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ORIGINAL REPORT

Medulloblastoma Comprises Four Distinct Molecular Variants

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See accompanying editorial on page 1395 and articles on pages 1400, 1415, and 1424

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ABSTRACT

Purpose
Recent genomic approaches have suggested the existence of multiple distinct subtypes of medulloblastoma. We studied a large cohort of medulloblastomas to determine how many subgroups of the disease exist, how they differ, and the extent of overlap between subgroups.

Methods
We determined gene expression profiles and DNA copy number aberrations for 103 primary medulloblastomas. Bioinformatic tools were used for class discovery of medulloblastoma subgroups based on the most informative genes in the data set. Immunohistochemistry for subgroup-specific signature genes was used to determine subgroup affiliation for 294 nonoverlapping medulloblastomas on two independent tissue microarrays.

Results
Multiple unsupervised analyses of transcriptional profiles identified the following four distinct, nonoverlapping molecular variants: WNT, SHH, group C, and group D. Supervised analysis of these four subgroups revealed significant subgroup-specific demographics, histology, metastatic status, and DNA copy number aberrations. Immunohistochemistry for DKK1 (WNT), SFRP1 (SHH), NPR3 (group C), and KCNA1 (group D) could reliably and uniquely classify formalin-fixed medulloblastomas in approximately 98% of patients. Group C patients (NPR3-positive tumors) exhibited a significantly diminished progression-free and overall survival irrespective of their metastatic status.

Conclusion
Our integrative genomics approach to a large cohort of medulloblastomas has identified four disparate subgroups with distinct demographics, clinical presentation, transcriptional profiles, genetic abnormalities, and clinical outcome. Medulloblastomas can be reliably assigned to subgroups through immunohistochemistry, thereby making medulloblastoma subclassification widely available. Future research on medulloblastoma and the development of clinical trials should take into consideration these four distinct types of medulloblastoma.

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INTRODUCTION

Brain tumors are the leading cause of cancer-related death in children, and medulloblastoma is the most common malignant pediatric brain tumor. Although overall survival rates have improved in recent years, the mortality rate remains significant, with survivors often suffering from neurologic, endocrinologic, and social sequelae as a result of current treatment options. Completely resected tumors from patients older than 3 years of age with no leptomeningeal dissemination at diagnosis are classified as standard risk, whereas all others are considered high risk. This stratification scheme does not adequately account for the prognostic variability that exists among patients ascribed to either of these risk groups. Rational molecular-based classification methods for medulloblastoma are vital to permit accurate patient stratification, improved clinical trial design, and the future development of molecularly targeted therapies.

Published molecular markers of prognostic value in medulloblastoma include nuclear β-catenin, ERBB2, TP53, and TRKC immunopositivity.[1-3] Cytogenetic and genetic events, including chromosome 17 aberrations, *CTNNB1* mutation/monosomy 6, and *MYC* family amplification, are informative predictors of patient outcome.[4-10] Recently, a molecular risk stratification approach based on multicolor fluorescence in situ hybridization (FISH) of *MYC* and *MYCN* loci, as well as chromosomal alterations on 17q and 6q, proved

more effective than conventional clinical criteria at predicting patient survival.[6] Although statistically robust, the generalization of this technique could be limited by the geographic availability of high-quality FISH laboratories.

In the present study, we integrated genome-wide DNA copy number and mRNA expression profiles from a large cohort of primary medulloblastomas to generate a novel molecular classification scheme that reliably predicts patient prognosis. Because this is an immunohistochemistry-based approach, we expect that it will have widespread utility in clinical settings around the world, leading to significantly improved patient stratification.

METHODS

Tumor Specimens and Genomic Data Sets

All tumor specimens were obtained in accordance with the Research Ethics Board at the Hospital for Sick Children (Toronto, Canada), as described,[11] and the N.N. Burdenko Neurosurgical Institute (Moscow, Russia).[6] Copy number and expression array data were generated and analyzed as described.[11,12] Content and construction of the medulloblastoma tissue microarrays (TMAs) have been described previously.[6,11,13,14]

