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ORIGINAL REPORT

Genomics Identifies Medulloblastoma Subgroups That Are Enriched for Specific Genetic Alterations

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ABSTRACT

Purpose
Traditional genetic approaches to identify gene mutations in cancer are expensive and laborious. Nonetheless, if we are to avoid rejecting effective molecular targeted therapies, we must test these drugs in patients whose tumors harbor mutations in the drug target. We hypothesized that gene expression profiling might be a more rapid and cost-effective method of identifying tumors that contain specific genetic abnormalities.

Materials and Methods
Gene expression profiles of 46 samples of medulloblastoma were generated using the U133av2 Affymetrix oligonucleotide array and validated using real-time reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry. Genetic abnormalities were confirmed using fluorescence in situ hybridization (FISH) and direct sequencing.

Results
Unsupervised analysis of gene expression profiles partitioned medulloblastomas into five distinct subgroups (subgroups A to E). Gene expression signatures that distinguished these subgroups predicted the presence of key molecular alterations that we subsequently confirmed by gene sequence analysis and FISH. Subgroup-specific abnormalities included mutations in the Wingless (WNT) pathway and deletion of chromosome 6 (subgroup B) and mutations in the Sonic Hedgehog (SHH) pathway (subgroup D). Real-time RT-PCR analysis of gene expression profiles was then used to predict accurately the presence of mutations in the WNT and SHH pathways in a separate group of 31 medulloblastomas.

Conclusion
Genome-wide expression profiles can partition large tumor cohorts into subgroups that are enriched for specific genetic alterations. This approach may assist ultimately in the selection of patients for future clinical trials of molecular targeted therapies.

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INTRODUCTION

Gene mutations are promising targets for new anticancer therapies.[1-3] However, so far, only a handful of genetic abnormalities have been targeted successfully in the clinic. If we are to extend these early successes to all cancers, we must identify additional targets and the patient populations in whom these drugs are likely to be most effective. Clinical experience in the use of molecular targeted therapies is at an early stage; nevertheless, available data suggest that these drugs are more effective against tumors that contain mutant rather than wild-type proteins. For example, gefitinib, an inhibitor of the epidermal growth factor receptor (EGFR), is highly effective against non–small-cell lung cancers containing mutations in the kinase domain of *EGFR*, but it is relatively ineffective against tumors expressing the wild-type protein.[2,4] Thus, to avoid rejecting effective new treatments of cancer, molecular targeted therapies must be tested in patients whose tumors harbor mutations in the corresponding drug target. Traditionally, mutations have been identified in cancer by direct gene sequence analysis; however, this technique is likely to prove too time consuming and laborious for selecting patients for trials of molecular targeted therapies. Thus, the oncology community must develop and validate alternative techniques that can rapidly and accurately detect mutations in tumor samples.

As well as developing tools to detect known molecular drug targets, we must also increase the rate at which we discover new mutations. The Human Cancer Genome Project has suggested that new

cancer-causing mutations could be identified in tumors by analyzing the coding sequences of groups of genes that are thought to play a role in cancer (eg, protein kinases).[5] However, such broadly applied direct sequencing strategies are laborious, extremely costly (sequencing the genomes of the most common human tumors will cost an estimated $12 billion[6]), and unlikely to uncover new molecular targets with equal efficiency in all cancers. For example, although a study of 340 serine/threonine protein kinases in colorectal carcinomas identified a number of gene mutations,[7] similar studies of 540 kinases in breast cancers,[8] lung cancers,[9] and testicular germ cell tumors[10] have failed to detect somatic mutations that might represent useful drug targets.

Here, by studying a large cohort of medulloblastomas, we demonstrate that genome-wide patterns of gene expression can detect the presence of underlying genetic alterations in tumors. Thus, gene expression signatures can provide clinicians with a more expedient tool to triage patients for trials of molecular targeted treatments of cancer.

