Validation and next-generation update of a DNA methylation-based recurrence predictor for meningioma: a multicenter prospective study

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Abstract

Background: We previously developed a DNA methylation-based risk predictor for meningioma, which has been used locally in a prospective fashion since its original publication. As a follow-up, we validate this model using a large prospective cohort and introduce a streamlined next-generation predictor compatible with newer methylation arrays.

Methods: Genome-wide methylation profiles were generated with the Illumina EPICArray. The performance of our next-generation predictor was compared with our original model and standard-of-care 2021 WHO grade using time-dependent receiver operating characteristic curves. An nomogram was generated by incorporating our methylation predictor with WHO grade and extent of resection.

Results: A total of 1347 meningioma cases were utilized in the study, including 469 prospective cases from 3 institutions and an external cohort of 100 WHO grade 2 cases for model validation. Both the original and next-generation models significantly outperform 2021 WHO grade in predicting early postoperative recurrence. Dichotomizing patients into grade-specific risk subgroups was predictive of outcome within both WHO grades 1 and 2 tumours ($p<0.05$), while all WHO grade 3 tumours were considered high-risk. Multivariable Cox regression demonstrated benefit of adjuvant radiotherapy in high-risk cases specifically, reinforcing its informative role in clinical decision making. Finally, our next-generation predictor contains nearly 10-fold fewer features than the original model, allowing for targeted arrays.

Conclusions: This next-generation DNA methylation-based meningioma outcome predictor significantly outperforms 2021 WHO grading in predicting time to recurrence. We make this available as a point-and-click tool which will improve prognostication, inform patient selection for RT, and allow for molecularly-stratified clinical trials.

Keywords: Meningioma, DNA methylation, outcome prediction, neuro-oncology, prognosis

Key Points:

1. We prospectively validate a DNA methylation-based meningioma outcome predictor
2. We develop a next-generation predictor compatible with all versions of the EPICArrays
3. We make this tool publicly available allowing for widespread use

Importance of the Study

We previously developed and retrospectively validated a DNA methylation-based risk predictor for meningioma, which was shown to outperform WHO grade at predicting postoperative recurrence/progression. In this follow-up, we prospectively validate this predictor using a cohort of 469 meningiomas which were resected since the model was developed. Additionally, using a cohort of 1347 meningioma, we construct and validate an updated predictor using nearly 10-fold fewer probes, which is fully compatible with all versions of the Illumina EPICArray, including the new EPICv2 array. We demonstrate that both our original and updated models significantly outperform 2021 WHO grading (which does include some molecular features) to allow for increasing within-grade outcome prediction granularity and can be used to directly inform patient selection for adjuvant radiotherapy. Our updated publicly available point-and-click tool can be readily integrated into existing DNA methylation-based workflows to increase personalization of patient care and inform molecularly-informed clinical trials for meningioma.

Background:

Meningiomas are the most common primary central nervous system (CNS) tumor in adults, but accurately prognosticating individual patient outcomes remains challenging.[1] Unlike other CNS tumors which have adopted a molecular taxonomy, contemporary grading for meningiomas still largely relies on histopathology for most cases, which may not sufficiently capture the significant heterogeneity of cases between patients[2]. Since 2021, the WHO grading criteria now include the rare TERT promotor mutations and CDKN2A homozygous loss as molecular features defining WHO grade 3.[3] Clinical prognostication on an individual patient level is needed given the variability in outcome for each patient and that many patients live with this disease over a long period of time. We previously developed a DNA-methylation based meningioma recurrence predictor using a total of 486 samples (N=228 in the discovery cohort, N=258 in the combined validation cohorts) that was retrospectively validated.[4] This predictor has since been in different institutions to help better understand prognostication and to counsel the patients accordingly and guide treatment decisions regarding the use of adjuvant radiotherapy (RT) following surgery. However, as is the case for all molecular models of disease, model refinement and performance updates that accommodate new technologies, and robust prospective validation are important steps to promote/ensure widespread clinical implementation.[5–7]

In this study we 1) utilized an updated retrospective cohort of now 778 meningiomas with at least 5 years of clinical follow-up to construct a next-generation DNA-methylation based predictor of meningioma recurrence that is compatible with both recent generations of Illumina EPIC methylation array (850K, and EPICv2.0), whereas the original version was published before the development of the EPICv2 chip and therefore is not compatible with this array, 2) benchmarked the performance of this updated next-generation predictor against our original predictor, and 3) validated this novel predictor in an independent, prospective cohort of meningioma cases (N=469) as well as an external validation cohort of WHO grade 2 cases (N=100).

Materials and Methods:

Clinical Cohort and Outcomes

A total of 1347 meningioma cases were identified for this study, comprising a retrospective training cohort training cohort (N=778, i.e. specimens collected before the outcome predictor was trained in 2017), a prospective validation cohort (N=469, i.e. specimens collected after the model was trained), and an independent external dataset (N=100 WHO grade 2 meningiomas) from a separate institution. The retrospective cohort was comprised of meningioma patients accrued from multiple institutions (Toronto, Indiana, Case Western Reserve University/University Hospitals of Cleveland, Seattle, Vanderbilt, Northwestern) in addition to previously published, publicly available datasets (from the University of California San Francisco (UCSF) and Baylor Medical College).[8,9] The prospective cohort was comprised of consecutively treated meningioma patients who underwent surgery since the development of our original DNA methylation-based risk classifier (2017) from 3 different institutions (University Health Network, Toronto, Ontario, Canada; The University of British Columbia, Vancouver, British Columbia, Canada; The University of Indiana, Bloomington, Indiana, United States). The external grade 2 cohort comprised of 100 WHO grade 2 cases from a single center.[10] Annotated clinical data including demographic information, tumor data including primary/recurrent tumor status, WHO grade and tumor location, treatment data including extent of resection, and receipt of adjuvant RT in accordance with predefined consensus core clinical data elements in meningioma.[11] Patients with less than 5 years of follow up without demonstrated tumor recurrence during that time period were excluded from the model training cohort. A subset of the complete prospective cohort (N=112) and external grade 2 cohort (N = 74) with similarly complete 5-year PFS data were used for sensitivity analysis and model testing. Consent was obtained from patients institutionally for their tumour samples to be utilized for research purposes including for all prospective cases. This study was approved by the University Health Network Institutional Review Board (#18-5820).