Biostatistics and Bioinformatics

Medulloblastoma samples were classified into molecular subgroups using TM4 Microarray Software Suite (MeV v4.4; Dana-Farber Cancer Institute, Boston, MA) by unsupervised hierarchical clustering (HCL) using the Pearson correlation metric (average linkage) and bootstrapping analysis of high–standard deviation genes. Subgroup-specific signature genes were identified by a multivariate permutation test restricted on the proportion of false discoveries and one-way analysis of variance. Principal component analysis (PCA) of gene expression data was performed using Partek Genomics Suite (Partek, St Louis, MO). Prediction analysis of microarrays was carried out as described,[15] using the current data set as a training data set and the data set reported by Kool et al[16] (Gene Expression Omnibus accession No. GSE 10327) as a test data set. Pathway analysis was performed using Ingenuity Pathway Analysis (v7.5; Ingenuity Systems, Redwood City, CA). Gene Set Enrichment Analysis (v2.0; Broad Institute, Cambridge, MA), non-negative matrix factorization (NMF), and subclass mapping (SubMap) were carried out as described.[17-20]

Immunohistochemistry

Antibodies against the following antigens were used: β-catenin (1:100; BD Transduction Laboratories, Franklin Lakes, NJ), DKK1 (1:100; Abnova, Taipei City, Taiwan), GLI1 (1:5,000; Millipore, Billerica, MA), SFRP1 (1:2,000; Abcam, Cambridge, MA), NPR3 (1:200; Abcam), and KCNA1 (1:2,000; Abcam). TMA staining was performed, evaluated, and scored as published.[13]

RESULTS

Transcriptional Profiling Identifies Four Subgroups of Medulloblastoma With Distinct Demographics

Unsupervised HCL of medulloblastoma expression data identified the following four unique sample clusters: WNT, SHH, group C, and group D (Fig 1A). Support tree analysis of the clustering data reveals high confidence for these four subgroups (≥ 96%; Data Supplement). The degree of separation among the four subgroups was further established by PCA, with WNT and SHH tumors showing clear separation from each other and group C and D tumors (Fig 1B). In contrast, group C and D tumors are more similar to each other and exhibited some proximity in the PCA. Desmoplastic medulloblastomas were found predominantly in the SHH subgroup ($P = .0023$), but they were also found in groups C and D (Fig 1A; see Data Supplement for all available clinical details).[21] Large-cell/anaplastic (LCA) medulloblastomas were found in SHH, group C, and group D. Known targets of the Wnt pathway (*WIF1*, *DKK1*, and *DKK2*) and Shh pathway (*HHIP*, *SFRP1*, and *MYCN*) all show clear, statistically significant differential expression in their respective subgroups (Fig 1A; Data Supplement). Group C and D tumors both express high levels of the known medulloblastoma oncogenes *OTX2*[22,23] and *FOXG1B*.[24] Group C and WNT tumors, but not SHH or group D tumors, have high levels of MYC expression ($P < .001$). SHH tumors, but not group C or group D tumors, have high levels of *MYCN* expression ($P < .001$), making group D the only subgroup lacking elevated MYC family expression. As expected, monosomy 6 and 9q deletion were restricted to WNT and SHH tumors, respectively (Fig 1A).[4,5,16,25,26] Isochromosome 17q is restricted to groups C and D ($P < .001$). *MYC* amplification (two of 103 medulloblastomas) is limited to group C, whereas *MYCN* amplicons (three of 103 medulloblastomas) are distributed among SHH, group C, and group D.

Demographic comparison reveals notable differences between the subgroups. SHH-driven tumors are most common in infants (≤ 3 years old) and adults (≥ 16 years old; $P < .001$; Fig 1C; Appendix Fig A1A, online only). Group C tumors peak in childhood (age 3 to 10 years) but are completely absent in individuals over the age of 10 years ($P = .0184$). Group D and WNT tumors show a more distributed age of onset (median age, 9 to 10 years), ranging from infancy to adulthood (Fig 1C and Appendix Fig A1A). The published male-to-female sex ratio for medulloblastoma is approximately 1.5:1; in our cohort, the ratio is 1.55:1. Notably, approximately 70% of males belong to Group C or D compared with only approximately 42% of females (Appendix Fig A1B; $P = .0142$). Conversely, approximately 47% of females are classified as having SHH tumors versus only approximately 24% of males ($P = .0312$). The incidence of WNT tumors in female patients (approximately 11%) is nearly double that of males (approximately 6%).

