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Methylation Profiling of Medulloblastoma in a Clinical Setting Permits Sub-classification and Reveals New Outcome Predictions

Musa Alharbi[1†], Nahla Mobark[1†], Yara Bashawri[2], Leen Abu Safieh[3], Albandary Alowayn[2], Rasha Aljelaify[2], Mariam AlSaeed[2], Amal Almutairi[2], Fatimah Alqubaishi[2], Ebtehal AlSolme[3], Maqsood Ahmad[4], Ayman Al-Banyan[4], Fahad E. Alotabi[4], Jonathan Serrano[5], Matija Snuderl[5], May Al-Rashed[6] and Malak Abedalthagafi[3*]

[1] Department of Paediatric Oncology Comprehensive Cancer Centre, King Fahad Medical City, Riyadh, Saudi Arabia, [2] Department of Biostatistics, Research Centre, King Fahad Medical City, Riyadh, Saudi Arabia, [3] Genomics Research Department, Saudi Human Genome Project, King Fahad Medical City and King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia, [4] Department of Neuroscience, King Fahad Medical City, Riyadh, Saudi Arabia, [5] Department of Pathology, NYU Langone Medical Center, New York, NY, United States, [6] Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia

Medulloblastoma (MB) is the most common childhood malignant brain tumor and is a leading cause of cancer-related death in children. DNA methylation profiling has rapidly advanced our understanding of MB pathogenesis at the molecular level, but assessments in Saudi Arabian (SA)-MB cases are sparse. MBs can be sub-grouped according to methylation patterns from FPPE samples into Wingless (WNT-MB), Sonic Hedgehog (SHH-MB), Group 3 (G3), and Group 4 (G4) tumors. The WNT-MB and SHH-MB subgroups are characterized by gain-of function mutations that activate oncogenic cell signaling, whilst G3/G4 tumors show recurrent chromosomal alterations. Given that each subgroup has distinct clinical outcomes, the ability to subgroup SA-FPPE samples holds significant prognostic and therapeutic value. Here, we performed the first assessment of MB-DNA methylation patterns in an SA cohort using archival biopsy material (FPPE $n = 49$). Of the 41 materials available for methylation assessments, 39 could be classified into the major DNA methylation subgroups (SHH, WNT, G3, and G4). Furthermore, methylation analysis was able to reclassify tumors that could not be sub-grouped through next-generation sequencing, highlighting its superior accuracy for MB molecular classifications. Independent assessments demonstrated known clinical relationships of the subgroups, exemplified by the high survival rates observed for WNT tumors. Surprisingly, the G4 subgroup did not conform to previously identified phenotypes, with a high prevalence in females, high metastatic rates, and a large number of tumor-associated deaths. Taking our results together, we demonstrate that DNA methylation profiling enables the robust sub-classification of four disease sub-groups in archival FFPE biopsy material from SA-MB patients. Moreover, we

show that the incorporation of DNA methylation biomarkers can significantly improve current disease-risk stratification schemes, particularly concerning the identification of aggressive G4 tumors. These findings have important implications for future clinical disease management in MB cases across the Arab world.

INTRODUCTION

Medulloblastoma (MB) is the most common malignant brain tumor in children, accounting for ∼12% of childhood cancer deaths around the globe (1–5). Over the last 10 years, integrative genomics has rapidly advanced our understanding of the molecular mechanisms governing MB pathogenesis, revealing the syndrome to be more heterogeneous than previously predicted (6–18).

Advancements in cancer genomics and genome-wide transcription profiling now show that MBs comprise at least four molecular subgroups, termed Wingless (WNT-MB: mutations in CTNNB1, DDX3X, Chromatin-remodeling genes, and TP53), Sonic Hedgehog (SHH-MB: mutations in PTCH1, SMO, SUFU, TERT promoter, and Chromatin-remodeling genes), Group 3 (G3: mutations in SMARCA4, chromatin-remodeling genes, and genes of the TGF-β pathway), and Group 4 (G4: chromatin-remodeling genes) (12). The WNT-MB and SHH-MB subgroups are characterized by gain-of-function mutations that activate oncogenic cell signaling (10, 13, 18, 19). G3 and G4 have a low incidence of recurring mutations but show recurrent chromosomal alterations (18). These distinct genetic features lead to diverse clinical outcomes (5, 7, 18, 20). Patients with WNT-MB have an excellent prognosis with current therapy schemes (5-years event-free survival ≥90%) and are considered for the controlled reduction of treatment to minimize radiation and chemotherapy exposure (21–25). The prognoses of SHH-activated MBs are less favorable; SHH-MBs have an intact blood–brain barrier (BBB) and are less responsive to chemotherapy compared to WNT-MBs (22, 26, 27). Many SHH-MBs are age-dependent, with those aged ≥17 months more likely to harbor SMO and PTCH1 mutations (18, 28–32). Subgroup-driven clinical trials are currently underway to assess the efficacy of SHH pathway inhibitors such as vismodegib at diagnosis or in recurrent or refractory SHH-activated tumors.

