overcoming the limitations of classical histology-dependent methods. Owing to its robust nature, it overcomes potential inter-observer variability in reporting and the hypothesis-driven nature of targeted testing. Furthermore, methylation profiling using methylation arrays offers prognostic information like copy-number data and MGMT promoter methylation status in addition to methylation classification in a single assay.

RESULTS

v12.8 reference set expands classification to 184 hierarchical subclasses

Building upon the reference set of the previously published Heidelberg methylation classifier v11 comprising 2,801 samples

A

B

C

Figure 1. Overview of the molecularneuropathology.org platform and its utilization
(A) Global distribution of sample uploads to molecularneuropathology.org from October 2016 to February 2025.
(B) UMAP projection of 97,213 CNS tumor samples, dots are colored by the v11 classifier confidence score levels for each sample, as indicated in the legend. The *x* and *y* axes represent the first and second dimensions of the non-linear UMAP projection, respectively.
(C) Flow diagram illustrating the Machine Learning Operations (MLOps) life cycle used for model training, validation, deployment, and maintenance for the MNP classifier.

and our large database of over 160,000 samples (Figure 1), we expanded the reference set for v12.8 to 7,495 CNS methylation profiles (Figures 2 and S1). Of these, approximately 19% derive from the previous v11 cohort, preserving continuity and consistency with established diagnostic categories. An additional 11% originate from user submissions via our publicly accessible web platform. While these represent a relatively small proportion of the final training dataset, the diversity of the uploaded samples played a valuable supporting role in identifying new tumor entities. The remaining samples were either diagnostic cases added to increase the sample size of previously underrepresented classes or sourced from institutional collaborations, particularly those focusing on well-characterized entities such as meningiomas,[8] posterior fossa (PF) A and PFB ependymomas,[9,10] and medulloblastomas.[11,12]

To leverage the growing volume of methylation profiles, we regularly performed non-linear dimensionality reduction analyses—specifically, t-distributed stochastic neighbor embedding (t-SNE) and uniform manifold approximation and projection (UMAP) (Figure 3A). These methods enabled unsupervised exploratory data analysis, revealing new, distinct clusters constituting samples that were previously unclassifiable under the original (v11) framework (Figures 3B, 3C, and S2). Visual inspection of t-SNE and UMAP plots, in combination with molecular characterization and clinical data of samples helped differentiate relevant emerging tumor clusters from 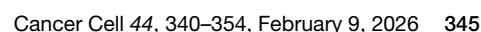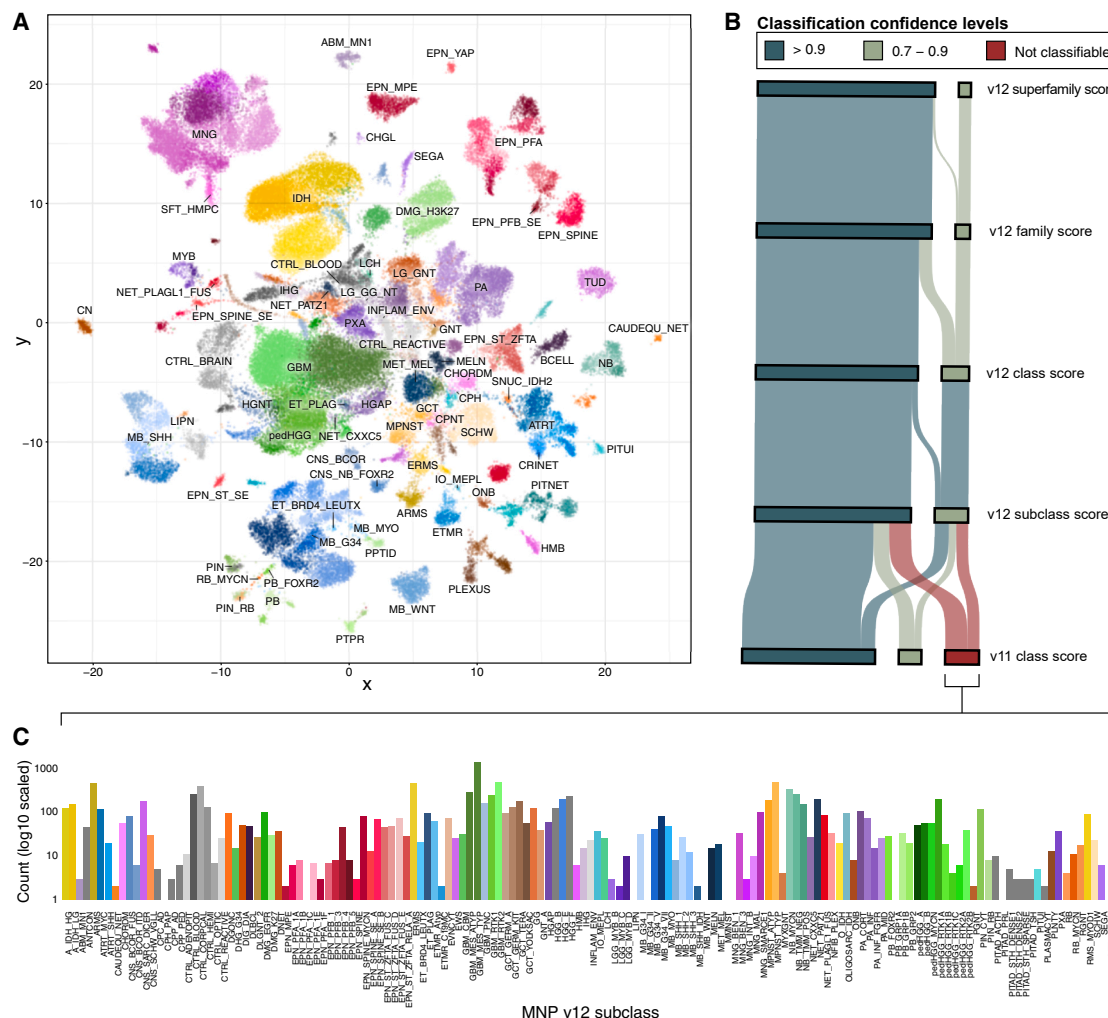known entities, prompting identification of novel subclasses and refinement of existing ones. In addition, methylation profiling using methylation arrays yields genome-wide copy-number variation (CNV) data and MGMT (methylated-DNA-protein-cysteine methyltransferase)