Molecular Classification of Medulloblastoma by Transcriptional Profiling

An 84-gene classifier of medulloblastoma subgroup signature genes derived from the current data set using prediction analysis of microarrays[15] was applied to a published data set of 62 medulloblastomas reported by Kool et al,[16] separating this cohort into the current four subgroups (Appendix Fig A2A, online only; Data Supplement). Comparison of the 20 most highly differentially expressed genes discriminating Groups C and D in both data sets demonstrates that 38 of 40 genes are concordantly differentially expressed ($P < .05$; Appendix Fig A2B; Data Supplement). Application of consensus NMF,[19] an unsupervised bioinformatic tool for determining the number of independent classes within an expression data set, strongly supports the existence of four medulloblastoma subgroups (Appendix Fig A2C; Data Supplement). Direct comparison of clustering results obtained by HCL and NMF confirmed strong concordance between the independent analyses (Rand index, 0.9309; adjusted Rand index, 0.8287).[27] Another bioinformatic algorithm, SubMap,[20] suggests subgroups C and D in the Kool data set both correspond to our group D ($P < .01$; Appendix Fig A2D). In the Kool data set, adult tumors (age ≥ 16 years) were devoid of group C cases. Notably, there was a high rate of M+ patients in group C in this data set (75%; $P = .0039$; Appendix Fig A2E).

