Simplified Molecular Subtyping of Medulloblastoma for Reduced Cost and Improved Turnaround Time

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Abstract: Molecular subtyping of medulloblastoma (MB) has become increasingly important for prognosis and management. Typically this involves detailed molecular genetic testing which may not be available in all centers. The purpose of the present study was to find a simplified approach to assign molecular subtypes of MB for routine use in centers with more limited resources. The molecular subtypes of MBs from 32 Thai patients, aged 0.5 to 35 years, were first determined by NanoString. These results were then compared with those obtained using a combination of limited immunohistochemistry (IHC) (β-catenin, GAB-1, YAP-1, p75-NGFR, OTX2) and *CTNNTB* exon 3 mutation analysis. By NanoString assay, there were 6 MBs (19%) in the wingless (WNT) group, 8 (25%) in the sonic hedgehog (SHH) group, 7 (22%) in group 3, and 11 (34%) in group 4. Although β-catenin immunostaining missed 4/6 WNT MBs, *CTNNTB* mutation analysis confirmed all WNT MB cases with amplifiable DNA. The IHC panel correctly assigned all the other molecular subtypes, except for 1 MB in group 4. Thus, our protocol was able to correctly categorized 31/32 cases or 97% of cases. Our study is the first to report molecular subtypes of MB in Southeast Asia. We found that molecular subgroups of MBs can be reliably assigned using a limited IHC panel of β-catenin, GAB-1, YAP-1, p75-NGFR, OTX2, together with *CTNNTB* exon 3 mutation analysis. This simplified approach incurs lower cost and faster turnaround time compared with more elaborate molecular methodologies and should be beneficial to centers with reduced laboratory resources.

Key Words: medulloblastoma, molecular subtype, immunohistochemistry, *CTNNB1*

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Medulloblastoma (MB) is a common malignant brain tumor in children worldwide. In our previous series of 132 brain neoplasms in Thai children, MBs accounted for 11% of malignant tumors, second only to germ cell tumors (13%).[1] Although all MBs are considered World Health Organization (WHO) grade IV tumors, they are heterogenous in terms of histology, molecular alterations, and prognosis. The most recent WHO recommendations for reporting includes not only the traditional histologic subgroups, including classic, nodular/desmoplastic/MB with extensive nodularity, anaplastic/large cell, but also molecular subgroups including wingless (WNT), sonic hedgehog (SHH), and group 3/4, along with *TP53* status in the SHH subgroup, although the methodology for molecular subclassification is not specified.[2] Among the molecular subgroups, WNT carries the best prognosis.[3,4] Diagnosing WNT and SHH subtypes of MB is becoming clinically important. For example, an ongoing phase II trial at St. Jude Children's Research Hospital, SJMB12 (ClinicalTrials.gov Identifier: NCT01878617) stratifies treatment of MB on the basis of both clinical risk and molecular subtypes. Patients with nonmetastatic, non–*MYC*-amplified WNT MB and gross tumor removal are treated with a reduced radiation dose in the tumor bed and craniospinal axis, whereas those with SHH MB receive the additional targeted drug, vismodegib, which is a smoothened receptor inhibitor. In Thailand, current treatment of MB is based on clinical risk classification but molecular subtyping is yet to be incorporated into upcoming protocols until this testing is more available countrywide.

Although a small number of clinical studies have involved MBs in Southeast Asia,[5–8] molecular subgroups have not been elucidated. Molecular subtyping typically involves laboratory analyses that can be costly, involving specialized equipment and personnel, not readily available in all laboratories, especially in countries where resources are more limited. Moreover, increasing regulations related to the shipping of human tissues in some countries may interfere with overseas laboratory outsourcing. An alternate approach involving immunohistochemistry (IHC) would be of great value if shown to be reliable, as this technology is readily available in most pathology laboratories. At least 3 IHC panels have been proposed for molecular classification of MBs with various combinations of antibodies that include β-catenin, GAB-1, YAP-1, filamin A, NGFR, and OTX2.[9–11] Problems with consistent