Only cases which passed DNA methylation quality control measures (see below) and classified as meningioma by the DKFZ DNA methylation-based central nervous system tumor classifier v12.5 (www.molecularneuropathology.org) were included in either cohort, thereby ensuring all cases were molecularly-defined meningiomas (in addition to being histopathologically defined as meningioma). All cases were graded according to the 2021 WHO classification, with WHO grade 3 including any tumor with homozygous *CDKN2A/B* deletions or *TERT*p mutations. Our primary outcome of interest was progression-free survival (PFS) defined as the time of surgical intervention to tumor progression, recurrence, or death and censored at end of follow-up.

DNA Methylation

DNA extraction and DNA methylation were performed centrally at the Princess Margaret Cancer Research Tower (PMCRT) as previously published[4]. Briefly, DNA was extracted from fresh frozen tumor tissue or formalin-fixed embedded tissue using the DNeasy Blood & Tissue Kit and QIAamp DNA FFPE Tissue Kit (Qiagen), respectively. A total of 250-500 ng of DNA was used for bisulfite conversion using the EZ DNA Methylation Kit (Zymo). DNA methylation profiling was performed using the Illumina 850K EPIC array (Illumina, San Diego, CA, USA) as per the manufacturer's instructions. Raw DNA methylation files (.idat) were imported and processed using the minfi package in R. For quality control (QC) purposes, the following probes were removed: those that failed to hybridize (detection p-value >0.05) in one or more samples, those that overlapped with known single nucleotide polymorphisms, cross-reactive probes, and probes located on X and Y chromosomes. Samples that failed QC were removed from the analysis. Post-processed β-values were used for downstream analysis.

Second-Generation DNA Methylation Model Building

Given the larger size of our updated retrospective cohort compared to the original training dataset used to build our original DNA methylation model and the advent of Illumina's Infinium MethylationEPIC v2.0 array, we constructed a next-generation DNA methylation model. The aims of this next-generation model were: 1) to generate a prognostic DNA methylation-based model that could be cross-compatible with both current generations of Illumina EPIC DNA methylation arrays (850K and v2.0), 2) to utilize as a few probes/features as possible for the model in order to move towards translatability using other microarray technology, and 3) to outperform standard of care WHO grading. Similar to the original DNA-methylation model, we used a gradient boosted model in the caret package, trained with 5-fold cross validation. Only probes common to the Illumina 850K, and V2 EPIC arrays were used in feature selection (n = 535 006). These probes were first filtered by univariate Cox regression analysis, selecting only probes with $p<0.0001$ (n = 309 746). To avoid highly correlated features, probes with Pearson correlation > 0.5 with at least one other probe were removed and differential methylation analysis was performed on these remaining probes between samples which did and did not recur 5 years after surgery using the limma package. Probes with adjusted $p < 0.05$ were ranked by moderated t-statistic and the 500 highest and lowest values were selected as features in the final model (n = 1000 unique probes). The new model was tested in both the complete prospective cohort (n=469), the complete grade 2 cohort (n = 100), and a subset of both with complete 5-year clinical follow-up (n=112 and 74, respectively) and compared with our previously published model as well as an analogous model built using WHO grade as the sole feature. Unless otherwise stated, DNA methylation risks presented in the manuscript were generated using our updated DNA methylation predictor.

Nomogram construction

To incorporate our DNA methylation predictor with known clinically prognostic features in meningioma, a nomogram was constructed using DNA methylation risk score, WHO grade, and extent of resection (gross total resection vs subtotal resection) using the *hdnom* package as a follow-

up to our previously published nomogram generated from the same variables. As in the DNA methylation model, training was done using the retrospective cohort only, and testing was done on the independent prospective cohort with complete 5-year follow-up. The nomogram was trained using an adaptive elastic net model with 10-fold validation. A second nomogram was constructed in the same way using only clinical variables (extent of resection and WHO grade). The performance (AUC) of each nomogram was computed for each nomogram and compared using bootstrapping with 10,000 resamples.

Copy Number Alterations

DNA copy number alterations were inferred from DNA methylation data using the conumee package in Bioconductor. Chromosomal arm level copy number variation (CNV) Plots were generated for each sample. Partial or complete losses or gains of the p- and q- arms of each chromosome were recorded at a log threshold of |0.2| as previously published and confirmed by manual inspect by two independent reviewers (J.Z.W, A.P.L.). Any discrepancies between the reviewers were resolved by discussion and consensus. Loss of the *CDKN2A/B* locus were confirmed by manual inspection of the CNV plots by the same independent reviewers and designated as heterozygous or homozygous loss as previously published.

RNA Sequencing

RNA extraction and sequencing were performed centrally at the Princess Margaret Cancer Research Tower (PMCRT) and The Centre for Applied Genomics (TCAG) respectively as previously published, from 150 cases in the prospective validation cohort. Briefly, RNA was extracted from fresh frozen tumour samples using the RNEasy Kit (Qiagen) and QC was performed using the Agilent Bioanalyzer 2100. Samples with RIN > 7.0 were selected to move forward with library preparation. cDNA libraries were created using the NEB Ultra II directional mRNA library prep kit in accordance with the manufacturer's instructions. Libraries were sequenced on the Illumina HiSeq 2500 high output flow cell (2x126 bp) to obtain 70 million reads per sample approximately. Raw RNA-

sequencing data were processed and aligned to the human reference genome (GRCh38) using the STAR aligner. SamTools was used to sort reads and remove duplicate reads. Raw gene expression counts were calculated for every sample using the Rsubread package. We used a combination of DNA methylation, inferred copy number profiles, and RNA sequencing to classify tumors into their representative Molecular Group.