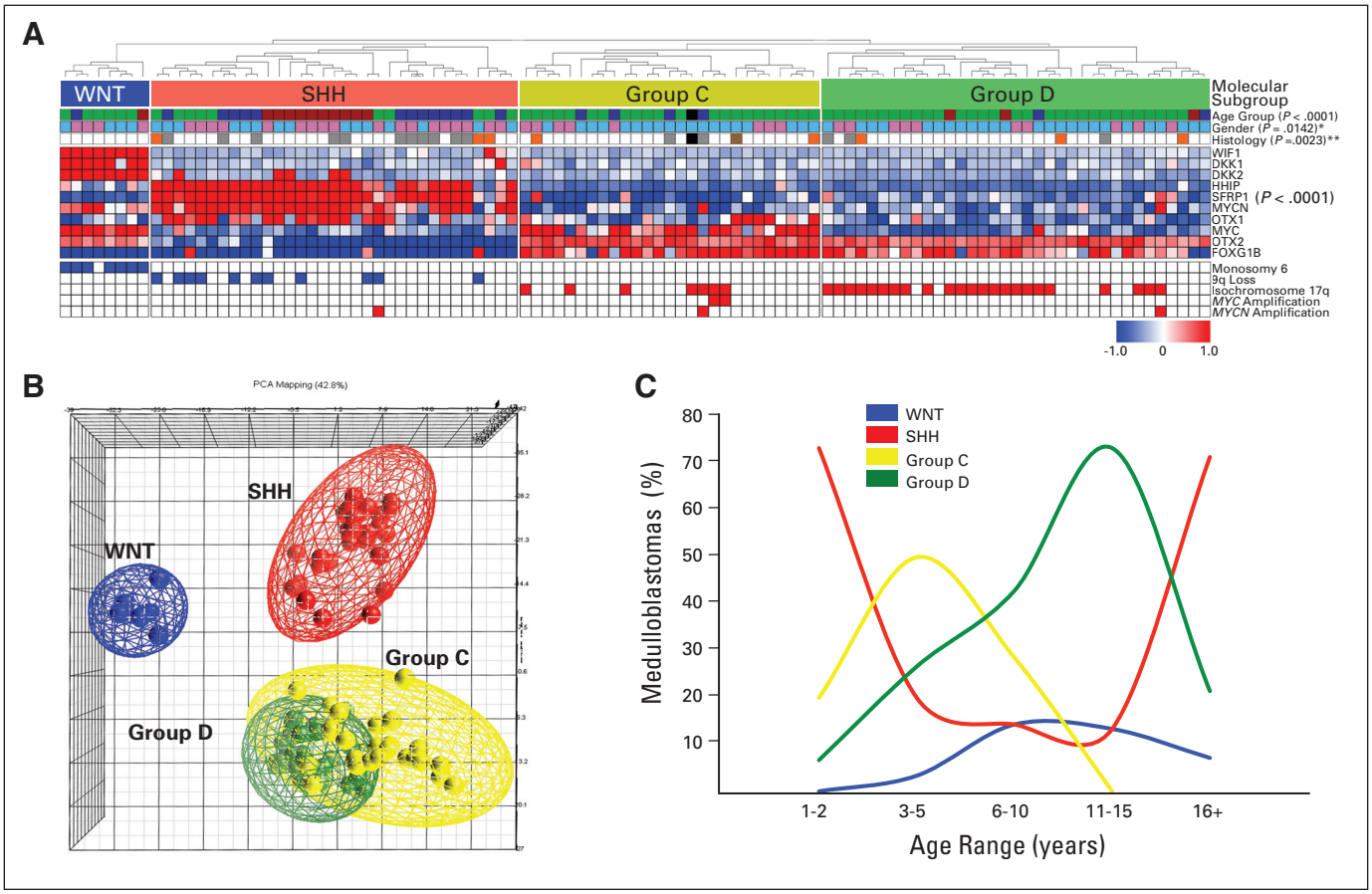

Fig 1. (A) Unsupervised hierarchical clustering of human 1.0 exon array expression data from 103 primary medulloblastomas using 1,450 high–standard deviation (SD) genes. Clinical features (age group, sex, and histology) for the 103 samples included in the study are shown below the dendrogram (see Data Supplement for summary of all clinical details related to the sample cohort). Age groups include infants (≤ 3 years; blue), children (4 to 15 years; green), adults (≥ 16 years; red), and unknown (black). Sex includes males (blue) and females (pink). Histology includes classic (white), desmoplastic (gray), large-cell/anaplastic (orange), medulloblastoma with extensive nodularity (brown), and unknown (black). Statistical significance for the different clinical features was determined using the χ^2 test (age group) and Fisher's exact test (sex and histology). (*) *P* value determined by comparing sex prevalence in WNT/SHH tumors versus group C/D tumors using Fisher's exact test. (**) *P* value corresponds to over-representation of desmoplastic tumors in the SHH subgroup as determined using Fisher's exact test. The heatmap below the dendrogram shows the expression profile for 10 genes well characterized in medulloblastoma and demonstrates their significant pattern of differential expression among the four subgroups (see Data Supplement for summary of all differentially expressed genes identified in the four subgroups). Statistical significance of differential gene expression was determined using one-way analysis of variance. Common genomic aberrations known to occur in medulloblastoma are shown below the heatmap. Blue boxes indicate loss/deletion, red boxes indicate gain/amplification, and white boxes denote balanced copy number state for the specified genomic aberration. (B) Principal component analysis (PCA) of the primary medulloblastomas described in (A) using the same 1,450 high-SD genes used in clustering. Individual samples are represented as colored spheres (blue = WNT, red = SHH, yellow = group C, green = group D), and ellipsoids represent two SDs of the data distribution for each subgroup. (C) Age at diagnosis distribution for each of the four medulloblastoma subgroups.

Ingenuity Pathway Analysis identified the top canonical signaling pathways over-represented in medulloblastoma subgroups (Table 1). As expected, Wnt signaling was enriched in the WNT group tumors ($P < .001$), and Shh signaling was enriched in the SHH subgroup ($P < .001$). However, Wnt pathway genes were also enriched in SHH and group C, but not group D. Both WNT and SHH group tumors had an over-representation of genes involved in axonal guidance. Both group C (phototransduction and glutamate signaling) and group D (semaphorin, cyclic adenosine monophosphate, G protein–coupled receptors, and β-adrenergic signaling) were characterized by an over-representation of pathways involved in neuronal development. Gene Set Enrichment Analysis[17] comparison of genes discriminating group C and group D demonstrated that genes upregulated in group C positively correlate with genes associated with medulloblastoma treatment failure identified by Pomeroy et al[28] and with genes associated with elevated MYC levels (Data Supplement).

Subgroup-Specific Genetic Events in the Medulloblastoma Genome

With the exception of i(17)q, which is reported in approximately 30% to 50% of patients,[6,11] most of the known chromosomal changes in medulloblastoma occur at low frequency. To determine whether certain genetic aberrations were more prominent when accounting for subgroups, we manually cataloged all chromosomal changes in our data set in a subgroup-specific manner (Appendix Fig A3A, online only; Appendix Table A1, online only; Data Supplement). Not surprisingly, monosomy 6 was found exclusively in WNT tumors,[4,12,16,26] and 9q loss was found only in SHH tumors.[16]

Multiple novel regions of genomic aberration were identified in SHH tumors, including chromosome 9p gain ($P < .001$), which often co-occurred with 9q loss [ie, i(9)p], as well as gains of 3q ($P = .0015$), 20q ($P = .0331$), and 21q ($P = .0124$). Chromosome 10q loss was primarily limited to SHH and group C tumors ($P = .0034$). Events