MATERIALS AND METHODS

Gene Expression Microarray Analysis

With the approval of the St Jude Children's Research Hospital Institutional Review Board (XPD01-092), 77 samples of snap-frozen pediatric (aged < 21 years) primary medulloblastomas were collected from patients at initial surgery before any adjuvant therapy. Samples were subject to central histopathology review. RNA was extracted using the Trizol reagent (Invitrogen Corp, Carlsbad, CA). Gene expression profiles were generated, as described previously,[11] using the U133Av2 array (Affymetrix, Santa Clara, CA) that analyzes the expression level of 18,400 transcripts and variants. Gene expression data were normalized using the MAS5 algorithm and Bioconductor version 1.5. The data were then transformed by taking the logarithm (base 2). Unsupervised hierarchical clustering of expression profiles based on Z scores of 22,212 probes was performed using the Pearson correlation. Probe sets that discriminated the tumor subtypes defined by unsupervised clustering were identified using analysis of variance and *t* statistics.

Real-Time Reverse Transcriptase Polymerase Chain Reaction

Transcript levels were measured in tumors using real-time reverse transcriptase polymerase chain reaction (RT-PCR) and the BioRad iCycler single color real-time PCR detection system (BioRad, Hercules, CA). Primer sequences are listed in Supplementary Table 1. Transcript levels were measured relative to those in control human cDNA (pooled from a variety of human tissues) and normalized to the expression levels of r18S.

Fluorescence In Situ Hybridization and Immunohistochemistry

Dual-color fluorescence in situ hybridization (FISH) and immunohistochemical (IHC) analyses of tumor samples were performed using routine methods. Primary antibodies used were directed against β-catenin (Cell Signaling Technology, Danvers, MA), Dickkopf (DKK) 1, DKK2, SFRP1 (all R&D Systems, Minneapolis, MN), and GLI1 (Rockwell Scientific, Thousand Oaks, CA). All FISH probes were validated on normal control metaphase spreads to verify chromosomal localization.

Gene Sequence Analysis

CTNNB1 (exon 3), *APC* (mutation cluster region, base pairs 1255 to 1513), *AXIN1* (exons 1 to 5), *PTCH1* (exons 1 to 23), and *SUFU* (exons 1 to 12) were amplified from tumor DNA by PCR using previously published primers.[12-16] PCR products were directly sequenced in sense and antisense orientation using an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA).

RESULTS

Gene Expression Profiling Identifies Clinically and Histologically Distinct Subgroups of Medulloblastoma

To uncover molecular subgroups of medulloblastoma, we performed unsupervised hierarchical cluster analysis of the gene expression profiles of 46 primary medulloblastomas. Five principal tumor subgroups were identified (Fig 1A). Analysis of variance suggested that clinical and histologic characteristics were not evenly distributed among these subgroups. For example, the medulloblastomas that comprised subgroup B occurred exclusively in patients older than 3 years and were all of the classic histologic subtype. All subgroup B patients were also alive and disease free at the time of analysis, although the limited patient numbers precluded formal survival analysis. In contrast, medulloblastomas within subgroup D occurred mainly in children aged less than 3 years and were predominantly of the desmoplastic histologic subtype.

Gene Expression Signatures of Medulloblastoma Predict Subgroup-Specific Patterns of Chromosomal Alteration and Gene Mutation

Previous studies of medulloblastoma have also linked patterns of gene expression to specific tumor histologies and clinical behaviors, although the molecular basis of these correlations is unknown.[17,18] We reasoned that medulloblastoma subgroups might display distinct clinical characteristics and gene expression signatures because they are caused by distinct sets of genetic alterations. Further, scrutiny of these gene expression signatures might reveal the nature of the underlying genetic alterations in the different tumor subtypes.

