Rapid Diagnosis of Medulloblastoma Molecular Subgroups


Abstract

Purpose: Microarray studies indicate medulloblastoma comprises distinct molecular disease subgroups, which offer potential for improved clinical management.

Experimental Design: Minimal mRNA expression signatures diagnostic for the Wnt/Wingless (WNT) and Sonic Hedgehog (SHH) subgroups were developed, validated, and used to assign subgroup affiliation in 173 tumors from four independent cohorts, alongside a systematic investigation of subgroup clinical and molecular characteristics.

Results: WNT tumors [12% (21/173)] were diagnosed >5 years of age (peak, 10 years), displayed classic histology, CTNNB1 mutation (19/20), and associated chromosome 6 loss, and have previously been associated with favorable prognosis. SHH cases [24% (42/173)] predominated in infants (<3 years) and showed an age-dependent relationship to desmoplastic/nodular pathology; all infant desmoplastic/nodular cases (previously associated with a good outcome) were SHH-positive, but these relationships broke down in noninfants. PITH1 mutations were common [34% (11/32)], but PITH1 exon1c hypermethylation, chromosome 9q and REN (KCTD11) genetic loss were not SHH associated, and SMO or SUFU mutation, PITH1 exon1a or SUFU hypermethylation did not play a role, indicating novel activating mechanisms in the majority of SHH cases. SHH tumors were associated with an absence of COL1A2 methylation. WNT/SHH-independent medulloblastomas [64% (110/173)] showed all histologies, peaked at 3 and 6 years, and were exclusively associated with chromosome 17p loss.

Conclusions: Medulloblastoma subgroups are characterized by distinct genomic, epigenomic and clinicopathologic features, and clinical outcomes. Validated array-independent gene expression assays for the rapid assessment of subgroup affiliation in small biopsies provide a basis for their routine clinical application, in strategies including molecular disease-risk stratification and delivery of targeted therapeutics. Clin Cancer Res; 17(7); 1883–94. ©2011 AACR.

Introduction

Medulloblastoma, a primitive neuro-ectodermal tumor of the cerebellum, is the most common malignant brain tumor of childhood. Five-year overall survival rates have increased over recent years to 70% to 80% for standard-risk patients. However, for high-risk patients (infants <3 years, cases with metastatic disease at diagnosis or incomplete surgical resection), current treatments only cure around 40% to 60% of cases. In addition, many survivors exhibit long-term therapy-associated late effects. The development of novel targeted treatments, alongside refined patient stratification, will be essential to increase survival rates and reduce adverse sequelae. Improvements in understanding of the molecular basis of medulloblastoma will be fundamental to such advances.

The constitutive activation of developmental signaling pathways plays a key role in medulloblastoma pathogenesis, and pathway components represent the major mutational targets identified in the disease to date. The Sonic Hedgehog (SHH) pathway plays an essential role in normal cerebellar development, is activated by PITH1 mutation in around 10% of human primary medulloblastomas, and promotes medulloblastoma development in mouse models of the disease (2–4). Similarly, mutations in components of the canonical Wnt/Wingless (WNT) signaling pathway have been described in up to 20% of cases (5–7). Importantly, these pathways appear to have therapeutic significance; WNT-active cases are associated with a favorable prognosis.
Translational Relevance

Understanding the molecular basis of medulloblastoma will be essential to improve clinical outcomes through guidance of molecular risk-adapted adjuvant therapies and delivery of molecularly targeted agents. Expression microarray studies indicate the existence of discrete medulloblastoma molecular subgroups associated with activation of specific developmental signaling pathways (i.e., SHH, WNT). However, any translational utility will require definition of subgroup clinical and molecular correlates in large cohorts, alongside biomarkers and assays for routine subgroup assignment before adjuvant therapy selection. We report development of diagnostic gene expression signatures, which can be applied rapidly and cost-effectively in small biopsies, using array-independent methods, to assign subgroup status. Using these signatures in 173 medulloblastomas, we demonstrate that disease subgroups are robust and reproducible and have distinct clinical, molecular, and outcome characteristics of therapeutic importance. These findings provide strong rationale and methodologies to support subgroup assessment as a basis for future medulloblastoma clinical trials and biological investigations.