Table 1. Ingenuity Analysis of Subgroup-Specific Genes in Medulloblastoma

Ingenuity Canonical Pathways	*P*	Molecules
WNT		
Axonal guidance signaling	< .001	EPHA7, FZD10, BMP4, WNT16, EPHA4, NFATC4, SLIT2, EPHA3, ADAM12, PRKCD, FZD6, ADAM19, PLCB1, SEMA3B
WNT/β-catenin signaling	< .001	FZD10, AXIN2, WIF1, FZD6, DKK4, WNT16, DKK2, LEF1, KREMEN1, DKK1
Factors promoting cardiogenesis in vertebrates	.00339	FZD10, BMP4, PRKCD, FZD6, LEF1, DKK1
O-glycan biosynthesis	.00417	GLT8D2, GALNT7, GALNT14, GALNT12, GALNTL2
Basal cell carcinoma signaling	.00776	FZD10, BMP4, FZD6, WNT16, LEF1
SHH		
SHH signaling	< .001	DYRK1B, ARRB2, GLI2, GLI3, HHIP, GLI1, PTCH2
Basal cell carcinoma signaling	< .001	TP53, GLI2, GLI3, LEF1, HHIP, GLI1, BMP5, FZD7, PTCH2
WNT/β-catenin signaling	< .001	TP53, SFRP4, TCF4, HDAC1, SOX2, SOX9, RARB, TGFB2, SFRP5, PPP2R2C, LEF1, SFRP1, TCF7L2 (includes EG:6934), FZD7
Axonal guidance signaling	< .001	GLI2, PLXNC1, CXCR4, SEMA6A, GNG3, HHIP, NFATC4, BMP5, ROBO1, PTCH2, PLCB4, GLI3, SRGAP1, SDC2, NTRK3, NGFR, EFNA5, ABLIM3, PDGFD, GLI1, PRKD1, FZD7, UNC5C
Human embryonic stem-cell pluripotency	.00295	SOX2, NTRK3, PDGFRA, SPHK1, TGFB2, LEF1, PDGFD, BMP5, FZD7
Group C		
Phototransduction pathway	< .001	GNB3, GNGT1, RCVRN, PDE6H
WNT/β-catenin signaling	.00933	MYC, NLK, TGFB1, TGFBR3, PPP2R2B
Glutamate receptor signaling	.01122	GNB3, SLC17A7, SLC1A7
Thyroid cancer signaling	.04074	MYC, RXRG
p38 MAPK signaling	.04266	MYC, TGFB1, EEF2K
Group D		
Semaphorin signaling in neurons	< .001	ARHGEF12, RND1, RHOT1, DPYSL4, DIRAS3, FNBP1
cAMP-mediated signaling	.00275	GRK4, GRM8, RGS7, ADCY1, STAT3, PDE4B, AKAP9, CHRM3, PDE1C
G protein–coupled receptor signaling	.00490	HTR2C, GRK4, GPR12, GRM8, RGS7, ADCY1, STAT3, PDE4B, CHRM3, PDE1C
p53 signaling	.00603	KAT2B, CCND2, GADD45G, C12ORF5, HIPK2, TP53BP2
Cardiac β-adrenergic signaling	.00617	ADCY1, CACNA1C, PDE4B, GNG2, AKAP9, CACNA1A, PDE1C

Abbreviation: MAPK, mitogen-activated protein kinase; cAMP, cyclic adenosine monophosphate.

significantly over-represented in group C included 1q gain ($P < .001$), distal 5q loss ($P = .0038$), and 16q loss ($P = .0055$). Multiple events were over-represented in both groups C and D compared with WNT/SHH tumors, including gain of chromosomes 17q ($P = .0014$) and 18 ($P = .0136$), i(17)q ($P < .001$), and loss of 11p ($P < .001$). Despite the commonalities between group C and D, these subgroups could be discriminated by several genetic events that are significantly more common in group C, including 1q gain ($P = .0021$), loss of distal 5q ($P = .0689$), and 10q loss ($P = .0168$; Appendix Fig A3B; Table 2). Isochromosome 17q was significantly more prominent in group D (23 of 35 tumors; 65.7%) than group C (seven of 27 tumors; 25.9%; $P = .0024$), and loss of the X chromosome occurred more commonly in group D females ($P < .001$; Appendix Fig A3B; Table 2).