As a first step to determine the molecular basis of the five medulloblastoma subgroups, we performed *t* tests to identify the signature genes that most discriminated these subgroups. Each tumor subgroup displayed a unique gene expression signature of between 400 and 800 transcripts that were significantly upregulated or downregulated, at the $P < .0003$ level, when compared with expression levels in the other tumors (see Supplementary Table 2). The gene expression signatures of subgroups B and D were especially robust. In this regard, the top 100 signature genes of subgroup B were differentially expressed in these tumors relative to the other tumors in the study cohort, with an average log ratio of 3.6 with an average significance level of $P = 5.5 \times 10^{-10}$. Similarly, the top 100 signature genes of subgroup D were differentially expressed, with an average log ratio of 2.4 with an average significance of $P = 1.5 \times 10^{-7}$.

Interestingly, significant proportions of the signature genes of subgroup B, C, and D tumors are located in just one or two regions of the genome (Fig 1B). Of particular note, 51% (n = 112) of 221 probe sets reporting downregulated gene expression in subgroup B tumors represent genes that are located on chromosome 6. Because chromosome 6 genes account for only 5.8% (n = 1,306 of 22,212) of all probe sets on the U133av2 expression array, this genomic region is significantly over-represented among the genes that are downregulated in subgroup B tumors ($P < .0001$). Thus, we reasoned that chromosome 6 might be deleted in subgroup B tumors. In contrast, subgroup C medulloblastomas displayed a gene expression pattern predictive of concurrent deletion of 17p and gain of 17q (which is compatible with an isochromosome of 17q, a common chromosome alteration in medulloblastoma[19]), whereas genes on 17q were relatively underexpressed among subgroup D tumors (Fig 1B).