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staining have been reported for both β-catenin and GAB-1, and filamin A was felt to offer no additional benefit over YAP-1. With this in mind, we set out to determine the reliability of a limited IHC panel combined with limited molecular testing as a substitute for the standard more complex molecular analysis of MBs. We found that a panel of just 5 antibodies (β-catenin, GAB-1, YAP-1, p75-NGFR, and OTX2) combined with *CTNNB1* mutation analysis was 97% as reliable for subtyping MBs as the more complex and costly NanoString analysis.

MATERIALS AND METHODS

Study Design

The goal of the study was to determine the reliability of a limited IHC panel combined with limited molecular testing in assigning a molecular subtype to MBs. All MB cases diagnosed between 2004 and 2017 were retrieved from the Pathology archives at the Institute of Pathology (IOP), Department of Medical Services, Ministry of Public Health, Bangkok, Thailand. All cases were reviewed by a neuropathologist (S.S.) and histologically classified according to the WHO classification.[2] All cases were subtyped by NanoString analysis, which then served as the gold standard for evaluating different IHC panels in combination with molecular testing that was limited to *CTNNB1* mutation analysis. This study was approved by the institutional review board at the IOP (No. IOP-KM-R60-005).

NanoString Analysis

Molecular subtypes were determined using formalin-fixed paraffin blocks and NanoString analysis performed at the Hospital for Sick Children, Toronto, Canada. As there were many long-term storage samples, Agilent 2100 Bioanalyzer was used to evaluate RNA quality. Only those cases with adequate RNA were analyzed further. Probe design and construction were performed in collaboration with NanoString Technologies (Seattle, WA). There were probes for WNT (*DDK2*, *EMX2*, *GAD1*, *TNC*, *WIF1*); SHH (*ATOH1*, *EYA1*, *HHIP*, *PDLIM3*, *SFRP1*); group 3 (*EGFL11*, *GABRA5*, *IMPG2*, *MAB21L2*, *NPR3*, *NRL*); and group 4 (*EOMES*, *KCNA1*, *KHDRBS2*, *OAS1*, *RBM24*, *UNC5D*) MBs, and probes for housekeeping genes (*ACTB*, *GAPDH*, *LDHA*).[12] NCounter Element reagents were purchased from NanoString and probes were synthesized by integrated DNA technologies (Coralville, IA). A 200 ng of RNA was mixed with the probes (integrated DNA technologies), nCounter Elements TagSet, and Hybridization Buffer (NanoString) following the manufacturer's protocol. The mix was incubated for 20 hours at 67°C. Sample processing was performed using the nCounter Prep Station (NanoString), and RNA counting was performed using the nCounter Digital Analyzer (NanoString). The geometric means of the housekeeping transcripts, ACTB, GAPDH, and LDHA were used to determine the RNA quality. Raw counts were subjected to normalization using the internal positive spike-in controls, followed by probe-specific background correction. The prediction analysis of microarrays classifier prediction method (pamr package in R programming) was used to determine the subtype on the basis of the different expression levels of the signature genes for each subtype. A confidence score was given for the subtyping to determine the accuracy of the prediction. The subtype determined by algorithm was also confirmed by visual inspection of the expression profile.