(\textgreater{}90\% overall survival; refs 8 and 9), whereas small molecule inhibitors of the SHH pathway show preclinical and early-clinical activity against the disease (10, 11).

Recent array-based genome-wide genomic and transcriptomic investigations in medulloblastoma have identified distinct molecular disease subgroups, which are distinguished by their gene expression profiles, and display related clinical disease features. Two disease groupings, characterized respectively by activation and mutation of the WNT and SHH signaling pathways, are consistently supported by these studies (12, 13). The WNT subgroup is best documented, and is distinguished by nuclear \(b\)-catenin immunostaining, CTNNB1 mutations, and chromosome 6 loss (5, 13–15), alongside its associated favorable prognosis (8, 9). The SHH subgroup is, however, less well characterized; \(P^TCH1\) mutations are only identified in a subset of SHH cases, indicating a role for other activating mechanisms and correlates. A series of putative mechanisms of SHH activation [e.g., \(P^TCH1\) hypermethylation, \(SUFU/SMO\) mutation, and \(R\).EN (KCDT11) genetic loss] have been reported in medulloblastoma (16–19), but any relationships to the SHH subgroup remain to be established. Moreover, SHH subgroup clinical features require clarification; SHH activation has been associated with the desmoplastic/nodular (DN) disease histologic variant in some studies, but not others, and associations with infant cases have also been postulated (12, 17, 20, 21).

Identification of these medulloblastoma molecular subgroups has significant potential to (i) improve clinical management, through molecular disease-risk stratification strategies and the identification of patients who could benefit from SHH and WNT targeted molecular therapeutics; and (ii) provide a basis for biological investigations to further understand disease molecular pathogenesis and its therapeutic applications. However, subgroup identification has so far relied on advanced genomics (i.e., microarray) technologies, which are relatively expensive and have used different gene expression data collection and analysis methodologies. The development of robust biomarkers and assays for subgroup detection, which can be routinely tested in clinical material across multiple treatment centers and are informative in small tumor biopsies, will therefore be essential for their future study and any clinical application. Moreover, studies in modestly sized cohorts have limited comprehensive investigation of subgroup clinical characteristics and their molecular basis.

In this study, we report the development and validation of minimal mRNA expression signatures, which are robust for the routine diagnosis of the SHH and WNT subgroups in clinical material and can be applied rapidly and cost-effectively using array-independent methodologies. Using these signatures, we show that equivalent disease subgroups are detected in 4 independent medulloblastoma cohorts, independent of gene expression assay used. We then use these signatures to assign subgroup affiliation in this large combined cohort of 173 medulloblastomas and use these data as the basis for comprehensive investigations to define the clinical and molecular characteristics of each subgroup, and their utility for improved disease management.

Materials and Methods

Tissue samples

A representative cohort of 55 medulloblastoma samples was analyzed, comprising 39 classic, 5 large cell/anaplastic (LCA), and 11 tumors of the DN histopathologic subtype (22). 21 female and 34 male cases, 11 infants (\(\text{\textless}3\) years), 41 children (3–15 years), and 3 adults (16–18 years). DNA (\(n = 55\)) and RNA (\(n = 39\)) were extracted from these snap-frozen tissues using standard methods. A panel of constitutional DNA samples from 100 normal individuals was obtained from the North Cumbria Community Genetics Project in the United Kingdom. Research Ethics Committee approval has been obtained for the collection, storage, and biological study of all material.