Patients with non-WNT G3 tumors have an unfavorable prognosis, particularly if associated with MYC amplifications (33), with ≥50% of cases metastatic at the time of diagnosis (18, 27). G3 MBs are more common in males and infants and currently lack defined precision therapeutics. In contrast, G4 patients (also known as glutamatergic) are the most common molecular MB subgroup (20, 34–37) and show excellent survival with current standard-of-care treatment (2, 19, 38–40).

Neuro-epithelial brain tumors are thought to affect younger populations in Saudi Arabia (SA) compared to Western countries; MBs are the second most prevalent tumors (∼13.3%) after Glioblastoma multiforme (∼32%) (41). Despite advances in the use of complementary molecular genetic techniques in SA, the molecular events in SA-MB cases have not been characterized,

and there is a lack of validated prognostic biomarkers. In this regard, studies assessing the DNA methylation profiles of adult brain tumors have demonstrated great promise in sub-classifying the disease and predicting clinical outcomes (42, 43). However, the methylation events in MBs have been restricted to specific genes and small cohorts, and its wider role in MB, specifically in the Arab world, remains poorly characterized.

In this study, we report the first examination of MB-DNA methylation patterns in SA using an extensive primary tumor cohort ($n = 49$). We assessed DNA methylation patterns in archival biopsy material (FPPE) to sub-classify SA-MBs and to explore the applicability of such testing to clinical applications. We herein establish methylation events as clinically useful biomarkers and demonstrate how their incorporation into current risk-stratification schemes could significantly improve the accuracy of survival predictions in SA. This holds potential for future precision therapeutic approaches aimed at improving the outcomes of afflicted SA-MB patients.

MATERIALS AND METHODS

Patient Material

Both patient material and clinical data ($n = 49$) were obtained from the KFMC according to protocols approved by the institutional review board. Tumors were histopathologically re-assessed according to the 2016 WHO classifications. Areas of high tumor cell content (≥70%) were selected for DNA extraction. We collected essential demographic and disease-specific characteristics from the patient's electronic medical charts and radiology images to assess the extent of tumor resection. Information on neurosurgical management was obtained from operative records and standardized neurosurgical reports. Archived pathology specimens were reviewed by a board-certified neuropathologist (MA). All relevant ethical regulations were followed.

DNA Methylation Profiling of the Saudi MB Cohort

The 450 k or EPIC (850 k) methylation array was used to obtain genome-wide DNA methylation profiles for FFPE tumor samples, according to the manufacturer's instructions (Illumina). To investigate sample stability, samples were assessed using the successor Methylation BeadChip (EPIC) array or whole-genome bisulfite sequencing. Established molecular characteristics of the WNT subgroup (CTNNB1 mutations, chromosome 6 loss), MYC and MYCN amplifications, and chromosome 17 status were assessed as previously described (13, 21–24, 27). Each MB subgroup was assessed by immunohistochemistry and mRNA

expression signature assays. Methylation array processing was performed on the 450 k array to obtain genome-wide DNA methylation profiles for tumor samples. Data were generated from formalin-fixed paraffin-embedded (FFPE) tissue samples. A total of 250 ng of DNA was used for all FFPE tissues. On-chip quality metrics of all samples were controlled. Copy-number variation (CNV) analyses from the 450 k methylation datasets were performed using the conumee Bioconductor package version 1.3.0. Control samples displaying a balanced copy-number profile from both male and female donors were used for normalization.

Bioinformatics and Statistical Analyses

Array data analysis was performed in R version 3.2.0 34, using a number of packages from Bioconductor and other repositories. A Random Forest classifier that was compatible with both 450 k and EPIC platforms was generated that calculated the class probabilities from Random Forest scores. Data (idat files) were uploaded to the Classifier (www.molecularneuropathology. org). Following the upload, the classification result was returned automatically. All datasets were submitted to the GEO database (GSE142627 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142627). For survival analysis, data were right-censored at 3 years. Overall survival (OS) and progression-free survival (PFS) were analyzed by the Kaplan-Meier (KM) method. P-values were found using the log-rank test. All statistical analyses were performed using SPSS 25.0 software (SPSS Inc., Chicago, IL, USA). A two-tailed p-value of 0.05 was considered significant.