IHC

Indirect immunoperoxidase staining was performed on 4-μm-thick sections using an autoimmunohistochemistry stainer (Leica BON-MAX, Leica Microsystems, Melbourne, Australia). Details of the antibodies are provided in Table 1. All MB cases with conclusive NanoString results underwent immunostaining for β-catenin, GAB-1, YAP-1, p75-NGFR, and OTX2. The expected results of each immunostain in MB subtypes are shown in Table 2 and illustrated in Figure 1. β-catenin was considered "positive" when at least 5% of tumor nuclei were positive. The remaining immunostains were recorded as either "positive" or "negative" as the staining pattern was typically diffuse. Expression of YAP-1 (nuclear and cytoplasmic) and OTX2 (nuclear) was found in WNT MBs. Neoplastic cells in SHH MBs expressed GAB-1 (cytoplasmic staining), YAP-1, and p75-NGFR (cytoplasmic). Tumor cells in the internodular regions of SHH nodular/desmoplastic MBs and MB with extensive nodularity express the markers to a greater degree than tumor cells within the nodules. Group 3/group 4 MBs were reactive with only OTX2. Vasculature was served as an internal positive control for GAB-1, YAP-1, and p75 NGFR immunostaining (negative for OTX2).

For the purpose of assessing the reliability of immunostaining in predicting the molecular subtype of MB, the 5 antibodies were grouped into 3 panels. IHC panel 1 consisted of β-catenin, GAB-1, and YAP-1; IHC panel 2 consisted of β-catenin, YAP-1, p75-NGFR, and OTX2; and IHC panel 3 consisted of YAP-1, p75-NGFR, and OTX2. IHC panels 1 and 2 have been used in previous studies,[10,11] whereas the panel 3 was devised by us on the basis of the NanoString results (see the Discussion section). The main difference in panel 3 is the omission of β-catenin. Each case was scored as to which molecular subtype (WNT, SHH, or group 3/4) each of the 3 panels

TABLE 1. Antibodies Used in Immunohistochemistry for Molecular Classification of Medulloblastoma

Antibodies	Company (Catalog No.)	Clone	Dilution	Localization
β-Catenin	Cell Marque (224M-16)	C14	1:500	Nuclear
GAB-1	Abcam (ab133486)	EPR375	1:1000	Cytoplasmic
YAP-1	Santa Cruz (sc-101199)	63.7	1:1000	Nuclear and Cytoplasmic
p75-NGFR	Thermo Fisher (MA5-13314)	NGFR5	1:30	Cytoplasmic
OTX2	Thermo Fisher (MA5-15854)	1H12C4B5	1:200	Nuclear

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TABLE 2. Expected Immunoprofile of MBs According to Molecular Subtypes

Molecular Subtype	β-Catenin	GAB-1	YAP-1	p57-NGFR	OTX2
WNT	+	−	+	−	+
SHH	−	+	+	+	−
Group 3/group 4	−	−	−	−	+

MB indicates medulloblastoma; SHH, sonic hedgehog; WNT, wingless.

identified them to be, and any case that did not match the expected IHC results for a molecular subtype (as set out in Table 2) was scored as "indeterminate" for that panel.