Mutation screening

All coding exons of the \(P^TCH1\) and SMO genes were PCR amplified using the primers and conditions shown in Supplementary Table S1. Mutation screening methods for the \(SUFU\) gene have been described previously (23). Mutation screening was performed by analysis of PCR products for heteroduplex formation, before and after ‘spiking’ with equal amounts of control wild-type DNA using denaturing high performance liquid chromatography (Wave DNA Fragment Analysis System, Transgenomic) according to the manufacturer’s instructions. Products detected as containing a heteroduplex were sequenced directly on an ABI...
and sequence variants (23). Mutation analysis of the PTCH1 gene, spanning exons 1a and 1c (16), were identified and characterized using the Emboss CpGPlot website (http://www.ebi.ac.uk/emboss/cpgplot/): 1a methylation status was determined by methylation-specific PCR (MSP), and 1c by bisulphite sequencing using previously published primers (16). A CpG island of the SUFU gene was also identified, and its methylation status analyzed by MSP (24). COL1A2 methylation status was assessed by bisulphite sequencing, and has previously been reported (25). Bisulphite treatment, methylated, and unmethylated controls have been described previously (23). Primers and conditions for analysis of PTCH1 CpG island 1a are shown in Supplementary Table S2. Methylation status was designated as methylated or unmethylated, as previously described (26). For loci assessed by MSP, any sample showing a visible PCR product using primers specific for the methylated sequence was classed as showing evidence of methylation (i.e., methylated). For loci assessed by bisulphite sequencing, the relative peak intensities at each CpG residue were determined. Samples in which the methylated peak represented >25% of the total peak height in greater than 25% of the analyzed CpG sites were classed as showing evidence of methylation (i.e., methylated).

Loss of heterozygosity analysis
Loss of heterozygosity (LOH) of chromosome regions 9q22.3 and the p-arm of chromosome 17 was analyzed using polymorphic microsatellite markers D9S1869, D9S1816, D9S287, D9S1809, D9S1786, D9S1851, and D17S2196, D17S936, D17S969, D17S974, D17S1866, D17S1308, respectively (www.ncbi.nlm.nih.gov/genome/), as previously described (27). LOH of chromosome 6 was analyzed using previously described markers and methods (14).

A multigene mRNA expression signature to identify SHH and WNT pathway activated tumors
A 13-gene multiplex mRNA expression assay (GeXP; Beckman Coulter; ref. 28) was developed and used to test tumors for membership of the SHH or WNT medulloblastoma subgroups in 39 cases. Detailed clinical and pathologic data for individual cases are summarized in Table 1. Two previously reported independent medulloblastoma expression microarray data sets (12, 13) were used to design SHH and WNT subgroup signatures. Data were respectively downloaded from the St Jude Research website (http://www.stjude.org/site/data/medulloblastoma) and the Gene Expression Omnibus (GEO; ref. 29). Data were 

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Statistical analysis
Fisher’s exact and $\chi^2$ tests were used to identify relationships between expression signatures and molecular and clinical features of the disease in the primary investigative
Table 1. Associations between molecular subgroups and medulloblastoma genomic, epigenomic, and clinical disease features

<table>
<thead>
<tr>
<th>Molecular subgroup</th>
<th>Genotypic mutation</th>
<th>Hypermethylation</th>
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<td>Noninfant (&gt;2yrs)</td>
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NOTE: Cases are shown arranged by signature status (SHH, WNT, WNT-/-SHH-independent) as determined in Fig. 1, and then by ascending age. "Molecular subgroup" indicates the mutation status, hypermethylation status, and chromosomal loss status of the cases. "Clinical features" include age, sex, pathology, and stage. Significant (* \( p < 0.05 \)) and marginally significant (** \( p = 0.051 \)–0.10) associations are marked. Diagonally hatched boxes indicate unavailable data.

Abbreviations: FS, frameshift; MS, missense mutation; ns, not significant.
A diagnostic multigene mRNA expression signature assay for SHH and WNT medulloblastoma subgroups