The bioinformatic tool Genomic Identification of Significant Targets in Cancer (GISTIC)[29] delineated statistically significant, subgroup-specific genetic changes in an unbiased manner. GISTIC confirmed all of the large genomic abnormalities identified by previous manual curation of the data set and revealed several novel subgroup-specific amplifications and deletions (Appendix Figs A3C and A3D). GISTIC identified gain of chromosome 2 (*Q*, .2412 to .0019) and deletion of chromosome 14 (*Q*, .7534 to .0017) as significant in the SHH subgroup. Focal *MYC* amplification on 8q24 was identified exclusively in group C tumors ($Q = .0011$; Appendix Fig A3C). Loss of chromosome 8p was highly significant in group C (*Q*, .5086 to .0005), whereas group D tumors exhibited significant loss on both 8p and 8q (*Q*, .1662 to .0010) Appendix Fig A3D).

Validation of Medulloblastoma Subgroups by Immunohistochemistry

We selected highly expressed, subgroup-specific signature genes based on our microarray expression data set (Appendix Fig A2A; Appendix Fig A4A, online only) and the availability of high-quality commercial antibodies. Staining two separate medulloblastoma TMAs using these antibodies, as well as β-catenin and GLI1, demonstrated robust staining in a subgroup-specific manner (Appendix Fig A4B). Remarkably, 288 (approximately 98%) of 294 tumor samples stained positive for a single marker protein, a failure rate of only approximately 2.1% ($P < .001$; Appendix Fig A4C). Demographics for the TMA patients validate the results from our microarray data set (Figs 1C and 2A; Appendix Figs A1A, A1B, A4D, and A4E), confirming that SHH tumors occur primarily in infants and adults ($P < .001$). Group C tumors were largely confined to childhood ($P < .001$), except for two patients both age 18 years (Fig 2A; Appendix Fig A4D). WNT group tumors were almost three times more common in females than males (17% *v* 6%, respectively; $P = .0046$; Appendix Fig A4E). Metastases were significantly over-represented in group C (46.5%; $P < .001$), followed by group D (29.7%), similar to the Kool data set (Fig 2B; Appendix Fig A2B). *MYC* amplification was demonstrated by interphase FISH in 11 of 98 group C tumors on the German Cancer Research Center TMA, but not in any SHH, WNT, or group D tumors (Table 2). LCA histology was much more common among group C tumors (23%) compared with group D tumors (8%; $P < .001$). Overall survival in the German Cancer Research Center

Table 2. Group C Versus Group D Medulloblastoma

Genotype/Phenotype	Group C		Group D		
	No.	Total No.	No.	Total No.	*P*
Demographics					
Infant (MB103/TMA)	10	106	8	114	NS
Child (MB103/TMA)	94	106	91	114	NS
Adult (MB103/TMA)	2	106	15	114	.0018
Gene expression					
MYC expression (mean signal intensity exon array)	9.424		6.787		< .001
NPR3 expression (mean signal intensity exon array)	9.195		6.280		< .001
KCNA1 expression (mean signal intensity exon array)	6.812		10.702		< .001
NPR3 positive (TMA)	80	80	0	80	< .001
KCNA1 positive (TMA)	0	80	80	80	< .001
Genetics					
1q gain (SNP array)	12	27	3	35	.0021
5q loss (SNP array)	6	27	2	35	.0689
10q loss (SNP array)	7	27	1	35	.0168
i(17)q (SNP array)	7	27	23	35	.0024
MYC amplification (SNP array/FISH)	11	98	0	98	< .001
MYCN amplification (SNP array/FISH)	8	98	6	98	NS
Histology					
Classic (TMA-DKFZ)	54	71	56	63	.0708
Desmoplastic (TMA-DKFZ)	1	71	2	63	NS
Large cell/anaplastic (TMA-DKFZ)	16	71	5	63	.0306
Outcome					
M+ (TMA-DKFZ)	33	71	19	64	.0527
PFS (TMA-DKFZ), %	32		63		< .001
OS (TMA-DKFZ), %	33		77		< .001

Abbreviations: MB, medulloblastoma; TMA, tissue microarray; NS, not significant; SNP, single nucleotide polymorphism; FISH, fluorescent in situ hybridization; DKFZ, German Cancer Research Center; PFS, progression-free survival; OS, overall survival.

cohort (n = 236; Appendix Fig A4F), the Johns Hopkins University cohort (n = 50, a single WNT tumor was excluded; Appendix Fig A4G), and both cohorts combined (n = 287; Appendix Fig A4H) demonstrates that group C tumors have the worst prognosis (log-rank $P < .001$). Replotting the overall survival curves by dividing each subgroup into M0 and M+ sub-subgroups shows that group C patients exhibit a dismal prognosis regardless of M stage (Appendix Fig A4I). A multivariate analysis evaluating age, M stage, histology,