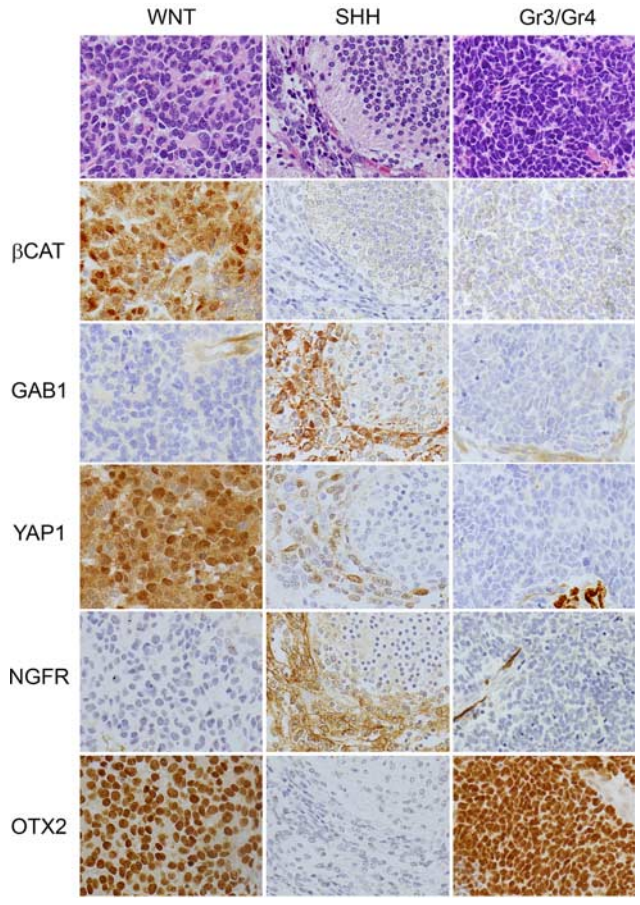

FIGURE 1. Immunoprofile of MBs according to molecular subtypes (Table 2). From left to right columns, WNT MB, with classic histology, SHH tumor with MB with extensive nodularity histology, and Gr 3/Gr 4 MB with classic histology are shown. β-Catenin (only nuclear staining is scored), YAP-1 (nuclear and cytoplasmic), and OTX2 (nuclear) are expressed in WNT MBs. SHH MBs express GAB-1 (cytoplasmic staining), YAP-1, and p75-NGFR (cytoplasmic). Tumor cells in the internodular regions of SHH MB with extensive nodularity express the markers to a greater degree than the tumor cells within the nodules. Gr 3/Gr 4 MBs express only OTX2. Vasculature serves as an internal positive control for GAB-1, YAP-1, and p75 NGFR immunostaining (negative for OTX2) (original magnifications ×400). Gr indicates group; MB, medulloblastoma; SHH, sonic hedgehog; WNT, wingless.

CTNNB1 Mutation Analysis

CTNNB1 mutation analysis was performed on gene exon 3 in all NanoString-verified WNT MBs, using direct sequencing. This choice was based on a previous study that showed a mutation in *CTNNB1* exon 3 was a molecular marker for WNT MBs.[3] DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue, using QIAamp DNA FFPE Tissue kit (Qiagen). DNA quantity and quality were measured using NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). Polymerase chain reaction (PCR) of *CTNNB1* exon 3 (phosphorylation domain, codons 30 to 48) was performed using 60 ng of DNA template, 1X Buffer II, 25 mM of $MgCl_2$, 10 mM of dNTP, 10 μM of forward primer (5′GTAAAACGACGGCCAGTTGATGGAGTTGGACATGGC3′) and reverse primer (5′GCGGATAACAATTTCACACAGGCTGTTCCCACTCATACAGG3′), and 0.2 U of AmpliTaq DNA polymerase (ThermoFisher Scientific). Thermal cycling was performed by Veriti Thermal cycler (ThermoFisher Scientific) as follows: initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 1 minute, extension at 56°C for 1 minute, and extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. Following this, the expected PCR product at 224 base pairs was visualized by using 8% polyacrylamide gel electrophoresis and SYBR Safe DNA gel stain (Life technologies) comparing with 20 bp DNA ladder (Lonza). PCR products were purified using ExoSAP-IT cleanup reagent (Affymetrix USB). Bidirectional direct sequencing was carried out at Macrogen, Korea. Mutation analysis was performed using Sequencher v5.4.6 (Gene Codes Corporation).

TP53 Status

According to WHO guidelines, *TP53* status is required to be reported for all MBs with the SHH subtype. Immunostaining for p53 protein was performed using Cell Marque (453M-96), clone DO7, dilution (1:450), only in SHH MBs and interpreted as previously detailed.[13] Both staining intensity (0 = none, 1 = weak, and 2 = strong) and distribution (< 25%, 25% to 50%, and > 50% of tumor cells) were scored in this scheme, and cases were considered positive when strong staining was observed in > 50% of tumor cells. To correlate with the result of p53 immunostaining, all cases of SHH MB with sufficient DNA quality were subjected to next-generation sequencing analysis (NGS) (Illumina/Qiagen) of the entire *TP53* gene and, if that failed, direct sequencing of mutation hotspots in exons 5 to 8 previously reported in MBs.[13–15]