RESULTS

Patient Demographics

Forty-nine patients were assessed (30 males, 19 females) with a median age at diagnosis of 90.61 months (range: 39–152 months). Histopathological assessment by a board-certified neuropathologist (MA) for all cases confirmed medulloblastoma, grade IV, using 2016 WHO criteria (**Figures 1A,B**). The majority of diagnoses (37/49 cases) had a classic histology; 8/49 patients were classified with desmoplastic/nodular tumors, 3/49 had LC/anaplastic tumors, and a single patient showed extensive nodularity (**Figure 1B**). Amongst the patients, 39/49 had Gross Total Resection (GTR), of whom 7/39 had relapse/progression (**Figure 1C**). In total, 4/49 patients received Sub-Total Resection (STR, relapse in 2/4 patients), 4/49 received near GTR (relapse in 2/4 patients), and 2/49 received Partial Resection (PR, relapse in 1/2 patients) (**Figure 1C**). Nine residual tumors were observed after surgery/radiation therapy (**Figure 1D**). The average radiation dose was 29.341 CSI, with an average radiation boost of 27.75 CSI, which did not differ across the subgroups. All patients, excluding a single patient, received the same chemotherapeutic regimen. Medulloblastoma DNA methylation subgroups were closely related in the SA cohort. All patient characteristics have been summarized in **Supplementary Table 1**.
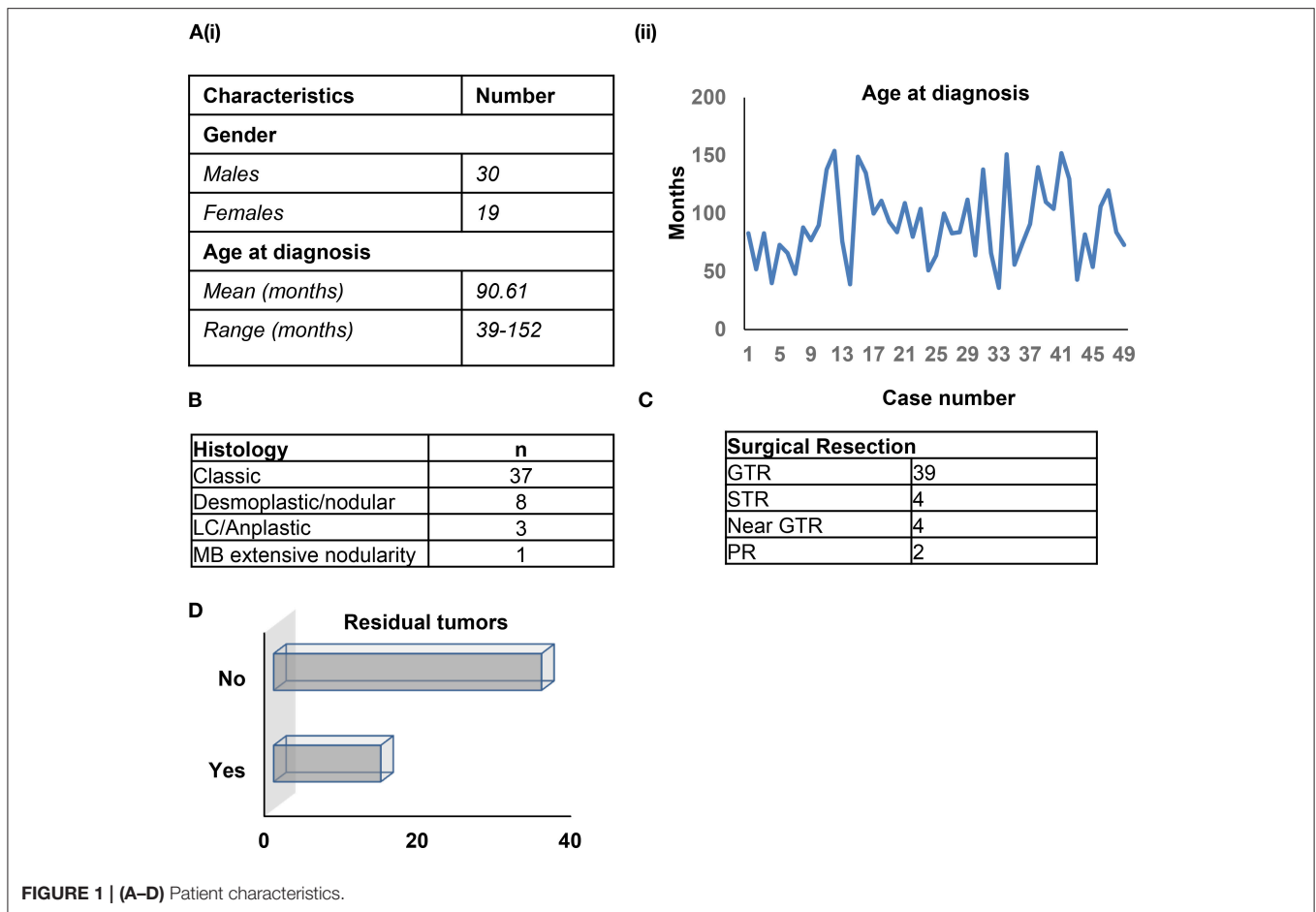
We next examined whether the SA cohort could be sub-classified by its DNA methylation patterns in archival diagnostic FFPE samples (11, 12, 18, 44–46). From next-generation sequencing (NGS) assessments, 45/49 tumors had been previously assigned to known WNT, SHH, G3,G4, and non-WNT-SHH subgroups (**Figure 2A**) using the NGS method and classification criteria as previously described (47, 48). Of the tumors, 15 were non-WNT-SHH, 1 failed due to sample quality, and 1 was unclassifiable. To assess the efficacy of methylation sub-grouping, DNA methylation events were then profiled using the Illumina Infinium HumanMethylation450 BeadChip (450 k) arrays using established molecular characteristics of DNA methylation events (42, 43). This permitted repeat analysis of the cohort to compare the NGS and methylation methods and to reclassify the "non-WNT-SHH cases" observed from NGS analysis.