promoter methylation status independently of methylation classification from the same assay. This unified assay is highly efficient and conserves precious tissue in addition to being essential for contemporary CNS tumor diagnostics. Copy number data robustly identifies pathognomonic and prognostic alterations in multiple entities particularly diffuse gliomas and meningiomas. This information is indispensable for characterizing emerging entities from unsupervised clustering of methylation data. For example, the newly described glioneuronal tumor with ATRX alteration (GTAKA) subclass is known to harbor homozygous *CDKN2A/B* deletions in ~50% of cases, *ATRX* alterations and importantly recurrent targetable NTRK fusions (Figure S3A).[13] In the same way, the novel entity DGONC was found to harbor a characteristic monosomy of chromosome 14 or homozygous *CDKN2A/B* deletion (Figure S3B).[7] Thus, methylation-based classification can directly guide the search for actionable therapeutic alterations. Similarly, the CNV profile can provide strong evidence for other targetable alterations, such as the characteristic tandem duplication at the *BRAF* locus indicative of a *KIAA1549:BRAF* fusion, which can be confirmed by transcript-level analysis (Figure S4). This multi-faceted approach, combining unsupervised clustering with detailed CNV and clinical and molecular analysis, allowed us to systematically incorporate these new entities, effectively doubling the total number of subclasses to 184, with the newly added entities detailed in Table 1.

Alongside the expansion of tumor classes, we introduced a four-tier hierarchical structure (Figures 2 and S1; Table S1) designed to reflect the complex biological relationships between tumor entities. While subclasses denote the highest granularity,

Figure 2. Training dataset for v12.8

(A) UMAP projection of 7,495 samples used for training the v12.8 classifier. The *x* and *y* axes represent the first and second dimensions of the non-linear UMAP projection, respectively.

(B) Legend indicating color code for subclasses shown in (A). Letters in rounded brackets before abbreviation of the subclass indicate "evidence level" for the respective subclass. Each broad category shown in bold in the legend is a superfamily, families are indicated using a solid bracket on the second level adjacent to the colored blocks, classes are indicated using a dotted-line bracket at the first level. Due to the large number of subclasses, only six superfamilies are highlighted here. The remaining superfamilies and their corresponding families, classes, and subclasses are shown in Figure S1.

See also Figure S1; Table S1.

established diagnostic categories, supported by robust clinical, histological, and molecular evidence, generally reside at the "family" or "class" level. Newly recognized entities, often defined by subtle epigenetic variations and with clinical relevance that may still be unresolved, are usually assigned to the subclass level. Moreover, superfamilies commonly correspond to the broad WHO categories. As a conservative approach, the classifier defaults to higher-tier assignments if subclass boundaries are not clearly defined. This hierarchical system is meant to provide a framework that mirrors the clinical and biological complexity of CNS tumors. Furthermore, we formulated evidence levels as annotations for each entity. These annotations, together with relevant publications, are listed in Table S1 and provide guidance on the available information about the entities and their alignment with the current WHO classification (Figures 2 and S1). Level *a* refers to tumor entities identical to the WHO 2021. Level *b* refers to entities defined by large single or more than one smaller dataset published describing the type/subtype as molecularly and/or clinically distinct, or the methylation class represents a distinct fraction of an established WHO 2021 tumor class. Level *c* refers to entities described by a single small dataset or case series. Level *d* refers to entities that are solely based on clustering signals in a t-SNE or UMAP. The annotation is typically provided at the most granular layer, and in addition at a higher layer if the latter matches a WHO type or subtype. The definitions, hierarchical levels and annotations

were curated and reviewed by an international group of neuropathologists.

v12.8 classifier achieves 95% cross-validated accuracy while providing well-calibrated, probabilistic confidence scores

To train the classification model, we followed the random forest-based approach described previously.[6,43] We evaluated the performance of the classifier using a 5-fold nested cross-validation scheme. All subclasses achieved a balanced accuracy greater than 0.75, with 175 out of 184 subclasses exceeding 0.9 in balanced accuracy (Figure 4A).