RESULTS

A total of 39 cases of MBs were retrieved that had paraffin blocks available. Of these, 7 did not yield adequate material for NanoString analysis, leaving 32 cases in the study. Demographic data, histologic and molecular subtypes, and IHC results are summarized in Table 3. There were 23 male individuals and 9 female individuals, with age ranging from 6 months to 35 years (mean = 6.9 y). The majority of MBs in our cohort had classic histology (27 cases, 84%). By NanoString analysis, the molecular subtypes were determined to be as follows: 6 WNT (19%), 8 SHH (25%), 7 group 3

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TABLE 3. Demographic Data, Subtypes, Immunoprofile, and Molecular Findings of 32 Medulloblastomas

#	Sex	Age (y)	Molec Subtype	Histol Subtype	β-cat IHC	GAB IHC	YAP IHC	NGFR IHC	OTX2 IHC	Panel 1	Panel 2	Panel 3	CTN
1	M	7	WNT	Classic	−	−	+	−	+	Indet	Indet	WNT	+
2	M	7	WNT	Classic	−	−	+	−	+	Indet	Indet	WNT	+
3	M	8	WNT	Classic	+	−	+	−	+	WNT	WNT	WNT	Fail
4	M	11	WNT	Classic	−	−	+	−	+	Indet	Indet	WNT	+
5	M	11	WNT	Classic	+	−	+	−	+	WNT	WNT	WNT	+
6	F	13	WNT	Classic	−	−	+	−	+	Indet	Indet	WNT	+
7	F	0.5	SHH	MBEN	−	+	+	+	−	SHH	SHH	SHH	ND
8	F	1	SHH	MBEN	−	+	+	+	−	SHH	SHH	SHH	ND
9	M	1	SHH	Classic	−	−	+	+	−	Indet	SHH	SHH	ND
10	M	2	SHH	Classic	−	+	+	+	−	SHH	SHH	SHH	ND
11	F	3	SHH	MBEN	−	+	+	+	−	SHH	SHH	SHH	ND
12	M	4	SHH	Classic	−	+	+	+	−	SHH	SHH	SHH	ND
13	F	9	SHH	Classic	−	+	+	+	−	SHH	SHH	SHH	ND
14	F	35	SHH	Nod/Des	−	+	+	+	−	SHH	SHH	SHH	ND
15	M	3	Group 3	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
16	M	3	Group 3	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
17	M	6	Group 3	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
18	M	6	Group 3	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
19	M	7	Group 3	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
20	M	7	Group 3	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
21	M	11	Group 3	Ana/LC	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
22	F	1	Group 4	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
23	M	3	Group 4	Classic	−	+	+	+	+	SHH	Indet	Indet	ND
24	M	3	Group 4	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
25	M	5	Group 4	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
26	M	5	Group 4	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
27	M	6	Group 4	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
28	M	7	Group 4	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
29	M	7	Group 4	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
30	F	8	Group 4	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
31	F	9	Group 4	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND
32	M	10	Group 4	Classic	−	−	−	−	+	Gr 3/4	Gr 3/4	Gr 3/4	ND

Panel refers to immunohistochemistry panel with panel 1 = β-catenin; GAB, YAP; panel 2 = β-catenin, YAP, NGFR, OTX2; and panel 3 = YAP, NGFR, OTX2.
Gray shades show cases in which molecular subtype was NOT identified by IHC.
Ana/LC indicates anaplastic/large cell MB; β-cat, β-catenin; CTN, CTNNB1 exon 3 mutation; F, female; Gr 3/4, Group 3/Group 4 (non-WNT/non-SHH); Histol, histology; IHC, immunohistochemistry; Indet, indeterminate; M, male; MB, medulloblastoma; MBEN, MB with extensive nodularity; Molec, molecular; N/D, not done; Nod/Des, nodular/desmoplastic MB; SHH, sonic hedgehog; WNT, wingless.