Statistical Analysis

PFS and OS analysis were performed using Kaplan-Meier estimates and fitting Cox proportional hazard models including variables such as age at diagnosis, sex, WHO grade, extent of resection, receipt of adjuvant radiotherapy, recurrent vs primary tumour, methylation risk group, and specific histopathologic features. The proportional hazards assumption was tested by plotting the scaled Schoenfeld residuals against time and computing a p-value for variables included in the model. Determination of an optimal cut-point for DNA methylation risk was done using the cutpointr package by maximizing the Youden-index for predicting PFS across 10 000 bootstrap resamples. Performance of the new predictor and WHO grade was calculated by independently computing the probability of recurrence at 5-years using a 10 000 bootstrap resampling approach. Receiver operating characteristic (ROC) curves were generated for each bootstrap using the nearest-neighbour time-dependent ROC curve method and area under the ROC curve (AUC) to determine the accuracy of the DNA methylation-based model in predicting tumour recurrence/progression compared to standard of care WHO grade. Propensity score matching between the adjuvant RT group and the observation group was performed using the matchit package in R using optimal matching.

Results:

Cohort Demographics and Outcomes

A total of 1347 meningiomas were utilized for this study: 778 cases in the retrospective training cohort, 469 in the full prospective validation cohort, and 100 in the external grade 2 cohort (**Figure 1A**). Baseline characteristics for each cohort are detailed in **Table 1**. In keeping with known epidemiologic data for meningiomas, most of our prospective cohort were female (N=339, 72%) and of older age at diagnosis (median age 60, interquartile range 50-70). The majority of meningiomas were primary tumors at the time of surgery (N=387/434, 89%) and were WHO grade 1 (N=333/469, 71%). The subset of the prospective cohort that had complete 5-year clinical follow-up (N=112**, Supplementary Table 1**) was enriched for WHO grade 2 meningiomas (N=54/112; 48%) but otherwise had similar baseline characteristics. WHO grade 2 and 3 meningiomas had expectedly worse PFS outcomes compared to WHO grade 1 (**Figure 1B**), and WHO grade 2 cases in our prospective cohort had similar outcomes to cases in the external grade 2 cohort (**Figure 1C).**[12]These findings were concordant in patients with complete 5-year follow-up data from both cohorts (**Supplementary Figure 1**).

Meningioma Methylation Risk Profiling Outperforms 2021 WHO Grade in Predicting Outcome

The performance of our next-generation predictor (AUC 0.78, 95% CI 0.71-0.84) was modestly higher than our previously published predictor (AUC 0.74, 95% CI 0.67-0.81) and both were significantly higher than standard of care 2021 WHO grade (AUC 0.65, 95% CI 0.57-0.73, p=0.01 vs next-generation predictor and p = 0.04 vs original predictor, **Figure 1D**) in predicting early tumor recurrence while utilizing nearly 10-fold fewer probes than our previous model (1000 vs 9529 probes; **Supplementary Figure 1A**). Results were concordant when model testing was performed in the subset of prospective samples (N=112) with complete PFS-5yr data (i.e. predicting 5-year PFS,

Supplementary Figure 1E). Similarly, an external cohort containing only WHO grade 2 cases yielded strikingly similar performance, with an AUC of 0.76 (95% CI 0.66-0.85) on the next-generation predictor and 0.71 (0.61-0.81) on the original predictor (**Figure 1E),** and similar performance on the 5-year subset of this cohort (**Supplementary Figure 1F**).

Meningioma Methylation Risk Profiling as a Clinical Tool

We have demonstrated through clinical use that our original DNA methylation-based risk predictor is able to resolve some of the recognized outcome heterogeneity within WHO grading of meningiomas on an individual basis (illustrative case in **Figure 2A**).[3] As expected, methylation risk scores were significantly higher among increasing WHO grade (**Figure 2B**) and even across complete molecular classification of meningiomas that have been previously described[5] (**Supplementary Figure 2**).

To generate clinically meaningful subgroups based on our model output, cases were dichotomized into High- and Low-Risk groups by maximizing the Youden's index on both the complete validation cohort and the subset with complete 5-year follow-up. Optimal cut points to split the cohort were determined to be 0.5056 for the full prospective cohort and 0.4717 on the 5-year prospective cohort; these same thresholds were applied to the external grade 2 validation cohort. Encouragingly, the High-Risk group had shorter PFS compared to the Low-Risk group in both the full prospective cohort (log-rank $p<0.0001$, **Figure 2C**) and the external grade 2 validation cohort (log-rank $p = 0.0041$, **Figure 2D),** with similar results on the 5-year cohorts (**Supplementary Figure 3**). While WHO grade 1 meningiomas are generally considered to be benign, 9% of these cases (N=30/333) were reclassified as High-Risk based on their methylation risk score (**Supplementary Table 2**). WHO grade 2 meningiomas were more heterogeneous, but while most are treated as "higher grade" lesions in clinical practice, a large proportion in our cohort (N=60/119, 50%) could be reclassified as Low-Risk using our predictor. Importantly, even within WHO grade 1 and WHO grade 2 meningiomas, methylation-defined High-Risk meningiomas had significantly shorter PFS compared to Low-Risk

cases (**Figure 2E**). All WHO grade 3 meningiomas were determined to be High-Risk, consistent with their universally poor prognosis. Further stratifying by extent of resection, methylation risk grouping was strongly predictive of outcome within WHO grade 1 cases regardless of extent of resection (**Figure 3A),** which is of critical importance as completely resected WHO grade 1 meningiomas are traditionally considered to be cured. Furthermore, our model remained predictive among WHO grade 2 cases which underwent GTR, a cohort associated with the highest clinical equipoise in current clinical practice (**Figure 3B**), and among patients >60 years old regardless of grade (**Supplementary Figure 4**).