We first developed and validated multigene mRNA expression signatures to facilitate routine identification of the SHH and WNT medulloblastoma molecular disease subgroups and assessment of their molecular basis, associated biomarkers, and clinical relevance. The 8-gene SHH (BCHE, GLI1, ITIH2, MICAL1, PDLIM3, PTCH2, RAB33A, and SFRP1) and 5-gene WNT (CCDC46, DKK2, PYGL, TNC, and WIF1) subgroup signatures developed were initially assessed by GeXP assay in our primary sample cohort (n = 39) and were unequivocal for all samples, independent of data analysis method used (stacked bar plots, PCA, or unsupervised hierarchical clustering; Figs. 1 and 2). Validation of these signatures in 3 independent medulloblastoma expression microarray data sets [Thompson and colleagues (n = 46; ref. 13); Kool and colleagues (n = 62; ref. 12); and Fattet and colleagues (n = 40; ref. 15)] showed the signatures could be successfully interrogated...
by hierarchical cluster analysis and PCA, were diagnostic in all cases, independent of cohort or gene expression assay used (Fig. 1), and showed close consistency with stacked bar plot data (Fig. 2). Signature positivity was concordant with the disease subgroups apparent after independent clustering of the most variable probes within each entire array data set, and correctly classified disease subgroup (i.e., as SHH, WNT, or WNT/SHH independent) in 99% (146/148) of cases overall (Fig. 1). Data sets similarly showed complete concordance in subgroup assignment, using all analytical methods, of the 14 cases (4 SHH, 2 WNT, and 8 WNT/SHH independent cases) where signatures were assessed in parallel by microarray and GeXP assays.

Integration of cohorts and gene expression data sets: molecular subgroup incidence

Based on our validated gene expression signatures and data analysis methods, the molecular subgroup data from all 4 cohorts could be combined for meta-analysis with the mutational and clinical data which were consistently reported for all studies. A total cohort of 173 cases was assessed in this analysis (Supplementary Table S5). Clinical demographics available for the combined cohort were consistent with previously reported groupwide estimates for medulloblastoma (1); male:female ratio (1.42:1), histology [115 classic (68%), 39 (23%) DN, 16 (9%) LCA, 3 cases with data not available; pathologic classifications were those reported in the original publications.
Genetic and epigenetic mechanisms of SHH and WNT activation

Genetic mutations in \textit{PTCH1} and \textit{CTNNB1} were exclusively detected in SHH and WNT subgroup cases, respectively, across all 4 cohorts, where data were available [combined cohort, \(P = 5.3 \times 10^{-8}\) and \(P = 0\) (\(\chi^2\) test), respectively; Fig. 1], further validating the fidelity of the gene expression signatures developed. \textit{CTNNB1} mutation was the primary mechanism and correlate of WNT pathway activation; in 20 cases where combined expression and mutation data were available, all except 1 WNT subgroup case (19/20; 95%) had concurrent \textit{CTNNB1} mutation. Consistent with this, no \textit{APC} mutations were found in the 55 cases tested within our primary cohort.

\textit{PTCH1} mutation was a major mechanism of SHH pathway activation; 34\% (11/32) of SHH subgroup cases investigated harbored a \textit{PTCH1} mutation (Fig. 1). We therefore next undertook a systematic investigation of alternative genetic mechanisms of pathway activation in the remaining majority of SHH cases in our primary cohort. Mutational analysis encompassed all pathway genes in which mutations have previously been reported; the complete coding sequences of \textit{PTCH1} [including exon 1B, which has been shown to code for protein (32), but has not been analyzed in previous studies (3, 20, 21)], \textit{SUFU} and \textit{SMO} [which has only been previously assessed for mutation at specific residues (18)]. In addition to \textit{PTCH1} truncating mutations (\(n = 5\)), only 1 further potentially pathogenic missense \textit{SUFU} mutation was identified, with no evidence of \textit{SMO} mutation found (Supplementary Table S6). Additional nonpathogenic variants discovered (e.g., polymorphisms or intronic changes) are summarized in Supplementary Table S7. Allelic loss of chromosome 17p, targeting \textit{REN (KCTD11)} at 17p13.2, has also been previously associated with SHH activation in medulloblastoma (19, 33), and was observed in 24\% (9/37) of cases.

Epigenetic mechanisms of pathway activation were additionally investigated as an alternative to genetic determinants. Two predicted promoter-associated CpG islands spanning exons 1a and 1c of \textit{PTCH1}, and a promoter-associated CpG island within \textit{SUFU}, were identified and investigated for evidence of DNA hypermethylation, which may lead to epigenetic transcriptional silencing. No evidence of DNA methylation of the \textit{PTCH1} exon 1a-associated or the \textit{SUFU} CpG island was seen in any tumor analyzed (\(n = 39\); Table 1), indicating no major role in disease development. Mixed patterns of methylation of the \textit{PTCH1} exon 1c CpG island were observed in 12 of 27 of tumors successfully analyzed (44\%; Table 1), suggesting a potential role for this epigenetic mechanism.