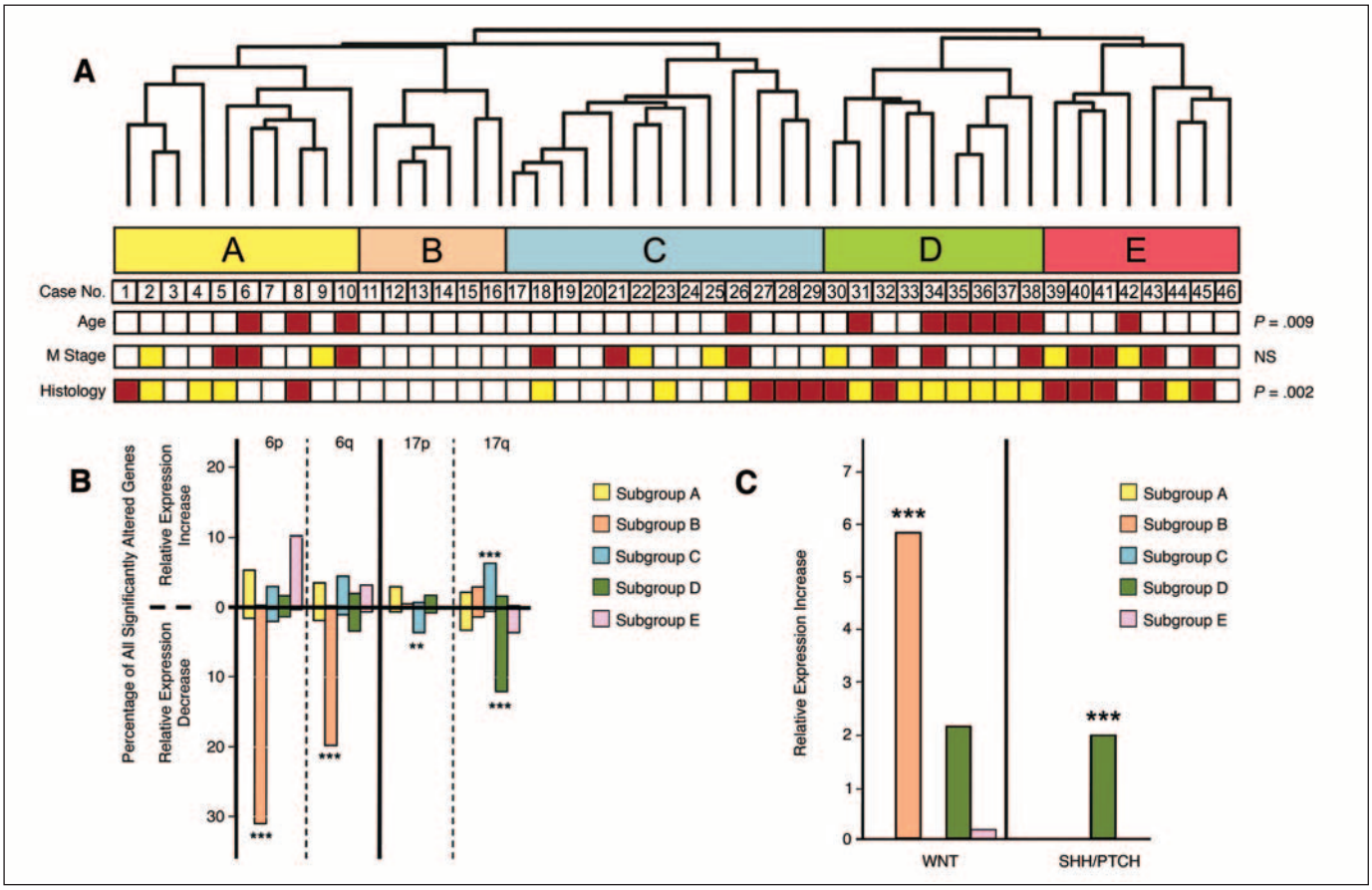

Fig 1. (A) Expression profiling. Age (white box, > 3 years; red box, < 3 years); M stage (white box, M0; yellow box, M1; red box, > M2); histology (white box, classic; yellow box, nodular/desmoplastic; red box, large-cell anaplastic). P[r] values report analysis of variance. Proportion of altered genes (B) on chromosomes 6 or 17 or (C) within the WNT or SHH signal pathways. (***) $P < .0001$; (**) $P < .001$.

We next studied whether aberrant activation of certain cell signal pathways might contribute to medulloblastoma gene expression signatures. To test this, we first reviewed all 33,000 probe sets on the U133av2 expression array and identified 392 that report the expression of genes that are either members or targets of the WNT (n = 240), SHH (n = 29), NOTCH (n = 20), or BMP (n = 103) cell signal pathways (see Supplementary Table 3 for a full list of genes). These four pathways have each been implicated in the development of medulloblastoma.[12,15,20-23] Furthermore, anticancer drugs that target the WNT,[24] SHH,[25] and NOTCH[26] pathways are currently being developed for use in the clinic. Thus, defining subgroups of medulloblastoma in which these pathways are aberrantly active is likely to have the most immediate clinical utility. We found that members or targets of the WNT and SHH pathways were significantly over-represented among upregulated genes in subgroup B and D tumors, respectively (Fig 1C). Gene expression signatures did not yield evidence of either NOTCH or BMP pathway activation among any tumor subgroup.