(22%), and 11 group 4 (34%). All of the nodular/desmoplastic MBs and MB with extensive nodularity cases belonged to the SHH subgroup, whereas the classic subtype was represented in all 4 molecular subtypes.

With respect to the MB cases that were the WNT subtype, IHC for β-catenin failed to identify 4/6 (66.7%) cases of this subtype (cases #1, #2, #4, and #6), all of which showed negative immunostaining. In the other 2 cases, nuclear β-catenin staining was seen in 50% of nuclei (case #3) and 70% of nuclei (case #5). As this antibody is a component of IHC panels 1 and 2, these four immunonegative cases were assigned an "indeterminate" molecular subtype by these panels, whereas IHC panel 3 (YAP1, p75-NGFR, and OTX2) correctly recognized all NanoString-verified WNT MBs. For confirmation, mutation analysis of *CTNNB1* exon 3 yielded positive results in all 5 WNT MBs that yielded sufficient DNA quality. Case 3 did not yield adequate DNA but this case was correctly identified as the WNT subtype by all 3 IHC panels. Details of the *CTNNB1* mutations are as follows: case #1, c.98C > T (p.Ser33phe); case #2, c.94G > A (p.Asp32Asn); case #4, c.98C > T (p.Ser33phe); case #5, c.94G > T (p.Asp32Tyr) and c.148G > A (p.Gly50Ser); and case #6, c.98C > G (p.Ser33Cys). Representative chromatograms (cases #2 and #5) are shown in Figure 2.

For the SHH subtype of MB, IHC panels 2 and 3 correctly identified all cases, whereas IHC panel 1 classified 1 case (case #9) as "indeterminate." All groups 3 and 4 (non-WNT/non-SHH) cases were accurately categorized by all 3 IHC panels except 1 (case #23, group 4 MB) that was missed by all 3 IHC panels and called "SHH subtype" by IHC panel 1 and "indeterminate" by IHC panels 2 and 3. Examining the 3 IHC panels overall, panel 1 correctly identified the molecular subtype in 26/32 (81.2%) cases (5 indeterminate cases and 1 misclassified case), panel 2 correctly in 27/32 (84.4%) cases (5 indeterminate cases), and panel 3 in 31/32 (96.9%) cases (1 indeterminate case).

With respect to *TP53* status in the SHH subtype, there were 8 cases analyzed. Overall, 6 had no p53 expression by IHC and 2 had <1% of positive cells; hence, all were scored as immunonegative. Only 2 cases had sufficient DNA quality to be analyzed, one by NGS and the other by direct sequencing as NGS failed, and no *TP53* mutations were found.

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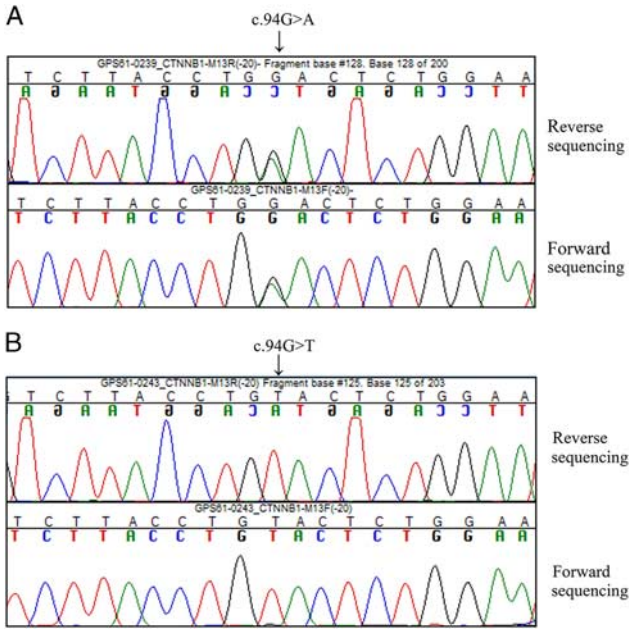

