Title

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Journal

Acta Neuropathologica, 135(4)

ISSN

0001-6322

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Publication Date

2018-04-01

DOI

10.1007/s00401-018-1819-x

Peer reviewed

Acta Neuropathologica (2018) 135:635–638
https://doi.org/10.1007/s00401-018-1819-x

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Deep sequencing of WNT-activated medulloblastomas reveals secondary SHH pathway activation

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Received: 14 December 2017 / Revised: 2 February 2018 / Accepted: 5 February 2018 / Published online: 12 February 2018

Initially described in 2011, it is now recognized that medulloblastomas (MB) can be stratified into four distinct molecular subgroups (WNT-activated, SHH-activated, Group 3, and Group 4) on the basis of underlying genetic alterations, transcriptional profiles, or genome-wide DNA methylation patterns that more accurately predict clinical outcomes than histologic features alone [4, 7]. The recurrent genetic alterations that characterize each of these four molecular subgroups have been described over the last few years [2, 5, 6, 8, 9]. WNT-activated MB, associated with favorable prognosis, are genetically defined by activating mutations in *CTNNB1* (encoding beta-catenin) often accompanied by monosomy 6 and alterations in chromatin regulatory genes. SHH-activated MB, associated with intermediate or poor prognosis depending on the status of the *TP53* tumor suppressor gene, are genetically characterized by alterations in components of the sonic hedgehog (SHH) signaling pathway including *PTCH1*, *SMO*, *SUFU*, and *GLI2*. Group 3 and Group 4 MB, associated with intermediate-to-poor prognosis, are characterized by activation of *MYC* or *MYCN* transcriptional networks often due to amplification or structural rearrangement involving these genes. Recent studies have suggested

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00401-018-1819-x) contains supplementary material, which is available to authorized users.

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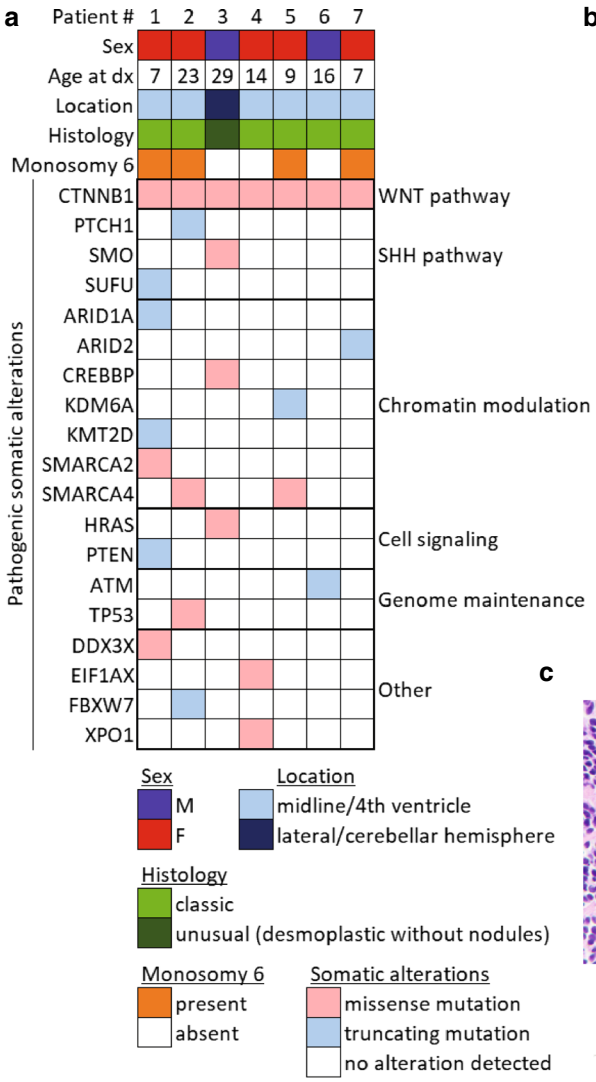