To confirm whether genes within the WNT and SHH pathways are upregulated in subgroup B and subgroup D medulloblastoma, respectively, we used real-time RT-PCR and IHC analyses to validate the gene expression signatures of these two subgroups. These studies confirmed that WNT pathway members, including *DKK1*, *DKK2*,[27] *DKK4*,[27] *WNT inhibitory factor 1* (*WIF*),[28] *LEF1*, and *GAD1*,[29] are indeed selectively upregulated in subgroup B tumors (Figs 2A and 2B). We also identified intense nuclear expression of β-catenin protein, which is frequently linked with stabilizing mutations in *CTNNB1*,[30] to be an exclusive feature of subgroup B tumors (Fig 2B). Conversely, real-time RT-PCR and IHC confirmed the upregulation of SHH pathway genes among subgroup D tumors (Figs 2A and 2B). These included *ATOH1*, *PTCH2*, *SFRP1*, and *GLI1* (Figs 2A and 2B). Each of these genes has been implicated previously in the development of mouse medulloblastoma in which the SHH pathway is activated.[31] Interestingly, our real-time RT-PCR and IHC analyses suggested that the SHH pathway is also activated in a proportion of subgroup C tumors (nos. 25 to 29; Figs 2A and 2B). These tumors were located predominately on a separate branch of the dendrogram in our initial hierarchical cluster analysis (Fig 1A) and are hereon referred to as subgroup C.