The classifier provides probabilistic confidence scores for each prediction. Our new hierarchical system leverages these scores by summing the probabilities of mutually exclusive subclasses to calculate parent-category probabilities. This approach provides more robust diagnostic guidance at higher tiers of the hierarchy. As observed with the v11 classifier, most "errors" occur between closely related subclasses or classes, such as among different subclasses (*) of posterior fossa group A (EPN_PFA_*) ependymomas (Figure 4B), between subclasses of group 3 and 4 medulloblastomas (MB_G34_*), or among benign meningioma subclasses (MNG_BEN_*). While some of these newly delineated subclasses indeed correlate with different prognostic outcomes, as described in their respective publications, most subclasses currently lack direct clinical

Figure 3. UMAP projection comparing classification performance of mnp_v11b6 and mnp_v12.8

(A) Methylation profiles of 97,213 CNS tumor samples, including the v12.8 training data, classified using the v12.8 classifier, where all samples achieve a classification score of ≥ 0.7 and are colored according to the v12.8 color scheme for the subclass. The labels are the abbreviation for the family level. The x and y axes represent the first and second dimensions of the non-linear UMAP projection, respectively.

(B) Sankey plot showing scores for predictions with the v11 classifier and hierarchical levels of the v12.8 classifier respectively for the samples illustrated in (A).

(C) Bar-plot indicates log10-scaled number of v12 subclass predictions for samples not classifiable using v11.

See also Figure S2.

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implications, making aggregated probability scores at higher hierarchical levels sufficient for guiding diagnostic decisions according to current knowledge.

Overall, the classifier achieved a 95% subclass-level accuracy and a Brier score of 0.028. In multiclass classification, the Brier score measures the mean squared difference between predicted probabilities (i.e., calibrated classifier scores) and observed class frequencies, indicating that the probability estimates are exceptionally well-calibrated and outperform those of the original v11 classifier (Figures 4C–4E).

The 0.9 confidence threshold provides a reliable cutoff for clinical use

Similar to the previous v11 classifier, we recommend a threshold of 0.9 across all tumor entities at the family level in v12.8. This

threshold was selected because it showed good overall performance in our cross-validation across all subclasses in both the v11 and v12.8 training datasets, and because it is straightforward to communicate in clinical practice. In addition, we performed one-vs-all receiver operating characteristics (ROC) analyses for each subclass against all others and selected the threshold that maximizes Youden's index, thus optimally balancing sensitivity and specificity (Figure 4D; Table S1). The highest Youden-based threshold was 0.77, indicating that a 0.9 cutoff is somewhat conservative for most subclasses. Nevertheless, applying a 0.9 threshold helps maintain high sensitivity for certain subclasses. Ultimately, the well-calibrated probability scores provided by our classifier allow users to make informed decision-making in the context of other available complementary clinical, histological, and molecular data.[44]

Table 1. Overview of newly added or expanded tumor classes in the v12.8 classifier