To facilitate clinical implementation of our model, we then combined DNA methylation risk scores with WHO grade and EOR in a clinical nomogram and showed that this combination of clinicomolecular factors significantly improved the prediction of 5-year PFS (AUC 0.81, 95% CI 0.72-0.89) compared to a nomogram utilizing clinical factors alone (AUC 0.73, 95% CI 0.63-0.82, p = 0.005, **Supplementary Figure 5**).

DNA Methylation Risk as an Independent Prognostic Factor

To determine if DNA methylation risk prediction could be prognostic independent of histopathological features, we first fit a univariable Cox regression with potentially prognostic clinical and histologic variables. Methylation risk score, both as a continuous variable and dichotomized risk group (High vs Low risk) was highly predictive of PFS. Additionally, male sex, recurrent tumor status, WHO grade 2 or 3 classification, subtotal resection (STR), receipt of adjuvant RT, and all meningioma-specific histopathologic features except small cell change (namely, mitoses per 10HPF, brain invasion, necrosis, sheeting, hypercellularity, and prominent nucleoli) were also associated with worse PFS on univariable analysis (**Table 2**).

In an effort to see if High Risk cases were associated with histopathologic differences compared to Low Risk cases, we directly compared relevant features between these groups, demonstrating that High-Risk tumours were associated with significantly higher average mitotic index (median 3.5 (IQR 1-6) versus 1 (IQR 0.5-1.5) mitoses per 10HPF, p<0.0001) we well as more of each atypical feature of meningioma (brain invasion, small cell change, necrosis, sheeting, hypercellularity, and prominent nuclei, **Supplementary Table 3).** Within WHO grade 1 cases, there was no difference between High-Risk and Low-Risk cases in terms of mitotic index (median 1 mitosis per 10HPF in both groups, p = 0.294) nor any individual atypical feature (p>0.05), suggesting that histopathologic features are fundamentally not sufficient to stratify risk within WHO grade 1 meningiomas. Among WHO grade 2 cases, the mitotic index was higher among High-Risk cases (median 4 vs 2 mitoses per 10HPF, p = 0.002) and necrosis was more common (p = 0.001), though all other atypical features were similarly common between risk groups (p>0.05).

On multivariable analysis of PFS, STR (HR 3.44, 95% CI 1.85-6.39, $p=9.78x10^{-5}$), recurrent tumour status (HR 2.27, 95% CI 1.12-4.60, p=0.023), WHO grade 2 (HR 2.54, 95% CI 1.06-6.11, p=0.037), mitoses per 10HPF (HR 1.06, 95% CI 1.00-1.10, p=0.032), brain invasion (HR 2.44, 95% CI 1.23-4.85, p=0.011), and High-Risk methylation group (HR 4.50, 95% CI 2.19-9.525, $p=4.41x10^{-5}$) were all independently associated with significantly shorter PFS (**Table 3**). Receipt of adjuvant RT, on the other hand, was associated with significantly improved PFS (HR 0.08, 95% CI 0.03-0.22, $p=1.72x10^{-6}$, **Table 3**). We also performed a sensitivity analysis whereby molecular group assignment was also added to the multivariable Cox regression, and found that the Proliferative group was independently associated with PFS (HR 4.07, 95% CI 1.08-15.34, p=0.038); in this model WHO grade, recurrence status, and mitoses per 10HPF were no longer significant (p>0.05).

DNA methylation can help refine selection of cases for adjuvant RT

There is considerable variability in which cases may be prescribed adjuvant RT following surgery, with ongoing randomized trials aiming to address this equipoise in gross totally resected WHO grade 2 meningiomas. Refinement of patient selection for RT is needed to avoid the adverse effects of overtreatment and to also not miss opportunities to treat patients who would benefit from adjuvant RT. When stratified into Methylation Risk Groups, significantly more High-Risk cases were prescribed adjuvant RT (46/106; 43%) than Low-Risk cases (25/363; 7%, p<0.0001, **Figure 4A**), though it is notable that nearly 60% of high-risk cases were not treated with postoperative RT, suggesting a significant opportunity to influence clinical decision making. On Kaplan-Meier analysis, cases that received adjuvant RT appeared to have poorer PFS compared to cases that were observed following surgery on the whole cohort, but not when stratified by risk group (**Supplemental Figure 6**). This, of course, is confounded by the fact that there was a much higher proportion of higher WHO grade and incompletely resected cases in the adjuvant RT group (**Supplementary Table 5**). When these covariates were controlled for using stratified multivariable Cox regression analysis, adjuvant RT was associated with significantly improved PFS in High-Risk meningiomas (HR 0.27, 95% CI 0.12-0.58, p<0.0001) but not in the Low-Risk cases (HR 0.60, 95% CI 0.17-2.14, p=0.43; **Figure 3A,E**). To further control for these group imbalances between treatment arms, we performed propensity-score matching to balance the key clinical covariates that differed between cases that received adjuvant RT vs those that did not in the methylation-defined Low-Risk (**Figure 4B**), and High-Risk cases (**Figure 4F; Supplementary Table 5**) respectively. Following optimal balancing of these covariates, adjuvant RT remained significantly associated with improved PFS in the High-Risk meningiomas only (**Figure 4G, H**) and not in the Low-Risk cases (**Figure 4C, D**). Importantly, even within the subgroup of WHO grade 2 meningiomas, for which there is the greatest equipoise surrounding prescription of adjuvant RT after surgery, methylation risk stratification remained independently prognostic (**Supplementary Table 6**).