Materials were available for 41/49 cases, all of which were classified, excluding a single sample that we deemed as a "non-match." Thus, 40/41 tumors could be sub-grouped (**Figure 2A**). In total, 68.42% of the tumors were in agreement between NGS and methylation status (**Figure 2B**). The fact that DNA methylation can control several genes that could contribute to MB progression may have accounted for the tumors that did not correlate. Of these, nine of the non-WNT-SHH cases from NGS assessments were classified by methylation as either G3 (five cases) or G4 (four cases). All WNT and SHH groups were matched by the two methods. Of note, a case that failed in NGS passed the methylation assay as a G4 tumor. This highlighted the accuracy for MB subgrouping to classify tumors that were deemed unambiguous molecular subgroups that could be neither assigned nor classified by the molecular neuropathology classifier. The high prevalence of G4 tumors was consistent with reports of this subtype having the highest occurrence in other cohorts (20, 34–37).

Methylation Subgroups Show Distinct Clinical and Pathological Features

We next investigated whether the clinico-pathological features of the methylation subgroups were consistent with those reported in previous cohorts (12, 18, 46). SHH-MB subgroups showed significant enrichment for desmoplastic/nodular (DN) pathology, which was consistent with previous studies (24) (**Figure 3A**). G3, G4, and WNT tumors were predominantly of classic histology (**Figure 3A**). All subgroups were predominantly males, excluding G4 tumors, which approached an uncharacteristic 50:50 male/female ratio (**Figure 3B**). G3 MBs are more common in males, consistent with what was observed in our cohort, but the high prevalence of female G4 sub-grouped patients has not, to our knowledge, been reported previously and may be unique to the SA cohort. WNT and SHH subgroups showed favorable outcomes, as previously reported from IHC studies, showing low levels of tumor recurrence (**Figure 3C**). Recurrence was most prevalent in G4 groups, which again was surprising. Dissemination via the *cerebrospinal fluid* (*CSF*) is indicative of tumor malignancy and was most prevalent in G3, followed by G4 (**Figure 3D**). Whilst the aggressive nature of G3 tumors would be expected (particularly when associated with MYC amplifications), the

A(i)

Characteristics	Number
Gender	
Males	*30*
Females	*19*
Age at diagnosis	
Mean (months)	*90.61*
Range (months)	*39-152*

(ii)

B

Histology	n
Classic	37
Desmoplastic/nodular	8
LC/Anplastic	3
MB extensive nodularity	1

C

Surgical Resection	
GTR	39
STR	4
Near GTR	4
PR	2

D

FIGURE 1 | (A–D) Patient characteristics.

high metastatic rates in G4 tumors were unexpected and again may represent a unique feature of the SA cohort.

Methylation Subgroups Can Dictate Survival Outcomes for SA-MB Patients

In view of the changes in clinical and pathological features observed, we investigated the prognostic potential of the individual DNA methylation subgroups in our SA cohort (**Figure 4**). We first examined the extent of tumor resection based on post-operative imaging, which we classified as gross total (GTR), near GTR (≥90%), subtotal (STR 51–91%), or partial (PR 10–50%). Whilst most patients underwent GTR, STRs were prevalent in the WNT subgroups (**Figures 4A,B**). There was no obvious benefit of GTR over STR, as previously reported, but such comparisons were limited by the small sample size (49). In terms of treatment, G4 and SHH subgroups received the most prolonged radiotherapy regimens (≥30 days, **Figure 4B**), though the radiation doses were comparable between the groups. The percentage of deaths were, as expected, highest in the G3-MB subgroup (**Figure 4C**). Consistent with the CSF findings, G4 tumors showed a surprisingly high percentage of deaths, exceeding those of the SHH subgroup, which typically respond poorly to MB therapy (**Figure 4D**). No deaths were observed in the WNT-MB subgroup, consistent with the limited

aggressiveness of these tumors. These findings were confirmed by Kaplan Meier (KM) analysis (**Figure 5**). The 3-years OS for the entire cohort was 0.707 (95% CI: 0.540–0.823) and the 3-years PFS was 0.685 (95% CI: 0.519, 0.804). We observed a significant difference in survival amongst the molecular subgroups from pooled analysis ($p = 0.028$). The 3-years OS for the SHH subgroup was 0.778 (95% CI: 0.365–0.939) compared to 0.375 for G3 (95% CI: 0.087–0.674) and 0.759 for G4 (95% CI: 0.670–0.975), which was again surprising. Additionally, the 3-years PFS for the SHH subgroup was 0.778 (95% CI: 0.470–0.939) compared to 0.375 for G3 (95% CI: 0.087–0.674) and 0.714 for G4 (95% CI: 0.472–0.860) ($p = 0.035$ across all four subgroups). No events were observed in the WNT group. It is therefore advised that patients with WNT-MB in SA be considered for the controlled reduction of treatment to minimize radiation and chemotherapy side effects (21–25).