Unlike \textit{PTCH1} mutations, other defects identified [\textit{PTCH1} exon 1c methylation, \textit{SUFU} missense mutation, and 17p allelic loss (\textit{REN; KCTD11})] were not associated with the SHH subgroup (Table 1), indicating that any role these mechanisms may play in medulloblastoma development is independent of the SHH pathway.

Genomic biomarkers of SHH and WNT pathway activation

We next investigated selected medulloblastoma chromosomal abnormalities (chromosome 6, 9q, and 17p loss) and epigenetic defects (\textit{COL1A2} status) of biological and/or prognostic significance (1, 25), for their associations with the SHH and WNT disease subgroups, and each other, to assess any utility as biomarkers of pathway activation.

Chromosome 6 loss, \textit{CTNNB1} mutation, and the absence of chromosome 9 and 17 abnormalities, were observed in all WNT cases in our primary cohort (Table 1), consistent with previous findings (12–15). Across the combined cohort, evidence of loss of an entire copy of chromosome 6 was associated with 88\% (14/16) of WNT cases with available data (\(P < 3 \times 10^{-18}\); Fisher’s exact); however, this relationship was not exclusive. Chromosome 6 loss was also detected in occasional non-WNT cases (2/145). Eight of 35 tumors tested (23\%) showed evidence of genetic loss at the 9q22.3 region surrounding the \textit{PTCH1} locus in our primary cohort (Table 1). Four of 8 were in the SHH subgroup and 2 of 4 tumors with \textit{PTCH1} mutations showed LOH of 9q22.3; however neither association reached significance (\(P = 0.09\) and 0.22 respectively, Fisher’s exact test). A significant inverse association between 17p loss and membership of the SHH and WNT subgroups was observed; 17p losses were exclusively observed in WNT-/SHH-independent cases [17p LOH in 0/12 SHH or WNT cases vs. 9/25 WNT-/SHH-independent cases (\(P = 0.02\), Fisher’s exact test)]. In addition, \textit{COL1A2} hypermethylation was detected in 76\% (25/33) of cases; an absence of \textit{COL1A2} methylation was significantly associated with the SHH subgroup (\(P = 0.01\), \(\chi^2\) test), but this relationship was not maintained when a correction for multiple hypothesis testing was applied (Table 1).

Medulloblastoma molecular subgroups display distinct clinical features

Analysis of the medulloblastoma molecular subgroups defined by our gene expression signatures, in all 173 cases of the combined cohort, revealed marked differences in their clinical disease features. WNT-/SHH-independent tumors made up the majority of cases, and had their peak incidence in the 3- to 6-year age group, but were extremely rare in the first 2 years of life (Fig. 3). In contrast, SHH subgroup tumors had their major peak of incidence in infancy [50\% (21/42) of SHH cases were \(\leq 3\) years of age]. The SHH subgroup tumors represented the majority of
the infant clinical group [62% (21/34) of cases ≤3 years of age], but were less common in noninfant children [>3–15 years; 15% (16/127)], and had a further increased incidence in adult cases [≥16 years (45%; 5/111); overall $P = 5.8 \times 10^{-9}$, $\chi^2$ test]. Almost all cases <2 years of age [92% (11/12)] were SHH-positive. WNT subgroup cases were not observed in infants (minimum age observed, 5 years) and had a bi-modal age distribution with major and minor peaks at 10 and 20 years, respectively.