Discussion:

In this study we utilized a large, multicentre cohort of consecutively treated, prospectively collected meningioma cases to validate a next-generation DNA-methylation based predictor of postoperative recurrence. We show that DNA methylation risk profiling provides a more granular and accurate method of prognostication compared to contemporary WHO grading alone, which does include molecular biomarkers and can be utilized as a useful clinical adjunct for guiding decisions around need for adjuvant RT after surgery.

While our previously published DNA-methylation based predictor was able to provide additive prognostic information to standard of care WHO grading and was robustly validated in retrospective cohorts, updates in methylation array technology, inclusion of molecular biomarkers in the 2021 WHO classification and the availability of new, molecularly profiled meningioma cases included prospectively collected samples provided an opportunity to refine our existing model and to evaluate its performance on real-world cases. Our updated risk predictor is trained on 778 retrospective meningioma cases with at least 5-years of clinical follow-up compared to our original model, which was trained on 228 cases. We validated both our next-generation model and our original model on a novel multi-institutional prospective cohort (N=469) as well as completely independent single-center cohort comprised entirely of WHO grade 2 cases, demonstrating strong prognostic ability in both cohorts. Additionally, we have reduced the number of prognostic probes from 9529 in our original model to only 1000 in our next-generation model, which is now compatible with all generations of the Illumina methylation arrays allowing for maximal translational utility. This reduction in features is an important step in improving the accessibility of our model across institutions whereby targeted methylation-specific sequencing or custom methylation arrays using these specific CpGs may be applied instead of genome-wide methylation profiling. Furthermore, we now make this new predictor publicly available as a simple point-and-click tool that can be used by clinicians, overcoming the potential for any limitations of external data analyses.

Importantly, we also demonstrate the ability of methylation profiling to select high-risk cases for consideration of radiation therapy. This has important clinical implications for refining the selection of patients for adjuvant RT that may be used in conjunction with or even independent of WHO grade. In our prospective cohort, 9% of WHO grade 1 meningiomas could be reclassified as High-Risk, 63% of which (19/30) were primary tumors that received GTR, a cohort that within the current treatment guidelines would not have been considered for adjuvant RT. Conversely, half of WHO grade 2 meningiomas could be reclassified as Low-Risk, including 22% of which received a STR, a cohort that often receives adjuvant RT. Our results provide data-driven rationale for adjuvant treatment escalation in the former group and de-escalation in the latter. By more accurately identifying benign meningiomas at low risk of progression even without adjuvant RT, these patients can be spared the potential treatment-associated adverse effects of RT. High-Risk cases, on the other hand, maybe have increased biological risk of recurrence despite their benign appearing histopathology. These cases may be selected for adjuvant treatment earlier instead of delaying treatment until recurrence, which may yield poorer outcomes.[12] This stratification of cases based on postoperative recurrence risk complements our recently published RT-specific outcome predictor, which is trained to specifically predict response to RT (post-RT PFS) among the subset of cases which receive it[13]. We therefore propose the following DNA methylation workflow for resected meningiomas: 1) a postsurgical risk score is generated based on the model we present here, stratifying cases into high vs low risk for early tumor recurrence; low risk cases can be monitored with serial imaging (in the absence of otherwise concerning clinical or histopathologic features) and 2) high-risk cases would need further investigation using the RT-specific predictor, to estimate the probability of RT-response with treatment. Those with a high probability of response are deemed strong candidates for radiotherapy, whereas those with a low probability of response should be counselled appropriately and alternative options may be considered (dose escalation, upfront repeat resection versus close observation and low threshold for additional intervention, clinical trials). Furthermore, the clinical-molecular nomogram presented here can be employed to further refine outcome prediction by incorporative WHO grade

and extent of resection, allowing increasingly granular outcome predictions. As the clinical benefit of routine methylation profiling in meningiomas continues to be reinforced, there is a need to expand the widespread availability of these approaches to allow for increasing personalization of patient care.

The results of our study should be considered in the context of some limitations. First, the retrospective nature of our training cohort and one of our external validation cohorts may limit generalizability, but this is in part addressed with a large prospective cohort used for rigorous model validation. Additionally, race was reported in only a minority of cases in both the retrospective training cohort and prospective testing cohort, making it impossible to draw rigorous conclusions about its influence on outcomes in the context of methylation risk scoring. This represents an important future avenue for future study that certainly warrants further investigation.

Conclusions

Overall, we validate the clinical utility of DNA methylation-based outcome prediction in meningioma using a large, multicenter prospective cohort. In addition, we construct and validate a next-generation model which lowers the barrier to widespread clinical implementation by using significantly fewer probes, being compatible with all generations of Illumina methylation arrays, and being made publicly available as a point-and-click tool ready for immediate clinical translation. This has the potential to significantly influence the clinical care of patients with meningioma and allow for molecularly-stratified clinical trials for this challenging and heterogeneous disease.

Data sharing statement

Our model is available publicly with a point-and-click, easy-to-use interface (https://www.meningiomaconsortium.com/models/). Source code and data used to generate the model is available on Zenodo (https://doi.org/10.5281/zenodo.12738510).

Authorship Statement

APL, JZW drafted the manuscript; APL, JL, VP, MY, JS performed all analysis; APL, JZW, CG ZP, RY, RK, PH, MW, AA, YE, SM, QW, OS performed all experimental procedures; CW, AACG, PV, NB, MB, JK, AA, JD, MAZ, GT, MT, FB, JSBS, AES, SC, LBC, AG, DST, AM, FE, DC contributed valuable samples and data from their respective institutions; FN, GZ oversaw all aspects of the project; APL, JWZ, KA, FN, GZ conceived the study; all authors critically revised the manuscript and approve its submission.