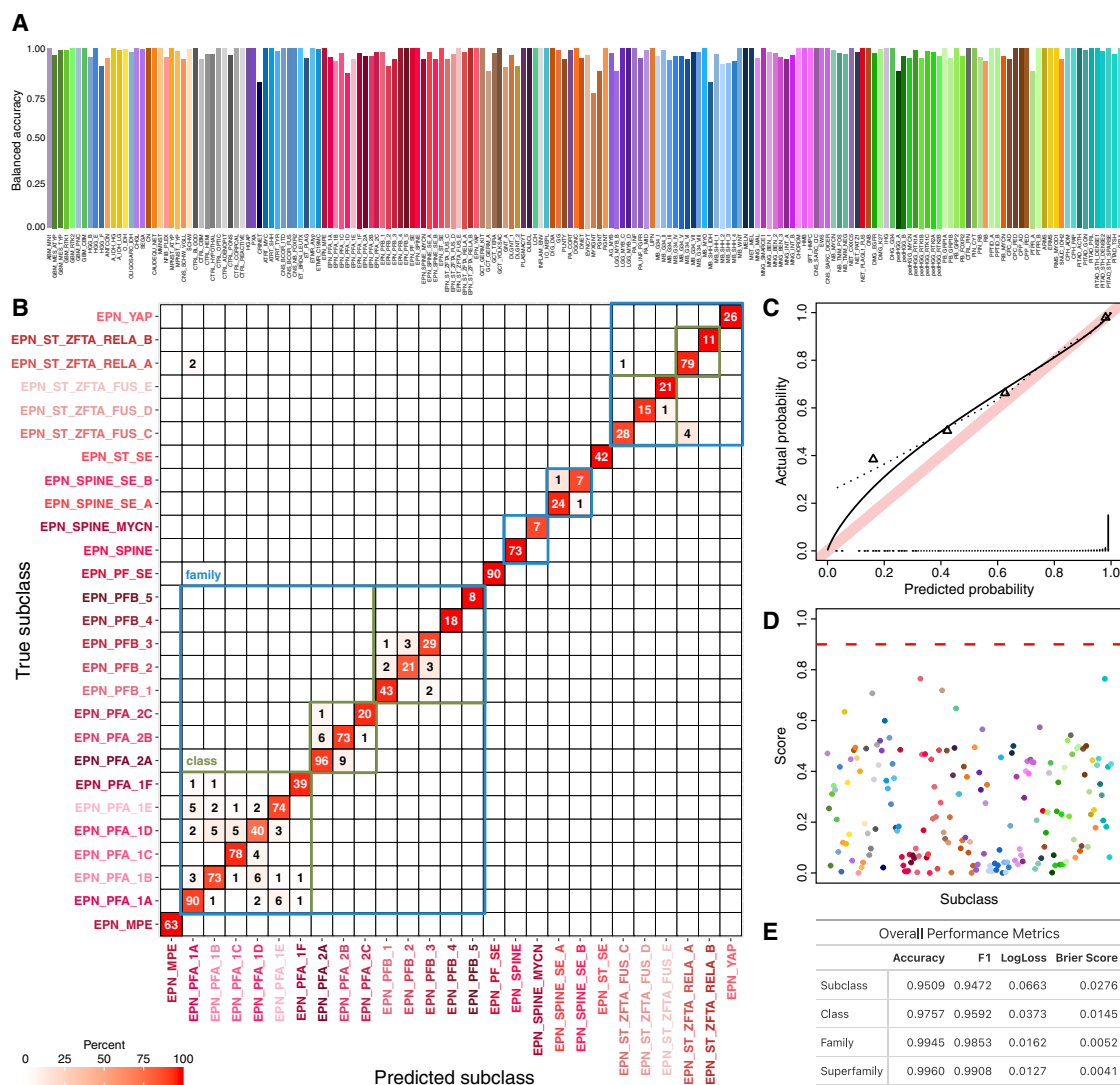| Broad Tumor Category	Tumor Class/Subclass and Key Features	Reference(s)
Meningiomas	Subclasses of Meningioma	Sahm et al.[8]
	Clear cell meningioma (*SMARCE1*-mutant)	Sievers et al.[14]
Ependymomas	Ependymoma, subtypes PFA and PFB	Pajtler et al.[9]; Cavalli et al.[10]
	Ependymoma, *ZFTA*-fused	Zheng et al.[15]
	Spinal ependymoma, *MYCN*-amplified	Ghasemi et al.[16]
Medulloblastomas	Wingless class (WNT), subtypes of Sonic Hedgehog (SHH) medulloblastomas and consensus subtypes of non-WNT/non-SHH medulloblastomas.	Cavalli et al.[11]; Sharma et al.[12]; Taylor et al.[17]; Hovestadt et al.[18]
Gliomas & Glioneuronal Tumors	Diffuse leptomeningeal glioneuronal tumors (DLGNT)	Deng et al.[19]
	Diffuse glioneuronal tumor with nuclear clusters (DGONC)	Deng et al.[7]
	IDH-mutant oligosarcomas	Suwala et al.[20]
	Glioblastomas with primitive neuronal component	Suwala et al.[21]
	Glioneuronal tumor with ATRX alteration (GTAKA)	Bogumil et al.[13]
	Gliomas with *MYB/MYBL1* alteration	Chung et al.[22]; Wefers et al.[23]
Embryonal & Neuroepithelial Tumors	Cribriform neuroepithelial tumors (*SMARCB1*-deficient)	Johann et al.[24]
	CNS neuroblastoma, *FOXR2*-activated	Tauziède-Espariat et al.[25]
	Embryonal tumors with *BRD4::LEUTX* fusion	Andreiuolo et al.[26]
	Embryonal tumors with *PLAG*-family amplification	Keck et al.[27]
	Neuroepithelial tumors with *PATZ1*-fusions	Alhalabi et al.[28]
	CNS Tumor with *BCOR* Internal Tandem Duplication	Sturm et al.[29]
	CNS tumors with *EP300::BCOR* fusion	Tauziède-Espariat et al.[30]
	Intraocular medulloepithelioma	Zheng et al.[15]
Retinal Tumors	Retinoblastoma, *MYCN*-activated	Ghasemi et al.[16]
Sarcomas & Mesenchymal Tumors	Rhabdomyosarcoma subtypes	Clay et al.[31]; Mahoney et al.[32]
	Malignant melanotic nerve sheath tumors	Terry et al.[33]; Koelsche et al.[34]
	Plexiform neurofibromas	Grit et al.[35]
	Langerhans cell histiocytosis	Koelsche et al.[36]
Other CNS & Related Tumors	Germ cell tumors	Fukushima et al.[37]; Williams et al.[38]; Kubota et al.[39]
	Sinonasal undifferentiated carcinoma, *IDH2*-mutant	Dogan et al.[40]
	Pineal parenchymal tumors of intermediate differentiation	Pfaff et al.[41]
	Neuroblastomas (subtypes)	Henrich et al.[42]

See also Figures S3 and S4.

v12.8 outperforms v11 and resolves previously unclassifiable tumors

Among the samples in our methylation database, 97,213 achieved a v12.8 classifier score of ≥0.7 at the subclass level (Figure 3A). When applying the v11 classifier to this cohort, only 79,749 samples (82%) could be classified with a confidence score of ≥0.7 (Figures 3B and 3C). These previously unclassifiable cases were successfully classified into newly identified subclasses as well as some existing classes, thus benefiting from the increased training data and refined classification scheme in v12.8. Of the samples in the latter category (v11 score <0.7), 2,128 (12%) were classified as glioblastoma, IDH-wild type, mesenchymal type, 1,422 (8%) as glioblastoma, IDH-wild type,

RTK1/RTK2, and 587 (3%) as IDH-mutant astrocytoma with scores ≥0.7, thus underscoring the higher confidence of the v12.8 classifier in previously established entities. Overall, we demonstrate the improved performance of v12.8, which accommodates newly discovered tumor types and provides more robust classification for previously recognized entities.