FISH and Gene Sequence Analysis Confirm the Genetic Alterations Predicted by Gene Expression Microarray Analysis

To confirm whether gene expression profiling predicted accurately the presence of chromosome alterations in subgroup B, C, and D tumors, we next performed FISH analysis of chromosome 6p22.1 (*CDKAL1*), 6q24 (*RAB32*), 17p13.3 (*DPH1*), and 17q23.2 (*RPS6KB1*). In agreement with our gene expression array data, we detected monosomy of chromosome 6 in four of five tested subgroup

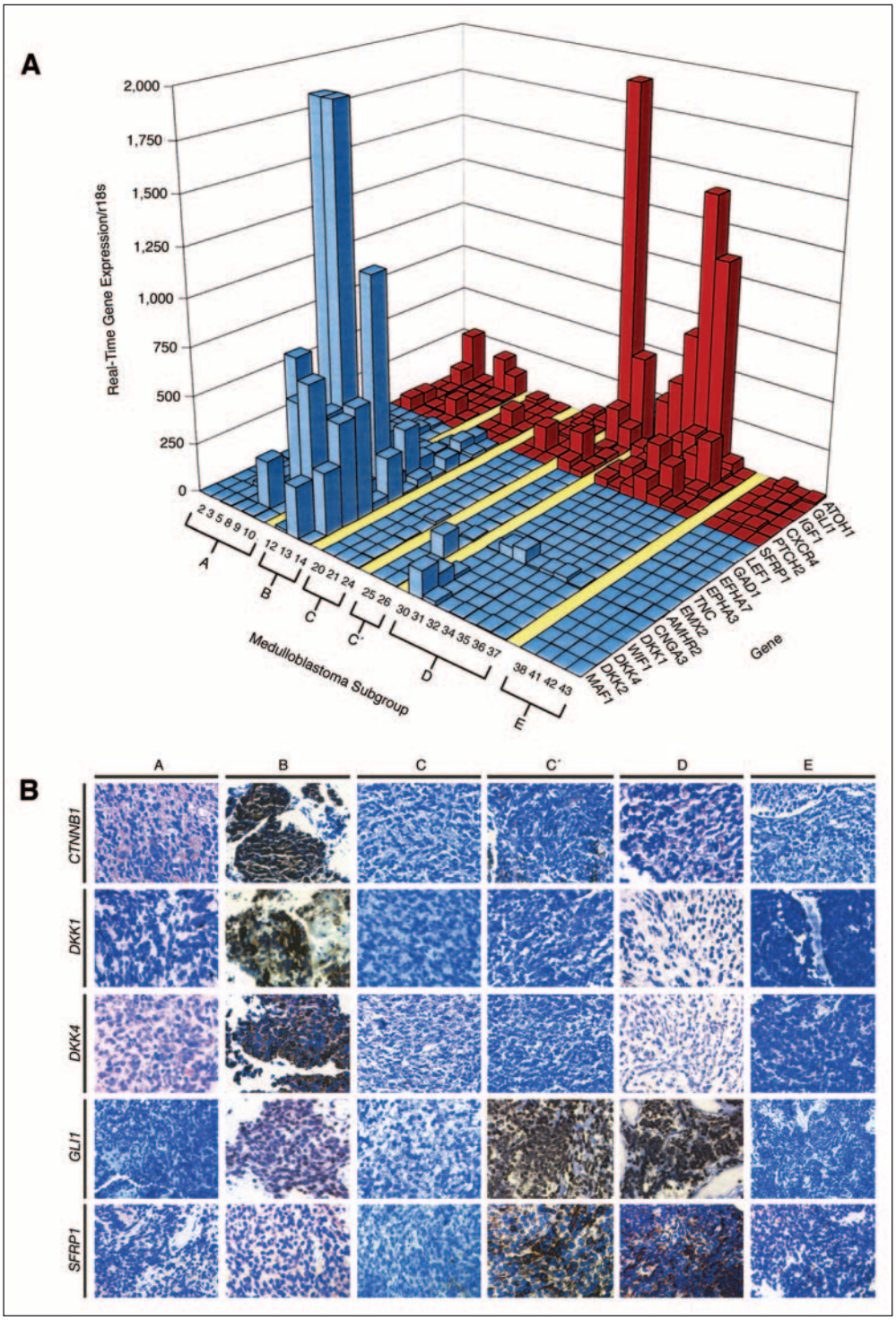

Fig 2. (A) Real-time reverse-transcriptase polymerase chain reaction and (B) immunohistochemical analyses of the expression of WNT and SHH pathway member genes among tumors in the study cohort. (A) WNT and SHH pathway genes are represented by blue and red bars, respectively. Note that C′ tumors share the SHH expression signature of subgroup D (see text).

B tumors but in only two of 36 tested tumors from the other subgroups ($P < .01$; Fig 3). FISH analysis also confirmed the predicted high incidence of chromosome 17 abnormalities among subgroup C tumors (Fig 3). Ten of 11 tested tumors in subgroup C deleted chromosome 17p; in four cases, this occurred in the context of an isochromosome of 17q. In contrast, deletion of chromosome 17p was relatively rare among the other tumor subgroups ($P < .0001$). In contrast to subgroups A, C, and E, no subgroup D tumor gained 17q, providing a potential explanation for the comparatively low expression of genes on chromosome 17q among tumors in this subgroup.

Next, we investigated whether the gene expression signatures of subgroups B, C, and D might also be driven by activating mutations in the WNT and SHH pathways. The majority of mutations that activate the WNT pathway in sporadic medulloblastoma target residues 32 to 34 of *CTNNB1*.[32,33] Other less frequent mutations occur in the mutation cluster region of *APC* and in the APC-binding domain of