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Figure Captions:

Figure 1. Overview of study cohort and model performance. A. CONSORT diagram of the retrospective model training cohort (N=778), the full prospective cohort (N=469) and the external grade 2 validation cohort (N=100). **B-C.** PFS outcomes of the prospective cohort (**B**) and the external grade 2 cohort (**C**) stratified by WHO grade. **D-E.** AUC ROC analysis for the next-generation methylation-based risk predictor versus the previously published first generation predictor, and standard of care WHO grade in predicting PFS at 5-years when all models were tested in the full prospective cohort (**D**) and the external grade 2 cohort (**E**). Notably, only cases profiled using the 850k array are included in this comparison, since the original methylation predictor is not compatible with the updated EPICv2 array.

Figure 2. DNA methylation risk prediction as a clinical tool. A. Representative MRI images of two sphenoid wing meningiomas in two different patients pre-operatively, post-operatively following gross total resection in both cases, and at last radiographic follow-up prior to tumor recurrence in Case 1 and interval stability in Case 2. Both cases were clinically graded as WHO grade 2, with mitoses exceeding 4 per 10 high-powered-fields, MIB1 of 10-15%, and nearly identical other histopathological features including the presence of hypercellularity, necrosis, sheeting, and prominent nucleoli without brain invasion. Respective genome-wide copy number variation (CNV) profiles generated from DNA methylation data including the resultant print-out of our web-based, institutionally available DNA methylation-based predictor (original model) for illustrative cases are shown to the right. The CNV plot for Case 1 demonstrated many poor prognostic CNV alterations, including losses of chromosomes 1p, 3p, 4q, 11p, 14q, and gain of 1q while, the CNV plot for Case 2 showed only losses of chromosomes 14q and 22q. Consequently, the estimated 5-year risk of recurrence for Case 1 was 0.995 while for Case 2 the probabilistic risk of recurrence was 0.467 based on its DNA methylation profile alone, agnostic to any clinical features. Notably, when combined with

Simpson grade and WHO grade, our molecular nomogram estimates a 4% probability of 5-year *recurrence-free survival* in Case 1 (therefore a *recurrence* probability of 96% CHECK) and 49% probability of 5-year recurrence free survival in case 2 (therefore a 5-year *recurrence* probability of 51%), further suggesting that Case 1 is particularly high risk compared to Case 2. **B.** Distribution of individualized methylation risks for each patient plotted by WHO grade. **C-D**. Distribution of WHO grades among risk groups and associated PFS outcomes on the full prospective cohort (**C**) and the external grade 2 validation cohort (**D**). **E**. PFS outcomes of the methylation defined High-Risk vs Low-Risk cases within WHO grade in the prospective cohort.

Figure 3: DNA methylation profiling identifies high-risk meningiomas among cases that would traditionally be considered "low risk". A: PFS outcomes among WHO grade 1 meningiomas, stratified by extent of resection. This demonstrates that DNA methylation identifies high-risk cases regardless of extent of resection, even among completely resected WHO grade 1 tumours which are traditionally considered to be cured. **B:** PFS outcomes among completely resected WHO grade 2 cases, a cohort associated with significant clinical equipoise, in both the prospective cohort and external grade 2 cohort.

Figure 4. Response to adjuvant RT in methylation-defined Risk Groups. A-E. Results of stratified multivariable Cox regression in the Low-Risk (**A**) and High-Risk (**E**) methylation group before propensity score matching. Love plot demonstrating covariate balance before (red) and after (blue) propensity-score matching of baseline clinical covariates in the Low-Risk (**B**) and High-Risk (**F**) methylation groups. PFS analysis of adjuvant RT vs observation after PSM in the Low-Risk (**C**) and High-Risk (**G**) methylation groups. Results of stratified multivariable Cox regression in the Low-Risk (**D**) and High-Risk (**H**) methylation group after propensity score matching.

Tables:

Table 1. Baseline demographic and tumor data for the retrospective training cohort, the prospective validation cohort, and the external grade 2 validation cohort.

	Retrospective Cohort (N=778)	**Prospective Cohort (N=469)**	**External Grade 2 cohort (N=100)**
Sex			
Male	271 (35%)	130 (28%)	53 (53%)
Female	494 (63%)	339 (72%)	47 (47%)
Unknown	13 (2%)	0	0
Median Age at Diagnosis (IQR)	57 (46-67)	60 (50-70)	59 (47-71)
Tumor Status (at time of index surgery)			
Primary	545 (70%)	387 (83%)	97 (97%)
Recurrent	148 (19%)	47 (10%)	3 (3%)
Unknown	85 (11%)	35 (7%)	0
WHO Grade			
1	486 (62%)	333 (71%)	0
2	217 (28%)	119 (25%)	100 (100%)
3	75 (10%)	17 (4%)	0
Molecular Group			
Immunogenic	126 (16%)	97 (21%)	21 (21%)
NF2-wildtype	238 (31%)	193 (41%)	18 (18%)
Hypermetabolic	153 (20%)	69 (15%)	23 (23%)
Proliferative	164 (21%)	61 (13%)	35 (35%)
Unclassified	97 (12%)	49 (10%)	3 (3%)
Extent of Resection			
GTR	504 (65%)	346 (74%)	78 (78%)
STR	265 (34%)	123 (26%)	14 (14%)
Unknown	9 (1%)	0	8 (8%)
Adjuvant RT			
Yes	129 (17%)	71 (15%)	15 (15%)
No	615 (79%)	398 (85%)	85 (85%)
Unknown	34 (4%)	0	0
Race			
White	156 (20%)	162 (35%)	0
Non-White	40 (5%)	91 (19%)	0
Unknown	582 (75%)	253 (54%)	100 (100%)

Table 2. Univariable Cox regression analysis for PFS in the complete prospective cohort.