The number of newly identified classes has steadily increased over the past years, now reaching a plateau state. To further explore the dynamics of rare subclass discovery, we performed an analysis of 14 subclasses with ≤50 cases each in the full cohort of 97,213 CNS tumors (Figure S2). The model projects that, given a throughput of 1,236 cases per month, it would take on average 2.9 years to identify 10 new cases of a typical

Figure 4. 5-fold nested cross-validation performance of the v12.8 classifier

(A) Bar plot showing the balanced accuracy for each of the 184 subclasses, as derived from 5-fold nested cross-validation.

(B) Confusion matrix focusing on the ependymoma superfamily, where the majority of misclassifications occur between subclasses that belong to the same class or family (indicated by green and blue rectangles, respectively).

(C) Calibration plot comparing predicted probabilities with observed outcomes, illustrating the degree of score calibration across subclasses.

(D) Scatterplot of subclass-specific Youden-optimal thresholds with color-coded tumor classes and a red dashed line at 0.9 marking the recommended threshold.

(E) Table summarizing overall performance metrics: accuracy, F1-score, log loss, and Brier score, evaluated at each hierarchical level (subclass, class, family, superfamily).
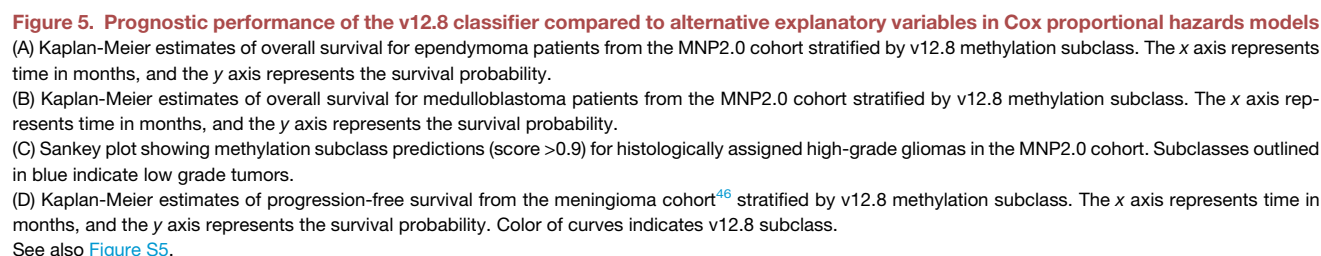
See also Table S1.

rare subclass. In light of nearly a decade of continuous data collection, we posit that the likelihood that entirely new and clearly distinct entities remain undiscovered is therefore low, although this cannot be excluded.

v12.8 subclasses show prognostic relevance in independent cohorts

To show the clinical potential of the v12.8 classifier, we analyzed data from the prospective, population-based Molecular Neuropathology 2.0 (MNP 2.0) study,[45] conducted within the German

pediatric neuro-oncology "Treatment Network HIT", which featured blinded central neuropathological review alongside molecular testing. In this cohort of over 1,200 newly diagnosed pediatric CNS tumor patients, the combined application of DNA methylation profiling and targeted panel sequencing improved the accuracy of tumor classification and identified cases where molecular data clarified ambiguous histology. Kaplan-Meier analysis of 80 ependymoma cases and 171 medulloblastoma cases, grouped by their v12.8 methylation subclass, revealed distinct survival curves for each subclass (Figures 5A and 5B),

Figure 5. Prognostic performance of the v12.8 classifier compared to alternative explanatory variables in Cox proportional hazards models

(A) Kaplan-Meier estimates of overall survival for ependymoma patients from the MNP2.0 cohort stratified by v12.8 methylation subclass. The *x* axis represents time in months, and the *y* axis represents the survival probability.

(B) Kaplan-Meier estimates of overall survival for medulloblastoma patients from the MNP2.0 cohort stratified by v12.8 methylation subclass. The *x* axis represents time in months, and the *y* axis represents the survival probability.

(C) Sankey plot showing methylation subclass predictions (score >0.9) for histologically assigned high-grade gliomas in the MNP2.0 cohort. Subclasses outlined in blue indicate low grade tumors.

(D) Kaplan-Meier estimates of progression-free survival from the meningioma cohort[46] stratified by v12.8 methylation subclass. The *x* axis represents time in months, and the *y* axis represents the survival probability. Color of curves indicates v12.8 subclass.

See also Figure S5.

highlighting meaningful prognostic differences. One important finding of the MNP2.0 study was that a subset of tumors originally diagnosed as high-grade gliomas (HGG) by conventional histopathology were classified as low-grade gliomas (LGG) when methylation results were taken into account; these patients showed more favorable outcomes during a median follow-up of 2.5 years, indicating that methylation-based classification could guide less intensive therapy. In line with this finding, Figure 5C shows a Sankey plot of 96 cases initially reported as "HGG". Of these, 22% were assigned to a lower grade tumor subclass with a score of at least 0.9 by the v12.8 classifier.

In another large study of meningiomas, the DNA-methylation family assignment was combined with WHO histological grading and chromosomal CNV data to develop an integrated molecular-morphologic score for risk stratification that significantly outperformed WHO grading alone.[46,47] To illustrate this potential of v12.8 methylation subclasses for risk stratification of meningiomas, Figure 5D shows the distinct Kaplan-Meier curves of pre-

dicted methylation subclasses for 958 meningioma patients included in this cohort. To further illustrate the clinical relevance of subclass annotations, we provide additional survival analyses in Figure S5. These include overall survival of patients from the HIT-2000 trial stratified by non-WNT/non-SHH medulloblastoma subclasses[48] and event-free survival of patients from the MNP2 study assigned to a specific subclass of the "low-grade glial/glioneuronal/neuroepithelial tumor" superfamily.