DISCUSSION

Medulloblastoma (MB) is the most common pediatric brain tumor in SA, with a high incidence amongst children aged ≤5 years (41). Disease dissemination is an early event in MB, with up to ∼40% of patients showing metastases at diagnosis, with poor survival. Metastatic disease and tumor recurrence are responsible
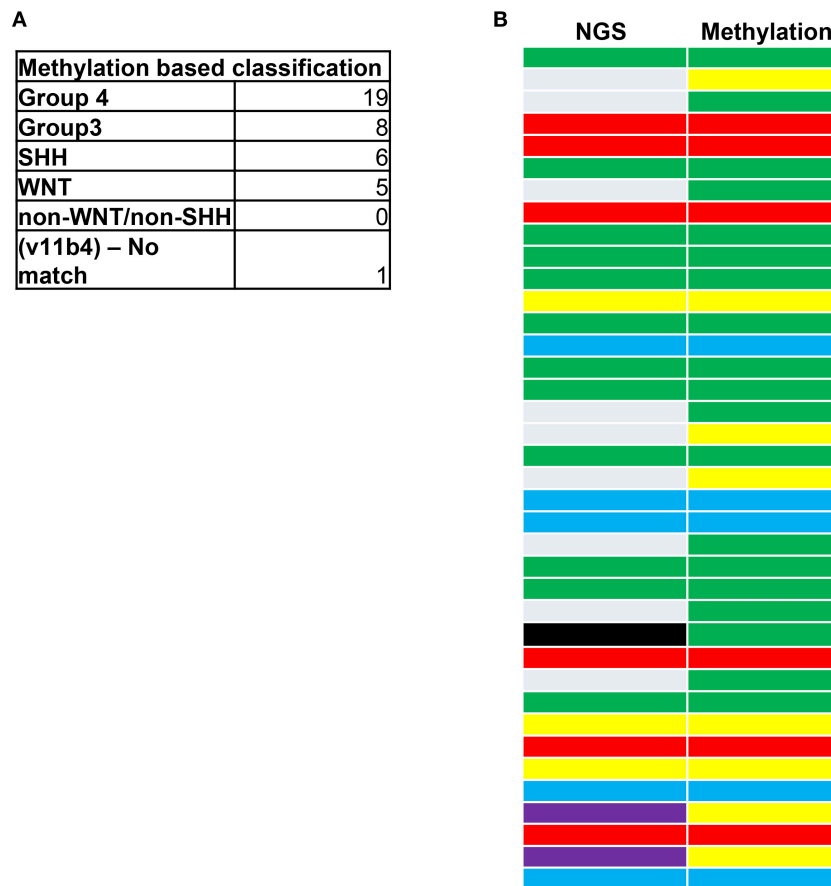
FIGURE 2 | (A) Medulloblastoma next-generation sequencing (NGS) and DNA methylation profiling. **(B)** Classifications are shown according to the accepted coding for MB tumors: G3 (yellow), G4 (green), SHH (red), WNT (blue), and non-WNT/SHH (gray). A sample not classified by NGS (black) was classed as a G4 tumor through methylation analysis. Purple represents G3/G4 subgrouping by NGS that were subclassed as G3 tumors through methylation profiling. The correlation between NGS and methylation status was 68.42%. A total of eight samples could not be classified for methylation subgrouping due to insufficient sample material.

for the low survival rates, and those who survive frequently show treatment-related adverse effects (5, 20, 35, 36, 38, 40, 50–53).

In this study, we report the first comprehensive investigation of gene-specific DNA methylation profiles in SA. Our hospital is a major referral center for pediatric oncology, and the cases represent most regions of SA. The SA MB cohort highlights the potential of epigenetics to improve disease management in the Arab world. Using cross-validated class-discovery approaches, we demonstrate that MBs in SA can be subdivided into the four major DNA methylation subgroups SHH, WNT, G3, and G4 (12, 18, 46). Independent assessments of each subgroup demonstrated a series of previously described relationships, exemplified by the excellent responsiveness and high survival of WNT patients under standard treatment protocols (13, 18, 21–25, 27, 45, 54, 55). Unique features were also identified, namely the aggressiveness of G4 tumors, leading to ∼1/3 deaths. This expands our knowledge of the molecular aberrations involved in MB tumorigenesis in SA.

DNA methylation assessments allowed the robust discrimination of subgroup status in 39/41 tumors in FFPE biopsies, which were comparable to the rates of transcriptomic methods (43/49). This offered accurate distinctions in the sub-classification of SA patients from archival cohorts (33). A subset of 8/49 samples could not be classified due to insufficient material for our analysis. Thus, our methylation assessments were more accurate than the ∼6% reported in previous cohorts (22, 24, 43, 55, 56). Importantly, methylation analysis permitted the classification of tumors that could not be sub-grouped through NGS assessments, highlighting its higher accuracy for MB sub-grouping.