	Progression-Free Survival			
Covariate	N	HR	95% CI	P-val
Age	469	1.01	0.99-1.03	0.245
Sex				
F	339	Ref	Ref	Ref
M	130	1.67	1.03-2.71	**0.038**
Tumor Status				
Primary	387	Ref	Ref	Ref
Recurrent	47	4.82	2.87-8.12	**3.09×10^{-9}**
WHO Grade				
1	333	Ref	Ref	Ref
2	119	4.33	2.59-7.26	**2.48×10^{-8}**
3	17	10.06	4.72-23.69	**9.94×10^{-9}**
EOR				
GTR	346	Ref	Ref	Ref
STR	123	2.75	1.72-4.41	**2.27×10^{-5}**
Adjuvant RT				
No	398	Ref	Ref	Ref
Yes	71	2.08	1.24-3.46	**0.0051**
Methylation Risk (continuous)	469	56.90	21.57-150.08	**3.17×10^{-16}**
Methylation Risk Group (discrete)				
Low-Risk	363	Ref	Ref	Ref
High-Risk	106	6.07	3.75-9.84	**2.10×10^{-13}**
Histopathologic Variables				
Mitoses (per 10 hpf)	371	1.06	1.04-1.08	**5.55×10^{-7}**
Brain Invasion (Yes/No)	58/350	3.43	2.02-5.80	**4.67×10^{-6}**
Necrosis (Yes/No)	93/315	2.71	1.65-4.48	**9.24×10^{-5}**
Small Cell Change (Yes/No)	54/354	0.80	0.38-1.68	0.558
Sheeting (Yes/No)	64/344	2.43	1.44-4.10	**8.41×10^{-4}**
Hypercellularity (Yes/No)	63/345	2.04	1.17-3.57	**0.0125**
Prominent Nucleoli (Yes/No)	73/335	2.10	1.23-3.58	**6.42×10^{-3}**

Table 3. Multivariable Cox regression analysis for PFS in the complete prospective cohort.

	Progression-Free Survival			
Covariate	N	HR	95% CI	P-val
Age	340	0.99	0.97-1.01	0.464
Sex				
F	234	Ref	Ref	Ref
M	106	1.38	0.73-2.61	0.320
Tumor Status				
Primary	299	Ref	Ref	Ref
Recurrent	41	2.27	1.12-4.60	**0.023**
WHO Grade				
1	225	Ref	Ref	Ref
2	101	2.54	1.06-6.11	**0.037**
3	14	4.06	0.93-17.76	0.063
EOR				
GTR	251	Ref	Ref	Ref
STR	89	3.44	1.85-6.39	**9.34×10^{-5}**
Adjuvant RT				
No	267	Ref	Ref	Ref
Yes	53	0.12	0.05-0.30	**9.18×10^{-6}**
Methylation Risk Group (discrete)				
Low-Risk	251	Ref	Ref	Ref
High-Risk	89	4.50	2.19-9.25	**4.41×10^{-5}**
Histopathologic Variables				
Mitoses (per 10 hpf)	340	1.06	1.00-1.10	**0.032**
Brain Invasion (Yes/No)	51/289	2.44	1.23-4.85	**0.011**
Necrosis (Yes/No)	86/254	1.28	0.62-2.64	0.502
Small Cell Change (Yes/No)	48/292	0.51	0.22-1.20	0.123
Sheeting (Yes/No)	62/278	1.06	0.51-2.17	0.884
Hypercellularity (Yes/No)	53/287	1.33	0.60-2.95	0.481
Prominent Nucleoli (Yes/No)	73/267	0.64	0.42-1.67	0.612