Overall, these three studies exemplify that DNA methylation-based profiling allows for more accurate classification and can be used for improved risk stratification of patients.

DISCUSSION

Identification of emerging clusters, designation as subclasses and classes, and their subsequent implementation into diagnostic guidelines is inherently an iterative process. The recent work of the cIMPACT-NOW consortium,[49] endorsed by the

International Society of Neuropathology, offers a framework for systematically evaluating emerging signals that suggest putative new tumor entities. Their guidelines recommend gathering comprehensive molecular, histopathological, and clinical outcome data before classifying any newly identified epigenetic cluster as a distinct diagnostic entity. The expanded Heidelberg Methylation Classifier (v12.8) described here embodies this iterative approach, wherein novel putative entities emerging from exploratory t-SNE and UMAP clustering undergo continuous scrutiny and validation by the international neuro-oncology community.

Although conceived primarily as a research tool, the Heidelberg Methylation Classifier has demonstrated profound diagnostic potential over time as reflected by its inclusion in multiple international neuro-oncology guidelines. The classifier possesses a distinct advantage owing to its scale and the comprehensive, meticulously curated reference data. While hypothesis-driven methods are inherently restrictive, our large data repository combined with an unsupervised approach enabled the precise classification of established tumor entities and the identification of previously uncharacterized, ultra-rare entities that would not be feasible with smaller cohorts.

The v12.8 expansion comes with a clear hierarchical structure and evidence level annotation, reflecting definitions and relevance of granular entities but also emphasizes the importance of shared terminology. Historically, subtle discrepancies have existed between formal guidelines and the subclasses output by the classifier. These discrepancies partly stem from the fact that diagnostic guidelines evolve on the basis of established clinical evidence, whereas classifier outputs may temporarily adopt more provisional (and often more granular) subclass designations when new molecular subgroups first emerge. To bridge this gap, a global panel of neuropathologists and molecular neuro-oncologists convened to refine and align subclass annotations, culminating in the updated nomenclature for both newly discovered and long-standing tumor entities identified by the classifier. Such efforts ensure that the classifier keeps pace with refinements in disease knowledge while maintaining coherence with diagnostic standards, ultimately improving acceptance and global dissemination.

Application of DNA methylation classification in routine diagnostics has advanced with unprecedented speed, catalyzed by the demonstrated clinical value of methylation-based tumor subtyping in independent prospective studies.[50–52] Adoption of the technology can be challenging for users new to molecular testing based on high-dimensional data, raising questions regarding robust quality control and cross-validation of results. Although multiple classifier tools may emerge—possibly using divergent statistical strategies—the potential redundancy could bolster procedural safety.[49] At the same time, discordant outcomes among classifiers may generate confusion, particularly if each tool adopts slightly different nomenclature or poorly calibrated confidence scores. Clear consensus, transparent communication, and adherence to regulatory guidelines will be essential for ensuring that the field continues to move toward improved and harmonized, rather than fragmented, diagnostic utility.

Recent work by Patel et al.[53] introduced the MNP-Flex classifier, trained on the same v12.8 reference dataset, which enables accurate classification from methylation data generated by different sequencing-based methods. Likewise, classifiers trained on v11 reference data were designed for use with ultra-sparse data obtained in intraoperative settings,[54,55] demonstrating that such pipelines can be applied to third-generation sequencing-derived methylation data. This demonstrates that methylation-based classification of CNS tumors is not limited to arrays but can potentially be applied across essentially all current and emerging methylation profiling platforms.

The impact of methylation profiling has also extended beyond CNS tumors. A DNA methylation classifier for sarcomas[36] was introduced shortly after the original CNS classifier and has recently been updated to version 13,[56] underscoring the broad applicability of this approach across tumor types. As an example, tumors with BCOR internal tandem duplications in the CNS and in sarcomas such as clear cell sarcoma of the kidney show highly similar DNA methylation profiles, reflecting shared lineage features.[57]

Overall, the Heidelberg Methylation Classifier v12.8 represents more than an incremental technical update, it embodies a collective effort to integrate clinical routine, cutting-edge translational research, and global equity. Ultimately, ongoing collaboration among researchers, clinicians and regulatory bodies will be crucial for realizing the full potential of methylation classification to improve patient care in neuro-oncology. Looking ahead, the impact of such developments will be defined not only by their precision but by the ability to democratize access to such advanced diagnostic methods for patients in diverse healthcare settings.

Limitations of the study

Despite its demonstrated diagnostic potential, the current approach has limitations, primarily rooted in its reliance on microarray technology. There are a substantial capital investment and high per-sample cost, thus creating significant access barriers, particularly for institutions with limited resources and throughput. This has resulted in an under-representation of data from low- and middle-income countries (LMICs) particularly in the global south, despite the high incidence rate in these regions. Furthermore, this reliance creates a dependency on a single product and its commercial life cycle. A key lesson from nearly a decade of providing this service is how challenging it is to maintain a stable platform when new array versions are released, as each update requires significant bioinformatic adaptation to ensure compatibility. This highlights the critical tension between technological advancement and the need for robust, validated clinical workflows. To begin addressing barriers of cost and global inequity, we have co-founded the Molecular Neuro-Pathology Outreach (MNP-Outreach) Consortium. The mission of this initiative is to facilitate the global adoption of methylation-based classification in LMICs. By fostering collaboration, we aim not only to improve diagnostic access but also to fill critical knowledge gaps regarding the molecular landscape of CNS tumors in underrepresented populations from the global South.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr. Martin Sill (m.sill@kitz.de).