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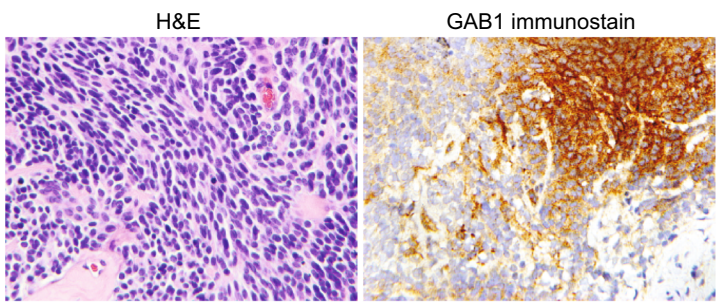
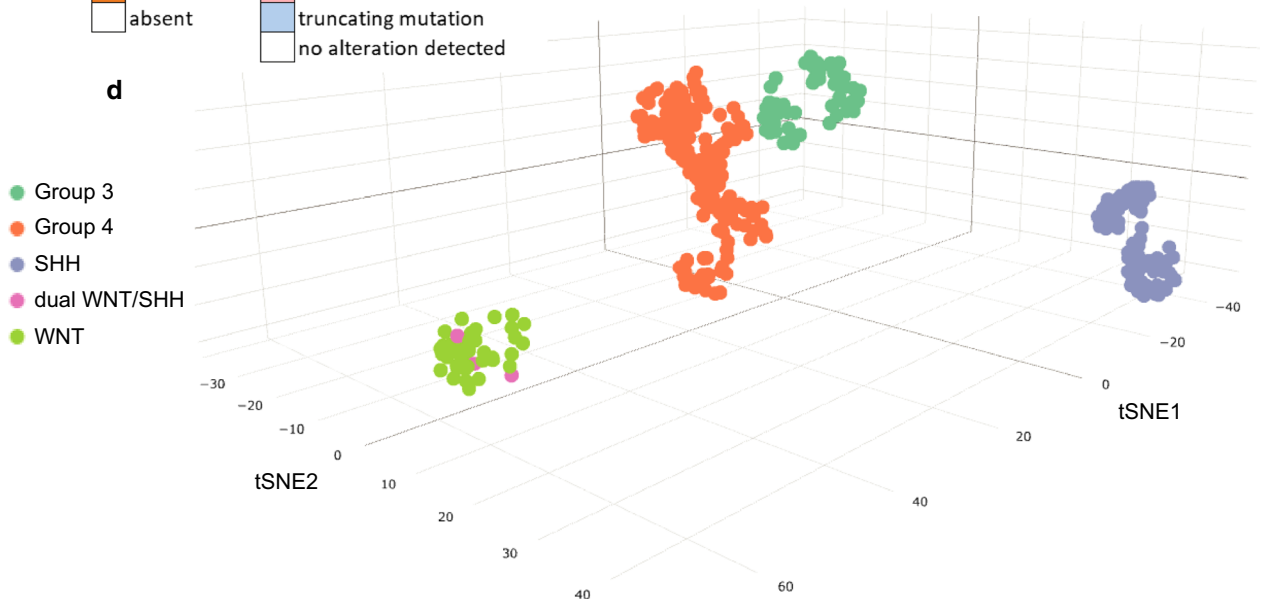
	Somatic Variants	Transcript ID	Mutant allele frequency	Predicted clonality
patient #1	**CTNNB1 p.D32Y**	NM_001904	50%	Heterozygous
	SUFU p.R146*	NM_016169	15%	Subclonal
	ARID1A p.V1561fs	NM_006015	32%	Subclonal
	PTEN p.E18fs	NM_000314	13%	Subclonal
	KMT2D p.P3487fs	NM_003482	11%	Subclonal
	SMARCA2 p.E1531K	NM_003070	10%	Subclonal
	DDX3X p.D506G	NM_001356	8%	Subclonal
	Monosomy 6	N/A	N/A	Heterozygous
patient #2	**CTNNB1 p.D32G**	NM_001904	44%	Heterozygous
	PTCH1 p.G38fs	NM_000264	53%	Heterozygous
	PTCH1 p.L923P	NM_000264	43%	Heterozygous
	SMARCA4 p.R1189Q	NM_001128844	49%	Heterozygous
	FBXW7 p.Q388*	NM_033632	44%	Heterozygous
	TP53 p.R158H	NM_000546	3%	Subclonal
	Monosomy 6	N/A	N/A	Heterozygous
patient #3	**CTNNB1 p.S33F**	NM_001904	46%	Heterozygous
	SMO p.S278N	NM_005631	23%	Subclonal
	SMO p.V210M	NM_005631	22%	Subclonal
	CREBBP p.H595fs	NM_004380	8%	Subclonal
	HRAS p.G13R	NM_001130442	4%	Subclonal





that there is heterogeneity within these four molecular subgroups, now with 12 distinct subtypes that can be separated by DNA methylation profiling and are each associated with distinct genetic alterations [1, 6]. Additional studies have shown that many of the underlying genetic alterations in MB may only be present in spatially restricted subclones within