FIGURE 2. Chromatograms of 2 wingless medulloblastoma cases with *CTNNB1* mutation are shown. The upper case (A) is negative for nuclear β-catenin immunostaining, whereas the lower case (B) is positive. full color online

DISCUSSION

MBs are heterogenous tumors and molecular subtype has become increasingly important for prognosis and treatment planning. In a large series of 787 patients,[4] the majority of cases were classified as group 4 (37%), followed by SHH (31%), group 3 (21%), and WNT (11%). Although the distribution of molecular subgroups of MB varies between studies, WNT MB is always the least common subtype.[3,4,12] This distribution of MB subtypes is similar to our cohort which included 34% group 4, 25% SHH, 22% group 3, and 19% WNT. The prevalence of WNT MBs is higher in our series compared with some others, but a similar prevalence has been reported in a Brazilian cohort that had 18% WNT in 104 MB cases.[16] In agreement with other series,[9,10] most MBs in our series had classic histology, and all cases of MB with extensive nodularity (3 cases) and desmoplastic/nodular (1 case) histology belonged to the SHH subtype. The age distribution in our series of MB patients was also in keeping with the literature,[17] with a bimodal peak of SHH MBs in infants and adults.

Although comprehensive molecular assays such as NanoString and methylation array have been shown to reliably assign molecular subgroups for MBs using FFPE samples,[3,12] the assays are not widely available. Moreover, cases need to be batched for optimal cost and this is not easy for uncommon tumors such as MB. For centers with limited resources, this type of testing is difficult to justify in-house and often requires referrals to other centers for testing, incurring additional expenses. Our goal was to devise a limited testing protocol that is as reliable as the detailed molecular assays but more practical for use in smaller centers. As we validated that our series of MB patients is typical of those in other series, the results we obtained using a simplified protocol should be applicable in other centers that see cases of MB.

Our simplified approach was based largely on IHC, as this technique is readily available, even in laboratories that lack molecular testing capabilities. We chose to test only 5 antibodies that were tested in our study including β-catenin, GAB-1, YAP-1, p75-NGFR, and OTX2. This choice was based on 2 IHC panels that have been used previously to classify MBs into molecular subgroups including[1] β-catenin, GAB-1, and YAP-1[10] (referred to as IHC panel 1 in our study) and[2] β-catenin, YAP-1, p75-NGFR, OTX2,[11] referred to as IHC panel 2 in our study. We also devised a third panel (IHC panel 3) derived from IHC panel 2 but without β-catenin. We created this panel after assessing the β-catenin IHC results in terms of the molecular subtyping results obtained by NanoString. We suspected the WNT cases with negative β-catenin IHC were truly WNT because the rest of the immunoprofile (YAP+ NGFR− OTX+) suggested the WNT subtype. To confirm this, we did *CTNNB1* exon 3 mutation analysis. All 4 cases with negative β-catenin IHC were found to have a *CTNNB1* mutation, confirming they were the WNT subtype. In other words, β-catenin IHC (included in IHC panels 1 and 2) missed two thirds of the WNT MBs (4/6 cases) in our series (false-negative staining). Hence, WNT MBs cannot completely be excluded by the basis of negative β-catenin IHC alone. Another study had the opposite problem of false-positive staining with β-catenin, and 3 cases in that study were incorrectly called the WNT subtype of MB on the basis of positive IHC for β-catenin.[3] This same study demonstrated that mutation in *CTNNB1* exon 3 was an excellent molecular marker for WNT MBs, and this is in line with our results which detected the WNT subtype by *CTNNB1* mutation testing even when IHC for β-catenin was (falsely) negative.