Significant differences were also observed in the distribution of medulloblastoma histologic subtypes between the molecular subgroups ($P = 3.1 \times 10^{-11}$, $\chi^2$ test; Figs. 4 and 5). WNT subgroup cases exclusively displayed classic histology ($P = 0.0003$, Fisher’s exact test), and WNT-/SHH-independent tumors were also predominantly of the classic subtype, but DN and LCA cases were also observed. Consistent with previous studies (3, 12, 13, 21), SHH cases were overall strongly associated with DN histology ($P = 1.1 \times 10^{-9}$, Fisher’s exact test). However, this relationship was not absolute and LCA and classic cases were also observed in the SHH group. Most notably, examination of this large cohort revealed the relationship between SHH activation and DN pathology to be age dependent (Figs. 4 and 5); DN cases made up the majority of infant (≤3 years old) SHH subgroup cases; all DN cases in this infant group displayed SHH activation. DN pathology may therefore serve as a surrogate marker of SHH activation in the infant group. In contrast, there were almost equal proportions of DN, LCA, and classic cases in SHH-expressing noninfant cases, and the majority of noninfant DN tumors were not SHH activated ($P = 8.6 \times 10^{-5}$, Fisher’s exact test). No significant evidence for differences in metastatic stage [WNT 6% (1/16) M stage 2/3, SHH 16% (5/32), and WNT-/SHH-independent 24% (20/82)] was observed between the different expression subgroups ($P = 0.20$, $\chi^2$ test).
Medulloblastoma Molecular Subgroups

Discussion

The identification of distinct medulloblastoma molecular subgroups (12, 13, 15) offers significant potential to improve our understanding of disease biology and clinical management. Here, we report the development and validation of minimal diagnostic gene expression signatures which can be routinely applied to identify the SHH, WNT, and WNT/SHH-independent medulloblastoma disease subgroups. These gene expression signatures are robust and informative for subgroup identification in RNA extracted from snap-frozen tumor material, and using different gene expression assays. In particular, the GeXP multiplex expression assay reported offers a number of significant advantages over microarray methodologies for the routine assignment of subgroup affiliation; analysis can be undertaken straightforwardly, rapidly (in 1 working day, compared with 2–3 days for a microarray experiment) and cost-effectively (approximately one tenth of microarray costs) and, importantly, can be performed on small amounts of total RNA (150 ng, compared with 500 ng to 5 μg for a typical microarray expression analysis), a common limitation when only small amounts of biopsy material are available. This removal of the need for relatively time-consuming, complex, and expensive array analysis platforms for subgroup assignment provides a strong basis for their clinical application; these methods are feasible for investigation in real time across multiple treatment centers during clinical treatment and in future clinical trials.

We have shown that the disease subgroups recognized by these signatures are equivalent and consistently identified in 4 independent medulloblastoma cohorts, allowing their assembly into a large combined cohort. Coupled with an extensive analysis of our novel primary cohort, this has allowed significant insights into the underlying molecular mechanisms, associated biomarkers, and clinical characteristics of these molecular disease subgroups.

Our systematic investigation of specific medulloblastoma genetic and epigenetic defects in this study has informed their roles as determinants or correlates of the molecular subgroups identified. Consistent with previous studies (12–14), CTNNB1 mutations were identified as the primary pathway-activating event present in almost all WNT subgroup tumors, with chromosome 6 losses also affecting the majority of these cases. PITCH1 mutation was the major mechanistic correlate of SHH activation, identified in ~34% of SHH cases. SHH-associated PITCH1 mutations were detected both in conjunction with chromosome 9q loss, and in the heterozygous state, indicating disruption of a single PITCH1 allele can be sufficient to cause SHH pathway disruption in medulloblastoma. An absence of COL1A2 hypermethylation was also significantly associated with SHH subgroup medulloblastomas, most strongly in infant cases. Notably, a number of the previously reported determinants of SHH activation that we examined [PITCH1 exon 1c methylation (ref. 16), SUFU missense mutation (ref. 17), and 17p REN (KCTD11; ref. 19) allelic loss] were not specifically associated with the SHH subgroup, indicating any role they may play in medulloblastoma is SHH-independent. It is similarly unclear whether the mixed methylation patterns observed for PITCH1 exon 1c in this study have functional significance, as this was not assessed. In addition, other SHH pathway defects examined [PITCH1 exon 1a hypermethylation], including events previously reported in medulloblastoma [SMO mutations (ref. 18) or SUFU truncating mutations (ref. 17)] were not observed at all, suggesting their roles are either less common than previously thought or are restricted to limited tumor subsets less well represented in our mutation screening cohort. This is likely the case for SUFU mutations, which appear to be associated with germline inheritance and have their peak incidence in infants (17, 23, 34). Further mechanisms of pathway activation remain to be identified in the majority of SHH cases. Chromosome 17 defects were the only events significantly correlated with the most common WNT/SHH-independent subgroup, suggesting a role for chromosome 17 genes in these cases. This disease subgroup, however, remains the least well characterized at the molecular level. Subdivision of this group has been proposed on the basis of its transcriptomic and genomic patterns; however, unlike the SHH and WNT groups, inconsistent results.
have been reported from different studies (12, 13), and the identification of specific genes and pathways associated with the pathogenesis of this subgroup will be critical to future advances in our understanding of its molecular basis and any underlying heterogeneity.