◂**Fig. 1** Deep sequencing of WNT-activated MB reveals frequent secondary SHH pathway activation. **a** OncoPrint table summarizing the clinicopathologic characteristics and likely pathogenic somatic alterations identified by targeted NGS of paired tumor-normal samples from seven patients with WNT-activated MB. **b** Somatic alterations identified from the three patients with WNT-activated MB harboring secondary SHH pathway activation. **c** H&E stain and GAB1 immunostain of the MB resected from patient #1 with clonal *CTNNB1* hotspot mutation and subclonal *SUFU* nonsense mutation. **d** Unsupervised clustering of DNA methylation patterns for the three dual WNT/SHH-activated MB alongside 367 previously characterized MB. Shown is a three-dimensional representation of pairwise sample correlations using the 10,000 most variably methylated probes by t-distributed stochastic neighbor embedding (tSNE) dimensionality reduction

the tumor [5]. However, in all patients studied to date, the molecular subgroup has been constant in all regions of the primary tumor, as well as at time of recurrence or metastasis, suggesting that the molecular subgroup is defined at the time of tumor initiation and is not affected by clonal genetic evolution, therapy, or other factors [5]. In addition, all MB studied to date have been classified into one of the four primary molecular subgroups that are thought to be mutually exclusive, and switching between these subgroups or tumors with dual activation of two or more of the signaling pathways that define the four primary molecular subgroups have not been reported. Herein, we report the results of deep sequencing on a cohort of WNT-activated MB and show that they often acquire subclonal genetic alterations that secondarily activate the SHH pathway, a finding that may have important prognostic and therapeutic significance.

Since 2015, genomic profiling has been performed on 33 patients with pathologically confirmed MB using the UCSF500 Cancer Panel, a targeted-capture next-generation sequencing assay performed on paired tumor and normal samples that assays approximately 500 cancer-associated genes for single nucleotide variants, structural variants, and copy number alterations on a quantitative basis with deep sequencing coverage of at least 500× to promote accurate diagnostic classification, molecular subtyping, and determination of germline versus somatic status of identified variants (Supplementary Table 1 [Online Resource 1]) [3]. Among these 33 MB, seven tumors (21%) demonstrated hotspot missense mutations in the *CTNNB1* gene known to cause activation of the WNT signaling pathway. Each of these *CTNNB1* mutations were present at allele frequencies near 50%, suggesting that they were clonal variants present in all tumor cells (Supplementary Table 2 [Online Resource 1]). These seven patients (5 female and 2 male) with WNT-activated MB ranged in age from 7 to 29 years (Fig. 1a). Six of the tumors had midline locations involving the cerebellar vermis/fourth ventricle and demonstrated classic histology, while one tumor had lateral location in the cerebellar hemisphere and demonstrated extensive desmoplastic histology without any nodules of neurocytic differentiation seen (Supplementary Figs. 1–3 [Online Resource 2]). Diffuse anaplasia or large cell histologic features were not seen in any of the cases. Four of the seven tumors demonstrated monosomy 6, while one tumor with monosomy 6 (SF-MB-WNT-7) also harbored the combination of 17p loss and 17q gain consistent with isochromosome 17q (Supplementary Table 2 and Supplementary Fig. 4 [Online Resources 1 and 2]), a cytogenetic finding common in Group 3 and Group 4 MB but not in WNT-activated MB [8]. The somatic mutations identified in these seven WNT-activated MB included genes involved in chromatin modulation (e.g., *ARID1A*, *CREBBP*, *KMT2D*, *SMARCA2*, and *SMARCA4*), cell signaling (e.g., *PTEN*), genome maintenance (e.g., *ATM* and *TP53*), and RNA metabolism (e.g., *DDX3X*), all of which are known to be recurrently mutated in WNT-activated MB (Fig. 1a and Supplementary Table 3 [Online Resource 1]). None of the seven patients harbored pathogenic germline alterations in known MB predisposition genes including *APC*, *TP53*, *PTCH1*, *SUFU*, *BRCA2*, or *PALB2*.

Three of these seven WNT-activated MB demonstrated somatic mutations predicted to cause activation of the SHH pathway (Fig. 1b). The radiographic and histologic features of these three tumors are shown in Supplementary Figs. 1–3 (Online Resource 2). Patient #1 is a 7-year-old girl, whose MB located in the midline/fourth ventricle exhibited an activating *CTNNB1* mutation at clonal allele frequency, monosomy 6, a subclonal nonsense mutation in *SUFU*, and a subclonal frameshift mutation in the *PTEN* tumor suppressor gene. Patient #2 is a 22-year-old woman, whose MB located in the midline/fourth ventricle exhibited an activating *CTNNB1* mutation at clonal allele frequency, monosomy 6, two mutations in the *PTCH1* tumor suppressor gene that were both present at clonal allele frequencies (one frameshift and one missense that were too distant to phase), and a subclonal missense mutation in the *TP53* tumor suppressor gene. Patient #3 is a 29-year-old man, whose MB located in the cerebellar hemisphere exhibited an activating *CTNNB1* mutation at clonal allele frequency and two missense mutations in the *SMO* oncogene that were both present at subclonal allele frequencies, but were too distant to phase or determine whether they affected the same or separate subclones. One of the two *SMO* mutations (p.S278N) localizes within the Frizzled domain at a codon that is recurrently mutated in SHH-activated medulloblastomas and basal cell carcinomas. The other mutation (p.V210M) localizes just N-terminal to the Frizzled domain and has been found as a confirmed somatic mutation in a couple of lung carcinomas [COSMIC database v83 release]. Also seen in this patient's MB were subclonal mutations in *CREBBP* and *HRAS*. Immunohistochemistry for GAB1 protein, a marker of SHH pathway activation, revealed focal staining in each of the tumors with multiple discrete clusters of positive