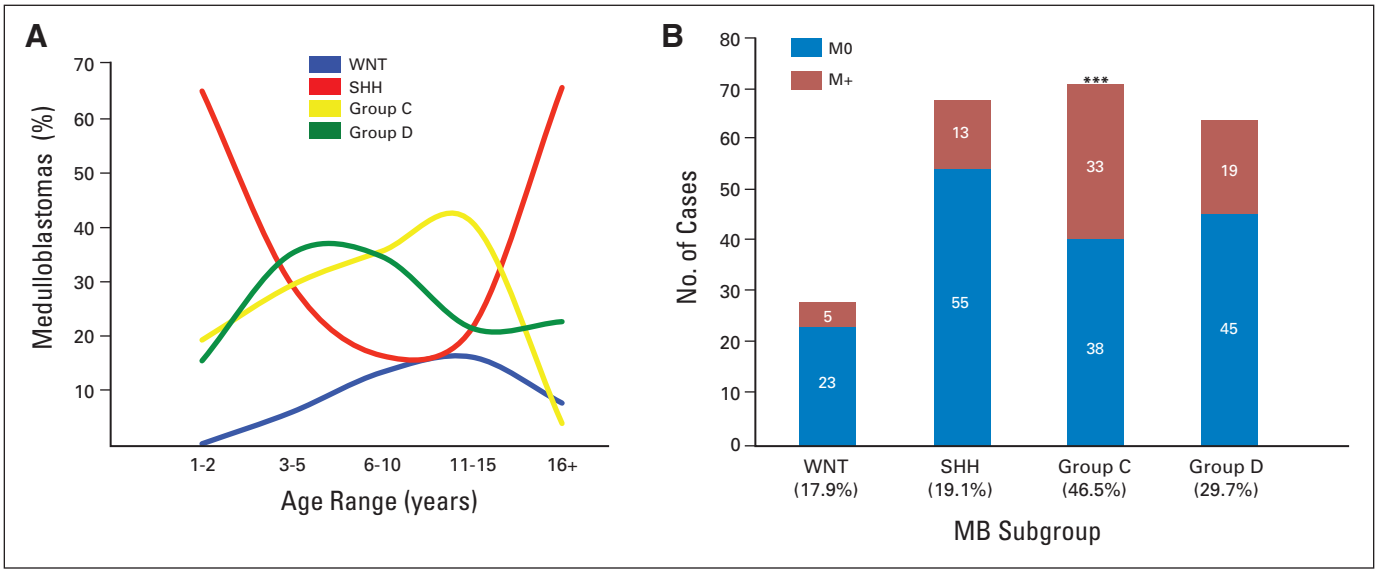

Fig 2. (A) Age at diagnosis distribution for each of the four medulloblastoma (MB) subgroups as determined by immunohistochemical staining of the MB tissue microarrays (TMAs). (B) Incidence of metastases by subgroup as determined by TMA staining. Significance was assessed by Fisher's exact test. (***) $P < .001$.

extent of resection, and subgroup (WNT, SHH, group C, or group D) revealed that only LCA histology and group C subgroup were prognostic (Data Supplement). Indeed, group C tumors with LCA histology have the worst prognosis (Appendix Fig A4J).

DISCUSSION

Prior attempts to subgroup medulloblastoma using genomics identified five[16] or six[26] subgroups. Both studies clearly show the WNT group of tumors clustering alone, whereas SHH-driven tumors did not always segregate as a single subgroup.[26] Both the current data set and the data set from Kool et al[16] show that although WNT tumors are classic medulloblastomas and most desmoplastic medulloblastomas are SHH tumors, the WNT and SHH subgroups always cluster together, suggesting that they are biologically similar. Although desmoplastic tumors were most commonly seen in the SHH group, they were also found in group C and group D, supporting the known difficulty in making a histologic diagnosis of nodular desmoplastic medulloblastoma.[21] We observed LCA tumors in SHH, group C, and group D tumors, and others have described the LCA phenotype in WNT tumors.[5] Interestingly, children with a WNT subgroup LCA medulloblastoma have a good prognosis.[5]