The significant associations observed between medulloblastoma molecular subgroups and specific gene, pathway, and chromosomal defects (i) strongly support the existence of molecularly distinct SHH and WNT subgroups, (ii) inform the contributory mechanisms involved in their pathogenesis, and (iii) provide potential biomarkers for subgroup identification. When assessed for suitability as primary biomarkers, only CTNNB1 mutations, which were specifically observed in all but 1 WNT subgroup case, have sufficient sensitivity and specificity to have utility. Nuclear localization of β-catenin has also been widely reported as a positive marker of WNT pathway activation (5, 8, 14), although its relationship to our WNT expression signature and CTNNB1 mutations could not be investigated in this study due to tissue limitations. Similarly, COL1A2 status may have utility in the identification of SHH subgroup infant desmoplastic medulloblastomas [this study and (25)], particularly in cases where biopsy limitations do not allow assessment of the DN pathologic variant. The remainder of gene and chromosomal defects investigated were not suitable as primary biomarkers for positive subgroup discrimination, as a result of their (i) non-exclusivity, (ii) limitation to subsets of subgroup cases, or (iii) inverse correlation with pathway activation. In comparison, gene expression signatures positively identified all subgroup cases and provide an accurate diagnostic test for subgroup membership. The genomic markers examined may therefore provide useful secondary or confirmatory markers, when used in conjunction with these signatures. It is not presently clear whether the expression signatures reported translate into subgroup-specific protein expression. Protein expression markers, which are testable by immunohistochemistry, may have utility for routine subgroup assignment in the diagnostic setting, however careful investigation and validation of their sensitivity, robustness, and reflection of expression array subgroup data, will be essential before their application.

The observation of 2 of 148 cases which were not consistently classified using the presently reported signatures and their respective array data sets (Fig. 1) indicates potential difficulties in the robust classification of a small subset (<2%) of cases. Inspection of the microarray expression data for these 2 cases revealed markedly reduced expression of pathway signature genes relative to the other pathway positive cases in their respective cohorts, despite their subgroup positivity using our signatures (refs. 12, 13, 15; Fig. 2). In addition, the stacked bar plot analyses revealed 2 further SHH-positive samples (T27, K452; Fig. 2) which, although clustered consistently between signature and array on hierarchical cluster and PCA analysis, could be classified as indeterminate based on the application of quantitative criteria to the individual expression data in stacked bar plot analysis [i.e., cumulative stacked bar plot expression score >8 (for WNT expression signature positive cases) or >4 (SHH cases)]. For such cases, additional markers of pathway activation (CTNNB1, PITCH1 mutation) could aid definitive subgroup assignment, and it is notable that 1 of these 2 samples also harbored a PITCH1 mutation, further supporting SHH subgroup membership. Silhouette analysis supported the robust assignment of subgroup status using our signatures (Supplementary Fig. S1) but, consistent with the other analytical methods applied, did not support the subgroup assignment made for 3 of the 4 discrepant cases described earlier, further highlighting difficulties in their classification. Thus, in diagnostic applications, a "nonclassified" designation could be reserved for these rare cases which do not classify consistently across all analyses performed on the signature data, particularly where subgroup assignment may impact clinical or therapeutic decisions.