Materials availability

This study did not generate new materials; all resources used are commercially or publicly available.

Data and code availability

- The v12.8 raw reference dataset, containing sensitive personal health information, is available through the German Human Genome-Phenome Archive (GHGA) under reference number GHGAS89861553411214. Access is controlled for data protection and granted for non-commercial research use following the execution of a Data Transfer Agreement (DTA). The v12.8 classifier is publicly accessible under: https://app.epignostix.com/. The code used to train the classifier is publicly available under: https://github.com/mwsill/mnp_training
- https://github.com/mematt/ml4calibrated450k.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

AUTHOR CONTRIBUTIONS

Conceptualization, M. Sill, D.S., D.T.W.J., F.S., A.v.D., S.M.P., and D.C.; methodology, M. Sill, D.S., D. Sturm, A.P., D.T.W.J., N.J., and P.S.; software, D.S.; classifier training, M. Sill; formal analysis, M. Sill, D.S., D. Sturm, A.P., D.T.W.J., N.J., and P.S.; investigation, all authors; data curation, all authors; critical review of class annotations for the v12.8 reference dataset and input on tumor nomenclature and evidence levels, D.C., F.S., C. Hawkins, C. Horbinski, C.T., K.A., P.W., D.R., A.v.D., S.M.P., M.K., and S.B.; supervision, D.T.W.J., F.S., S.M.P., and A.v.D.; writing – original draft, M. Sill and A.P.; writing – review and editing, M. Sill, A.P., D.S., D.T.W.J., F.S., A.v.D., D.C., and S.M.P.; resources, all authors. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

M. Sill, D.S., M. Snuderl, A.v.D., S.M.P., D.C., D.T.W.J., and F.S. are co-founders and shareholders of Heidelberg Epignostix GmbH, a company that develops and commercializes DNA methylation-based classifiers for CNS and other tumors, including the Heidelberg CNS Classifier described in this manuscript. M. Sill and N.J. became full-time employees of Heidelberg Epignostix in July 2024, A.P. in December 2024, and D.S. became a part-time employee in November 2024. M. Sill, A.v.D., D.S., D.T.W.J., D.C., V.H., and S.M.P. report patent EP16710700.2 and EP4384959A2. These patents cover the intellectual property for the DNA methylation-based CNS tumor classification method, which is the specific technology described and advanced in this manuscript. M.A.K. was funded in part through the NIH/NCI Cancer Center Support Grant P30 CA008748 to Memorial Sloan Kettering Cancer Center. G.F. and S.T. were funded by the DKS2020.02 research grant for HIT-REZ-Registry, German Childhood Cancer Foundation. M.W. has received research grants from Novartis, Quercis, and Versameb, and honoraria for lectures, advisory boards, or consulting from Anheart, Bayer, Curevac, Medac, Neurosense, Novartis, Novocure, Orbus, Pfizer, Philogen, Roche, and Servier. M. Snuderl is supported by NINDS grant R01-NS122987 and is scientific advisor/shareholder of Halo Dx, and advisor to Arima Genomics and InnoSIGN, and has received research funding from Lilly USA. The German HIT-LOGGIC-Registry was supported by the Deutsche Kinderkrebsstiftung (DKKS 2019.06, 2021.03,

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the authors used ChatGPT (OpenAI) in order to refine language, enhance clarity and coherence, and improve overall readability of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ccell.2025.11.002.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Illumina Infinium HumanMethylation450 (450k) BeadChip Kit	Illumina	Cat# WG-314-1003
Illumina Infinium MethylationEPIC (EPIC) BeadChip Kit	Illumina	Cat# WG-317-1003
Illumina Infinium MethylationEPIC v2.0 (EPICv2) BeadChip Kit	Illumina	Cat# 20020459
Deposited data		
v12.8 CNS Tumor Reference Dataset (raw IDAT files and metadata)	GHGA	GHGA: GHGAS89861553411214; https://data.ghga.de/browse?q=GHGAS89861553411214
Software and algorithms		
MNP Classifier Training Code	this paper	https://github.com/mwsill/mnp_training
R	The R Project for Statistical Computing	v4.3.3; https://www.r-project.org/
minfi R package	Aryee et al.[54]	v1.21.4; Bioconductor: https://bioconductor.org/packages/minfi; RRID: SCR_012830
limma R package	Bioconductor	v3.30.11; https://bioconductor.org/packages/limma; RRID: SCR_010943
uwot R package	CRAN	v0.2.3; https://cran.r-project.org/package=uwot
randomForest R package	CRAN	v4.7–1.2; https://cran.r-project.org/package=randomForest; RRID: SCR_015718
glmnet R package	CRAN	v4.1-8; https://cran.r-project.org/package=glmnet; RRID: SCR_015505
mltest R package	CRAN	v1.0.1; https://cran.r-project.org/package=mltest
rms R package	CRAN	v6.8-2; https://cran.r-project.org/package=rms; RRID: SCR_023242
pROC R package	CRAN	v1.18.5; https://cran.r-project.org/package=pROC; RRID: SCR_24286

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

This study utilized a large international cohort of human central nervous system (CNS) tumor samples for the development and validation of a DNA methylation-based classifier. All procedures involving human participants were conducted in accordance with the Declaration of Helsinki and relevant institutional and national guidelines. Written informed consent was obtained from all patients or their legal guardians. The study protocol was reviewed and approved by the Ethics Committee of the Medical Faculty Heidelberg (reference S-318/2022, approval date 09.05.2022). Sample collection and molecular analyses were additionally approved by the respective local institutional review boards or ethics committees at each participating center.