G4 MBs are the most prevalent biological subtype, comprising ∼40% of all MB tumors, which predominantly occur in those aged 3 to 16 years (22, 23, 50, 57, 58). G4 is reportedly 3-fold more frequent in males than females across all age groups, with an intermediate prognosis and 5-years survival reaching ∼80% with standard therapy, although non-metastatic tumors with chromosome 11 loss have an excellent prognosis, with > 90% survival (18, 44, 59). Up to 30–40% of G4 MB patients have metastases at diagnosis and are treated as high risk (7, 49). Adults with G4 MBs have a significantly poorer prognosis compared to the SHH- or WNT-subtypes (22, 23, 57). In our cohort,
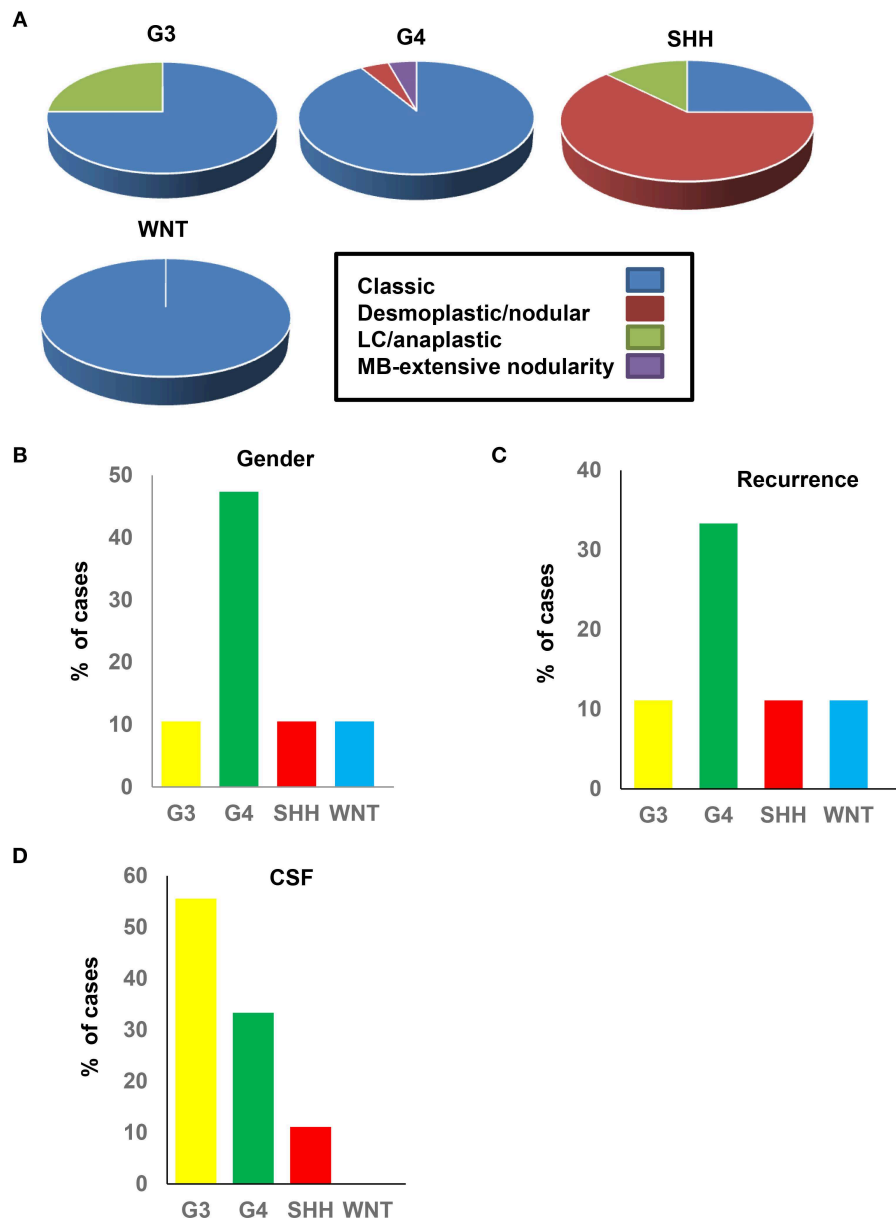
FIGURE 3 | Clinical and pathological data of the medulloblastoma methylation subgroups. **(A)** Histological classification in each subtype. **(B)** Gender percentage in each subtype. **(C)** Percentage of recurrence in each subtype. **(D)** CSF percentage dissemination in each subtype.