The combination of molecular and clinicopathologic data from 4 independent cohorts for meta-analysis, totaling 173 cases, has facilitated clear and significant insights to the clinical features of the medulloblastoma molecular subgroups, which have either not been apparent or not shown statistical significance in individual analyses of the smaller component cohorts reported to date (12, 13, 15). The SHH (24% of cases), WNT (12%), and WNT-/SHH-independent (64%) groups show different age distributions and relationships to disease histopathology. SHH subgroup tumors peak in infancy and are intimately correlated with DN pathology in this group, to the extent that DN pathology may be considered as a surrogate marker for SHH activation in medulloblastomas arising in infants <3 years old at diagnosis, although classic and LCA cases also constitute a minority of SHH subgroup cases in this age group. This relationship breaks down in noninants (>3 years at diagnosis), where SHH tumors are less common, and shows equivalent proportions of DN, classic, and LCA disease; SHH-independent DN cases are also commonly observed in this age group. These data strongly indicate that (i) SHH subgroup and (ii) DN tumors, arising in the infant and noninfant age groups, have different biological and clinical characteristics, and that SHH-positive DN tumors of infancy represent a unique disease subgroup associated with a favorable clinical behavior (35–37), and a characteristic molecular pathogenesis [COL1A2 unmethylated (ref. 25)] and mutational spectrum [SUFU (refs. 17, 34)]. Conversely, WNT tumors display classic pathology and occur in noninfants. Notably, both the SHH and WNT subgroups show at least 2 different incidence peaks in their age distribution (both have second peaks in adults), suggesting additional clinical and molecular heterogeneity within these groups. The inclusion of cohort-wide central pathology review in future studies may aid the further refinement of the associations observed.

The lack of association between M stage and molecular subgroups (P = 0.20) is in disagreement with the previous study by Kool and colleagues (12), who reported metastatic tumors being less common in WNT and SHH pathway-activated medulloblastomas. This could be due to the
different measurement criteria for metastasis between the studies (this study compared M0/1 vs. M2/3, whereas Kool and colleagues compared M0 vs. M1/M2/M3). Alternatively, the increased numbers in this study (130 vs. 58 with M stage data in the Kool and colleagues study) may have enabled a more accurate characterization of the relationship between signaling pathway activation and metastasis, and future large clinically controlled studies which include central review of metastatic status should be informative in this regard.

The identification of medulloblastoma molecular subgroups has significant prognostic and predictive potential to improve therapeutic delivery and disease outlook in the clinical setting and could represent a first step in the molecular diagnostic triage of medulloblastomas to guide treatment decisions. In addition to distinct clinical features, molecular subgroups also appear to have characteristic clinical behaviors; the favorable prognosis of WNT subtype medulloblastomas is now established in multiple clinical cohorts (8, 9, 13, 38) and will form the basis of treatment reductions in forthcoming international molecularly driven clinical trials (1). Combined data from this and other studies indicate SHH-positive DN tumors arising in infants represent a similarly favorable prognosis subgroup with a distinct molecular basis (25, 35–37). The nonavailability of outcome data for the 4 cohorts assessed in our meta-analysis have limited any direct assessment of survival associations in these cohorts. Moreover, their retrospective, heterogeneously treated nature would likely confound such analyses. Robust assessment of the prognostic impact of the medulloblastoma molecular subgroups will therefore now be essential, ideally within the context of adequately powered, centrally reviewed, and uniformly treated clinical trials cohorts, to determine their utility to direct the selection of adjuvant therapy. The molecular signatures we report will have significant utility in this regard. Molecularly targeted SHH inhibitors are also currently under preclinical and clinical development, and have shown early evidence of activity in medulloblastoma (10, 11). The ability to accurately diagnose the SHH molecular subgroup will thus be important for the targeted delivery of these novel agents, and our findings have identified SHH-positive subgroups of medulloblastomas which would be predicted to benefit most from SHH inhibition strategies. However, the SHH pathway plays a key role in normal, including cerebellar, development, and its transient inhibition in young mice causes permanent defects in growth plate formation and bone structure (39). In view of such potential toxicities, we recommend caution in the application of our data, particularly in the infant age group where SHH subgroup tumors predominate.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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