METHOD DETAILS

DNA-methylation array processing

The Illumina Infinium HumanMethylation450 (450k) array, Illumina Infinium MethylationEPIC (EPIC) array and Illumina Infinium MethylationEPICv2 (EPICv2) were used to obtain genome-wide DNA methylation data for tumor samples and normal control tissues according to the manufacturer's instructions (Illumina, San Diego, USA). Data not gathered through molecularneuropathology.org, were generated at the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ, Heidelberg, Germany) and processed accordingly on an iScan device (Illumina). DNA methylation data were generated from fresh-frozen and formalin-fixed paraffin-embedded (FFPE) tissue samples. Input DNA quantity for most fresh-frozen samples was >500 ng, while 250 ng was used for most FFPE tissues. FFPE-derived DNA was processed using the Infinium FFPE DNA restoration kit. All samples underwent strict on-chip quality control. Inclusion in the mnp_v12.8 reference dataset required samples to meet two criteria: (1) a median log2 signal >8 for both the methylated and unmethylated channels, and (2) $\geq$ 90% of probes achieving a detection P-value <0.05.

All computational analyses were performed in R version 4.3.3 (R Development Core Team, 2024). Raw signal intensities were obtained from IDAT files using the minfi Bioconductor package version 1.21.4.[58] Illumina EPIC, EPICv2 and 450k samples were merged

into a combined dataset by selecting the intersection of probes present on both arrays. Each sample was individually normalized by performing a background correction (shifting the 5th percentile of negative control probe intensities to 0) and a dye-bias correction (scaling of the mean of normalization control probe intensities to 10,000) for both color channels. Subsequently, a correction for the type of material (FFPE/frozen) and array type (450k/EPIC(v2)) was performed by fitting univariable linear models to the log2-transformed intensity values (removeBatchEffect function in the limma package version 3.30.11). The methylated and unmethylated signals were corrected individually. Beta-values were calculated from the retransformed intensities using an offset of 100 (as recommended by Illumina).

CNV analysis
CNV analysis was performed as described previously[6] (R-package conumee v1.42.0 and conumee2 v2.1 for EPICv2 arrays).

MGMT status prediction
The methylation status of the MGMT (Methylated-DNA–protein-cysteine methyltransferase) promoter was inferred as previously described[6] (R-package mgmtstp27 v0.7).

Non-linear dimension reduction
To perform unsupervised non-linear dimensionality reduction, the 10,000 CpG probes with the highest standard deviation were selected, and a UMAP projection was calculated using the umap() function available in the R-package uwot v0.2.3.

Classifier training
Classifier training was performed as described in Capper et al.[6] and Maros et al.[43] First, we applied a permutation-based variable importance measure (R-package randomForest v4.7–1.2) to select the 10,000 most informative CpG probes as features for the final Random Forest (RF). Unbalanced class sample sizes were taken into account by down sampling each bootstrap sample to the minority class. Next, a ridge-penalized multinomial logistic-regression model (R-package glmnet v4.1-8) was fitted to calibrate the RF output, mapping raw prediction scores to probability estimates. An optimal penalization parameter was chosen by a 10-fold cross-validation. Combining classifier outputs with a logistic regression model is an ensemble strategy known as stacking.[59]

Classifier validation
To evaluate the classifier, a 5-fold nested cross-validation scheme generated out-of-sample RF scores that enabled us to fit and validate the calibration models in each fold. To measure the performance of the classifier the following metrics and figures were generated: Accuracy, Balanced Accuracy, F1, Matthews Correlation Coefficient (R-package mltest v1.0.1), Confusion Matrix, multiclass Log Loss, multiclass Brier Score, Calibration Plots (R-package rms v6.8-2). In addition, receiver operating characteristics (ROC) curves and accompanying areas under the curve (AUC) are generated using R-package pROC v1.18.5.

RNA sequencing and fusion calling
RNA sequencing for the purpose of gene fusion calling was performed on a NextSeq 500 or NovaSeq 6000 instrument (Illumina) at the Department of Neuropathology Heidelberg as previously described.[60]

QUANTIFICATION AND STATISTICAL ANALYSIS

All computational analyses were performed in R version 4.3.3. To measure the performance of the classifier, the following metrics were generated: Accuracy, Balanced Accuracy, F1, Matthews Correlation Coefficient (R-package mltest), Confusion Matrix, multiclass Log Loss, multiclass Brier Score, and Calibration Plots (R-package rms). Receiver operating characteristics (ROC) curves and accompanying areas under the curve (AUC) were generated using R-package pROC. Survival analyses in Figures 5 and S5 were visualized using Kaplan-Meier estimates for descriptive purposes. The number of patients (n) for each subgroup is provided in the corresponding figure legend.