tumor cells (Fig. 1c and Supplementary Figs. 1–3 [Online Resource 2]). Sanger sequencing confirmed the presence of the dual *CTNNB1* and *SUFU*, *PTCH1*, or *SMO* mutations in these three tumors (Supplementary Fig. 5 [Online Resource 2]). No subclonal WNT or SHH pathway genetic alterations were identified any of the 16 Group 3 or Group 4 MB in this cohort, nor were there subclonal WNT pathway genetic alterations identified in any of the 10 SHH-activated MB.

Genome-wide DNA methylation profiling was performed using the Illumina MethylationEPIC BeadChip (850k array) to further characterize these three MB with apparent dual WNT and SHH pathway activation. Unsupervised clustering of DNA methylation patterns alongside 367 previously characterized MB revealed that each of the three tumors closely clustered with WNT-activated MB (Fig. 1d) [6]. These data support the conclusion that these three tumors originated as WNT-activated MB and subsequently acquired secondary SHH pathway activation, in keeping with the clonal allele frequency of the *CTNNB1* mutation and subclonal *SUFU* or *SMO* mutations in two of the cases, while the third case (patient #2) had an uncertain genetic origin with *CTNNB1* and *PTCH1* mutations that were both present at clonal allele frequencies. These data further support that the epigenetic state of a MB is dictated at the time of tumor initiation and remains stable despite the acquisition of additional genetic alterations during tumor progression [5].

To better assess the frequency of secondary SHH pathway activation in WNT-activated MB, we re-evaluated sequencing data from the cohort of 36 WNT-activated MB included in a recent large international MB genomics study [6]. Among these 36 MB belonging to the WNT subtype as defined by genome-wide methylation profiling, three cases (8%) demonstrated clonal *CTNNB1* hotspot mutations and secondary subclonal somatic mutations predicted to activate the SHH pathway (Supplementary Tables 4 and 5 [Online Resource 1]). Of note, however, is that the mean sequencing coverage of these 36 tumors was only 57×, suggesting the possibility that more cases could potentially harbor subclonal mutations that were beyond the detection limits of this low sequencing coverage.

The prognostic and therapeutic significance of secondary SHH pathway activation in WNT-activated MB is uncertain at present. While WNT-activated MB are typically associated with favorable prognosis, the presence of SHH pathway activation is associated with standard risk/intermediate prognosis (except for poor prognosis for cases with concurrent *TP53* mutation) and therefore, the presence of both might portend a different biology. In addition, these patients may be considered for targeted therapy using smoothened inhibitors (if the alteration in the SHH pathway is involving either *PTCH1* or *SMO*), but with the understanding that only a subset of the tumor cells may be inhibited for cases with subclonal mutations in these genes. All three of the patients with dual WNT/SHH-activated MB in this cohort underwent gross total resection followed by craniospinal radiation and chemotherapy with cisplatin, vincristine, and cyclophosphamide as per Children's Oncology Group protocol ACNS0332. While patient #2 died 12 months following resection due to complications of chemotherapy (refractory neutropenia, sepsis, and multi-organ failure with no evidence of tumor recurrence at autopsy), patients #1 and #3 remain recurrence-free at 16 and 11 months following resection, respectively.

The findings in this cohort of WNT-activated MB suggest a potential benefit of targeted deep sequencing for molecular subtyping of medulloblastomas over other methods, as it yields information about both the primary clonal and secondary subclonal genetic alterations acquired during tumor progression, including occasional tumors with dual WNT and SHH pathway activation.

Acknowledgements J.B.I. is supported by the Arthur Purdy Stout Stipend Award. B.C.B. is supported by an NCI Outstanding Investigator Award (R35 CA220481). D.A.S. is supported by NIH Director's Early Independence Award (DP5 OD021403).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests related to this report.

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