On the basis of our results, it might be concluded that the β-catenin stain and *CTNNB1* mutation test did not provide additional value for assigning MB molecular subtypes. At present, a *CTNNB1* mutation is considered as a defining feature of the WNT subtype. Immunostaining for β-catenin is a simple and inexpensive way to assess this, but false negatives can occur, as seen in our study. Hence, *CTNNB1* mutation testing provides a backup for immunonegative cases. To eliminate *CTNNB1* mutation testing altogether might be considered in the future, but first would require a consensus to redefine the WNT subtype without requiring this information.

For the other molecular subgroups (SHH, non-WNT/non-SHH MBs), all 3 IHC panels worked reasonably well with only 1 case missed by all panels (case #23). This case was misclassified as SHH by IHC panel 1 and called "indeterminate" by IHC panels 2 and 3. The specimen was re-examined, and the diagnosis of MB was confirmed. Evaluating overall the 3 IHC panels in this study, panel 1 correctly identified the molecular subtype in 81.2% of cases with 5 indeterminate cases and 1 misclassified case, panel 2 correctly in 84.4% of cases with 5 indeterminate cases, and

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panel 3 in 96.9% of cases with only 1 indeterminate case. Misclassifying the subtype is likely to have greater clinical implications, especially when treatment is different for specific subtypes, such as treating the SHH subtype by adding an smoothened receptor inhibitor such as vismodegib, or reducing radiotherapy for the WNT subtype. Calling a case "indeterminate" instead of a specific subtype would result in the case being treated on clinical grounds instead of molecular subtype, which could result in less specific therapy for the subtype of MB.

TP53 status is required to be reported in all SHH MBs.[2] Accumulation of p53 (strong intensity immunostaining in >50% of tumor cells) was shown to have a 100% sensitivity in predicting *TP53* mutation in MBs.[13] Total absence of p53 expression has, however, been found in the setting of nonsense mutations in *TP53* gene in ovarian cancers.[18] Of 54 *TP53*-mutant MBs from 3 studies, 50 were found to have missense mutations, 3 carried small deletions with no functional study to support loss of function, and only 1 had a nonsense mutation.[13–15] For the 8 SHH MBs in our study, no cases were scored as immunopositive. Only 2 cases had sufficient DNA quality for analysis and no mutations were identified. Therefore, our data are too limited for us to be able to recommend whether sequencing can be restricted to cases with p53 accumulation by immunostaining, or whether all SHH MBs should be sequenced.

To conclude, we propose that a limited immunostaining panel using just 5 antibodies (β-catenin, GAB-1, YAP-1, p75-NGFR, and OTX), combined with mutation analysis of *CTNNB1* exon 3 would provide a reliable system for molecular subtyping of MBs. This approach would reliably subtype 97% of MBs. Given the central role of *CTNNB1* mutations in WNT MBs, we still feel β-catenin IHC could be included in a diagnostic IHC panel for MB subtyping. However, knowing the concerns about false-negative and false-positive staining with β-catenin, we recommend that β-catenin IHC only be used in combination with other IHC markers and mutation analysis of *CTNNB1* exon 3 to diagnose WNT MBs. The cost to the laboratory would be considerably less than more elaborate molecular testing protocols. For example, in Thailand, a panel of 5 immunostains costs US$100 with a turnaround time of 3 days. *CTNNB1* exon 3 mutation testing adds US$100 to the cost with a turnaround time of 7 days. In contrast, NanoString costs about US$90 per case but only when 12 cases are batched, which delays the turnaround time to accumulate this number of cases. Otherwise, a single case can be run in 4 days but at a cost of US$1080 per case. We do recognize that the molecular classification of MB is continually evolving, as evidenced by a recent study that stratified the 4 molecular subtypes of MB further into 12 distinct subgroups,[19] and therefore our simplified protocol might need to be modified in the future. But at present, our simplified approach meets the requirement of the current WHO classification[2] and would be within the budgets of smaller laboratories with more limited resources.

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