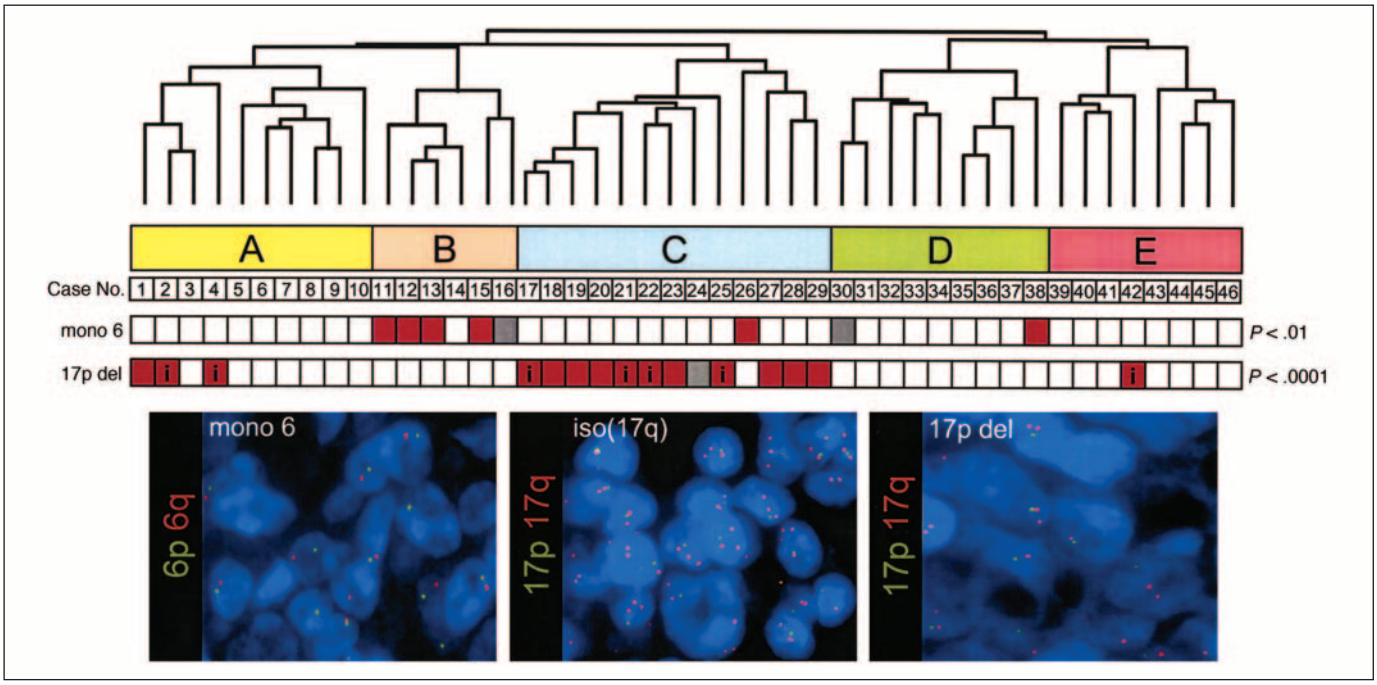

Fig 3. Fluorescence in situ hybridization (FISH) analysis of tumor subgroups A through E. Dendrogram and tumor numbers are the same as in Figure 1A. (red box, monosomy 6 (middle row) or 17p deletion (bottom row); i, denotes 17p deletion in the context of iso(17q); gray box, missing data). Examples of FISH results are shown at the bottom.

AXIN1.[32] Therefore, we sequenced the coding regions of *CTNNB1*, *APC*, and *AXIN1* in all tumor samples in the cohort. We identified activating mutations in residues 32 to 34 of *CTNNB1* in all tested subgroup B tumors but in no other medulloblastoma samples ($P < .0001$; Fig 4). Thus, the WNT pathway gene expression signature displayed by subgroup B tumors is driven by activating mutations in *CTNNB1*. Three tumors outside of subgroup B contained mutations in *APC* (Fig 4). However, these included two nonconservative missense mutations of unknown functional significance (nos. 25 and 30) and one truncating mutation with preservation of the wild-type allele