SHH tumors are seen in both infants and adults, whereas WNT and group D tumors are distributed across all age groups. Group C tumors peak in childhood, are rarely seen in older teenagers, and are never seen in adults. The paucity of group C tumors and predominance of group D tumors seen in adolescence may account for the distinct clinical course and pattern of relapse that has been described in adolescents with medulloblastoma.[30] The St Jude Medulloblastoma-96 study showed that the male-to-female ratio for high-risk medulloblastoma was 3.9:1, and several reports have suggested that females with medulloblastoma have a better outcome than males.[30-32] We believe this is attributable to a higher female incidence of WNT tumors and perhaps SHH tumors. Indeed, the improved survival among females is only seen among older children and adults (in whom WNT tumors are more prevalent), highly supporting a WNT subgroup prevalence as an explanation for the improved outcome in females.[32] Prior clinical trials studying medulloblastoma have tended to divide patients by age group, with infants often being studied separately. Our results suggest that infants with medulloblastoma should be broken down by subgroup and that infants with group C/D tumors may account for the children who respond poorly to current therapies.[33] This is consistent with identified prognostic factors of desmoplasia (good) and metastatic status (poor), which might be identifying SHH and group C tumors, respectively.[33]

Analysis of 62 medulloblastomas by Kool et al[16] identified three non-SHH/WNT medulloblastoma subgroups, whereas our analysis of 103 medulloblastomas revealed only two non-SHH/WNT subgroups. The statistical support for three non-SHH/WNT subgroups as reported by Kool et al[16] was either 21% or 41%, depending on the number of genes used in support tree analysis. In the current study, both HCL and PCA demonstrate that group C is distinct from group D with strong statistical support. NMF supports the existence of four subgroups of medulloblastoma. The initial obvious difference between group C and group D is that although *MYC* is highly expressed in group C and WNT tumors and *MYCN* is highly expressed in SHH tumors, neither *MYC* nor *MYCN* is highly expressed in group D tumors. Additionally, our data show that group C and group D tumors have different demographics, rate of metastases, genetic profiles, and clinical outcomes (Table 2). Our immunohistochemical analysis of medulloblastoma TMAs revealed that only one of 294 medulloblastomas stained positive for both group C and group D signature genes, demonstrating the lack of overlap between the two groups. NMF and SubMap analysis also support the existence of two non-SHH/WNT groups of medulloblastoma. Taken together, all of these data highly support the existence of two non-SHH/WNT subgroups that we have designated as group C (childhood tumors with high MYC levels and frequent CSF dissemination) and group D.

Our novel four-antibody approach to subgroup medulloblastoma should be broadly applicable across the globe because the technique does not require RNA, FISH, PCR, microarray technology, or any other techniques from molecular biology, but rather uses immunohistochemistry on archival specimens, which is in routine use in most neuropathology laboratories around the world. Furthermore, because marker staining is quite robust, we hope that there will be little interobserver variability.

Analysis of overall survival demonstrates a marked reduction in survival for children with Group C medulloblastoma regardless of metastatic stage. This suggests that some children with average-risk medulloblastoma who subsequently experience recurrence likely belong to group C. Indeed, the failure of metastatic stage to predict prognosis in our multivariate analysis suggests that the published association between poor prognosis and metastatic stage may be attributable to the high rate of metastases in group C tumors.

Historically, small round blue cell tumors of the cerebellum have been grouped under the rubric of medulloblastoma. Recently, atypical teratoid and rhabdoid tumors were shown to represent a distinct subgroup of blue cell tumors based on different histopathology and, subsequently, a lack of expression of the *hSNF5/INI1* tumor suppressor gene.[34,35] Our data suggest that the histologic entity of medulloblastoma comprises four distinct molecular variants that are demographically, clinically, transcriptionally, and genetically distinct and can be distinguished through the application of four commercial antibodies on formalin-fixed, paraffin-embedded tumor material. Preliminary analysis of the SHH subgroup tumors in this study suggest the existence of three transcriptionally and genetically distinct clusters (data not shown). We anticipate that analysis of much larger cohorts of medulloblastoma will likely reveal subclusters within the four main subgroups. Future clinical trials should prospectively validate our four-antibody immunohistochemical approach. Most importantly, because of the nonoverlapping character of the four types of medulloblastoma, we suggest that targeted therapies may need to be developed against each subtype individually because they are biologically distinct. Finally, prospective investigation into the cellular origin(s) and specific genetic events found in each subgroup will be necessary to determine whether these molecular variants constitute four distinct diseases.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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