Figure 1

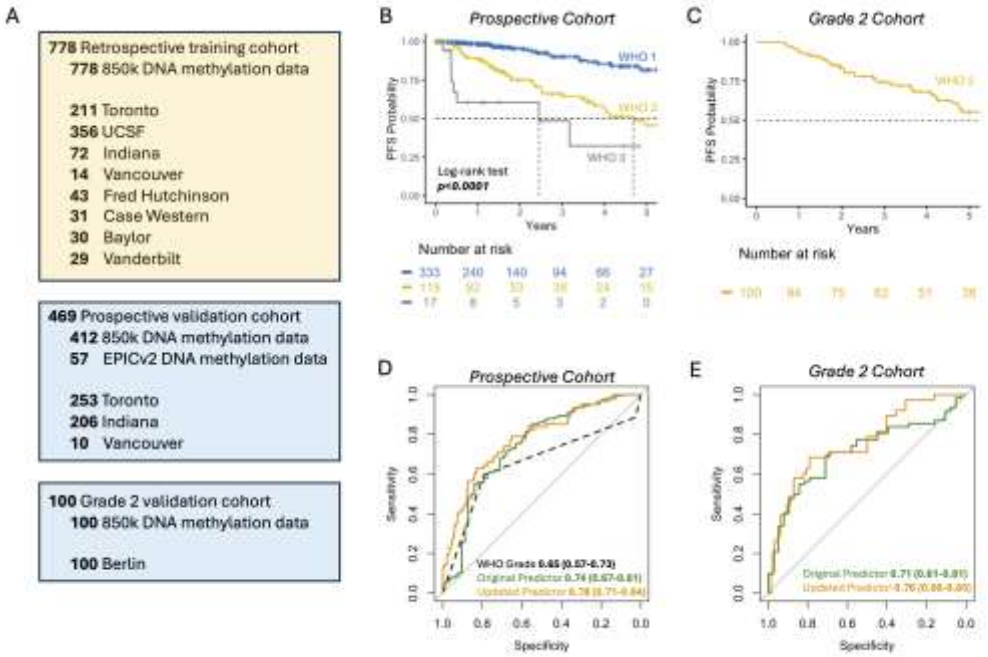

Figure 2

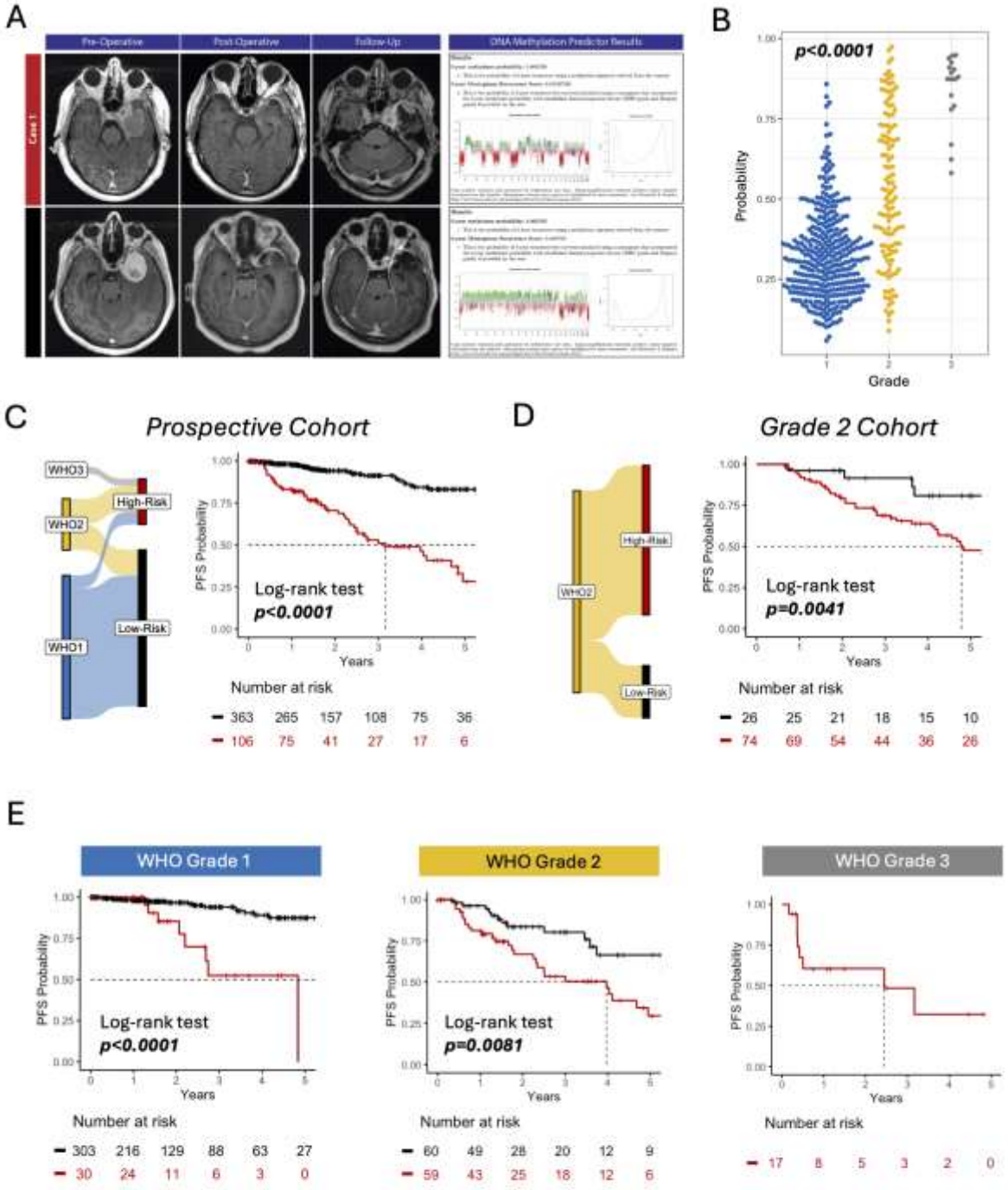

Figure 3

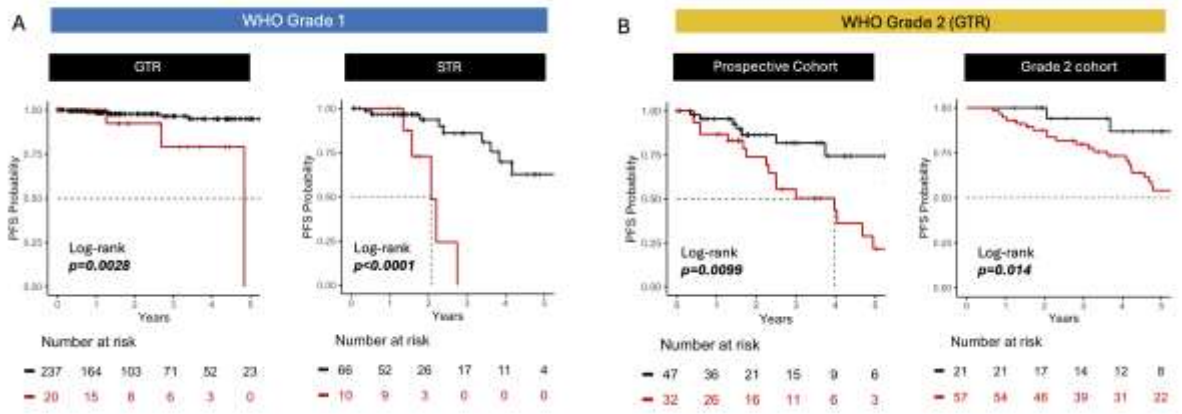

Figure 4