the male/female ratio was close to 50:50 in the G4 subgroup, arguing against a high gender preference in the SA population. This was discordant with the male/female ratios reported in previous cohorts, in which 80–90% of G4 cases are males. The majority of G4 tumors were of classic histology, with the rest of large cell/anaplastic histology 2, consistent with previous findings (7, 10, 18, 44, 49, 59). A high number of tumors showed CSF dissemination, suggestive of metastasis. The number of deaths in the G4 subgroup was surprisingly high (36.36%), indicative of a lower prognosis that the > 90% survival reported in other cohorts (7). These results imply a more aggressive nature of G4 MBs in SA carriers that may result from both genetic and environmental

factors. The discrepancies may also be, in part, due to study limitations, including our small sample size ($n = 21$ G4 tumors), lack of adjustment for disease characteristics, cancer treatment modalities, or the length of follow-up. In addition, we did not re-evaluate the cases for additional molecular diagnostics such as copy number profiling, and such analyses may further explain the unique features observed in G4-SA tumors. Future studies needed to further evaluate this observation.

Our study was performed in an all-SA cohort where all study participants underwent the same methylation-based assessments. The follow-ups were extensive, and tumor characteristics and outcome data were collected in a prospective manner. The
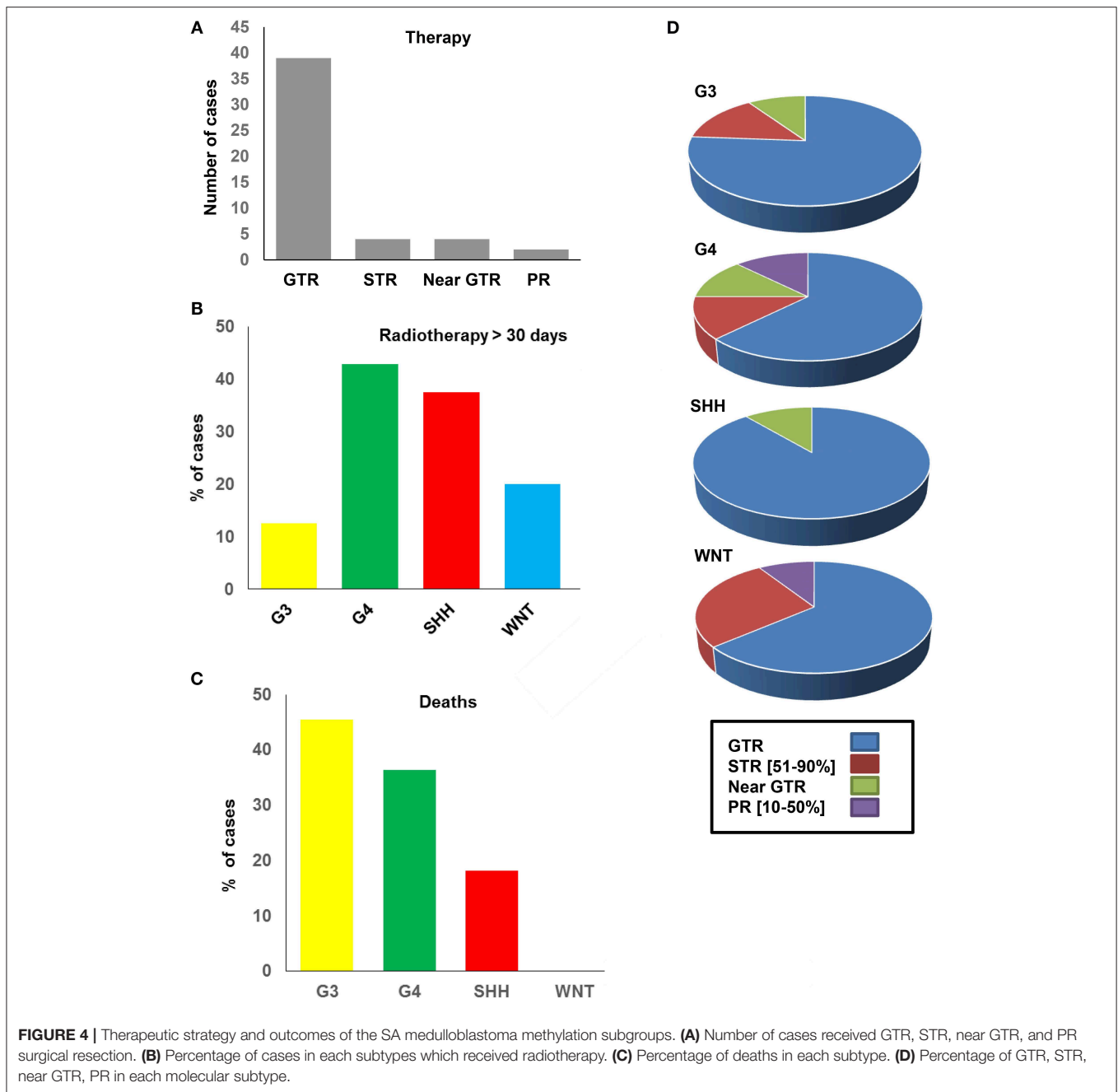
FIGURE 4 | Therapeutic strategy and outcomes of the SA medulloblastoma methylation subgroups. **(A)** Number of cases received GTR, STR, near GTR, and PR surgical resection. **(B)** Percentage of cases in each subtypes which received radiotherapy. **(C)** Percentage of deaths in each subtype. **(D)** Percentage of GTR, STR, near GTR, PR in each molecular subtype.

majority of MB patients received treatment in a single cancer center with consistent treatment guidelines. This homogeneity of data collection strengthens the validity of our prognostic analysis, revealing G4 tumors in SA to represent an aggressive MB subtype.

Our cohort displayed similar features to previous studies regarding the aggressiveness of G3 tumors (23, 30, 50, 57, 58, 60). G3 and G4 MBs were more closely related than WNT and SHH and appear as non-WNT/non-SHH in the revised 2016 WHO classification, but historically these tumors are molecularly and clinically heterogeneous (10, 18, 44, 49, 50, 59, 61). G3 MBs cause ~25% of all cases of MB (16.32% in our cohort) predominantly amongst infants, with a peak diagnosis between ages 3–5 years.

In our cohort, the mean age at diagnosis was 90.61 months, suggestive of delayed disease progression. The male-to-female ratio of these tumors has been reported as 1:2, with a ~60 5-years overall survival in children and a 45% 5-years overall survival in infants (7). The poor prognosis of G3 MBs is related to the young age of metastases (~50% of patients vs. 55.55% in our cohort) at diagnosis, large cell/anaplastic (LCA) histology (25% of tumors in our cohort), and MYC amplification (33). G3 MYC-amplified tumors confer an especially short survival, with only 1 in 5 patients surviving 5 years. G3 MBs also rarely recur at the original tumor site, consistent with the ≤10% recurrence observed in this study. G3 tumor metastases are frequent, but the
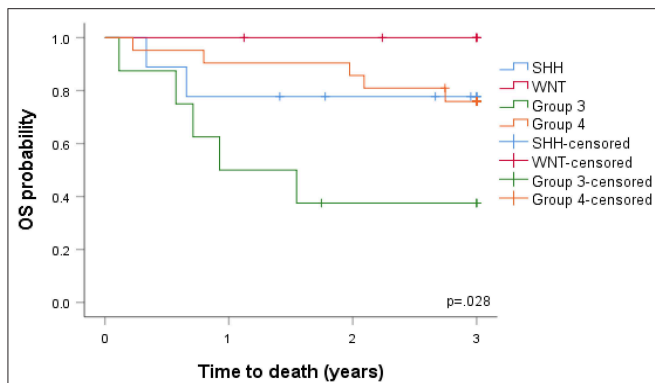
FIGURE 5 | Overall survival (OS) and progression-free survival (PFS) analyzed by the Kaplan-Meier method.

rates do not dictate survival. Significant differences in survival amongst the molecular subgroups from pooled analysis ($p = 0.028$) was also evident; the 3-years OS for the SHH subgroup was 0.778 (95% CI: 0.365–0.939) compared to 0.375 for G3 (95% CI: 0.087–0.674) and 0.759 for G4 (95% CI: 0.670–0.975), highlighting the aggressive nature of G4 tumors in the cohort. The 3-years PFS for the SHH subgroup was 0.778 (95% CI: 0.470–0.939) compared to 0.375 for G3 (95% CI: 0.087–0.674) and 0.714 for G4 (95% CI: 0.472–0.860) ($p = 0.035$ across all four subgroups). Given the close association of SA G3 cohorts to those previously reported, we recommend that SA G3-MB children in the presence/absence of disease spread should be assigned to standard-risk groups to avoid under-treatment. Precision therapies are yet to be developed for G3 tumors due to our limited understanding of tumorigenesis. A consistent finding was the low number of deaths observed in WNT subgroups, which we confirmed through KM analysis (18, 25, 62). Given these low rates, we recommend that this tumor subgroup should receive reduction therapy in SA, thus avoiding unnecessary treatments that have physical consequences for patients and their families and place a substantial financial burden on SA healthcare centers. The development of rational treatment approaches should also be focused on high-risk and metastatic non-WNT/non-SHH patients to suppress the poor survival rates of these subgroups in SA. As precision therapeutics for G3 and G4 tumors improve, the addition of DNA methylation biomarkers is likely to significantly improve survival predictions at diagnosis in SA, as described for previous cohorts (63–65). Our SA cohort further highlights the essential role of the methylation array as a prognostic tool to improve clinicians' ability to manage MB patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the All datasets submitted to GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142627).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by King Fahad Medical City IRB 16-310. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MAlh, NM, and MAb collected, analyzed the clinical data, and wrote part of the manuscript. MAlS, JS, and MA-R performed experiments. LA, EA, AAlo, RA, MAh, AAlm, and FA helped in the experimental studies and data analysis. MAb and AA-B collected the clinical samples. MAb designed the study, analyzed the data, and wrote the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00167/full#supplementary-material

Supplementary Table 1 | Detailed clinical and molecular data of the SA MB cohort.

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