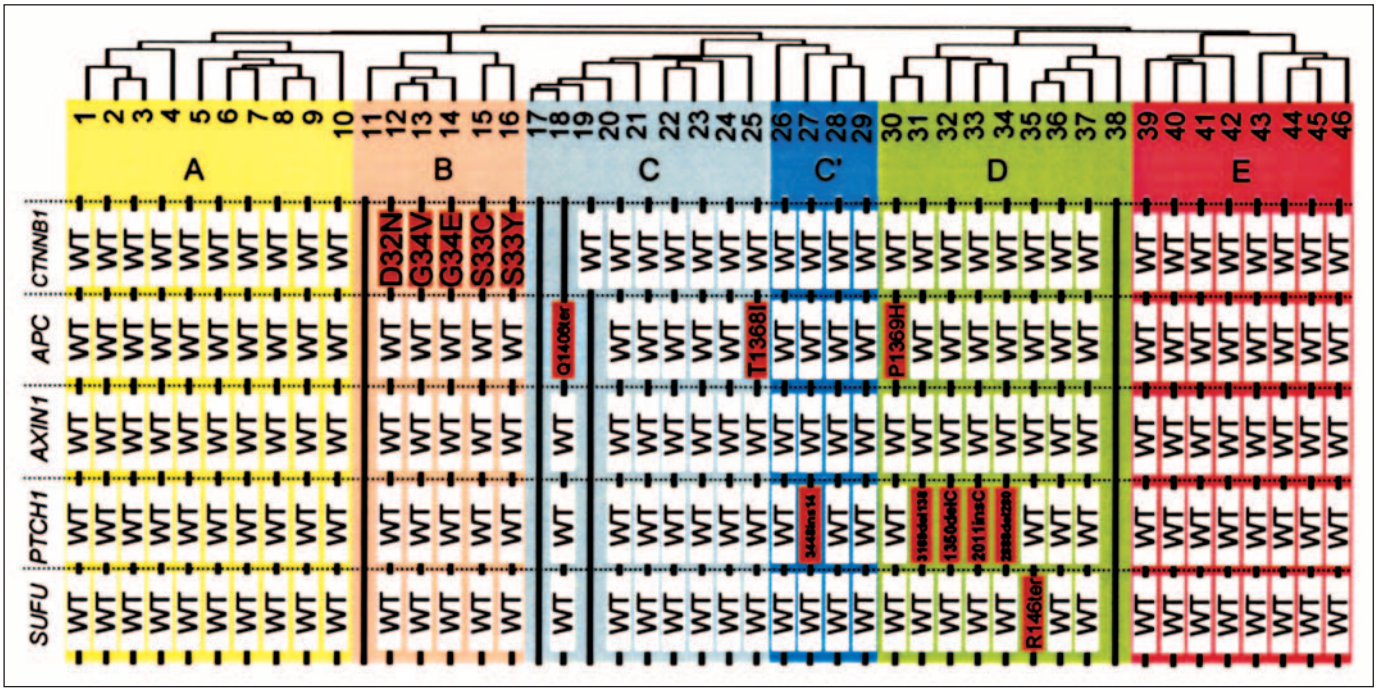

Fig 4. Gene sequence analysis of WNT and SHH pathway genes in tumors in subgroups A through E. Gene names are shown to the left. White boxes denote wild-type (WT) sequence. Red boxes denote mutations and the corresponding mutant sequence. Blank spaces indicate unavailable sequence results.

(no. 18). Thus, it remains unclear whether these *APC* mutations impact the WNT cell signaling pathway. Indeed, these tumors did not share the WNT pathway gene expression signature of subgroup B tumors (Figs 1 and 2A), and they also did not display β-catenin nuclear immunoreactivity or deletion of chromosome 6 (Figs 2B and 3). Thus, these data suggest that *CTNNB1* and *APC* mutations are mutually exclusive and molecularly distinct in medulloblastoma.

Activating mutations in the SHH pathway predominantly target *PTCH* and *SUFU*.[32] Five tumors from subgroups C and D contained frameshift mutations in *PTCH*, and one subgroup D tumor contained a truncating mutation in *SUFU* (Fig 4). In contrast, we did not detect mutations in either *PTCH* or *SUFU* in any other tested tumors ($P = .001$). Thus, the SHH pathway gene expression signature of most subgroup C and D tumors is driven by inactivating mutations in *PTCH* and *SUFU*. Six of 12 tested subgroup C and D tumors did not contain detectable mutations in either *PTCH* or *SUFU* (Fig 4). Thus, alternative mechanisms, including mutations in *SMO*,[34] are likely to activate SHH signaling in these tumors.

Real-Time RT-PCR Analysis Rapidly Identifies Medulloblastomas With Mutations in the WNT and SHH Pathways

We have identified gene expression signatures that correlate closely with the presence of activating mutations in either the WNT or SHH cell signal pathways in medulloblastoma. Therefore, we reasoned that these gene expression signatures might provide a rapid and accurate means of identifying additional tumors that contain underlying mutations in these pathways. To test this, we used real-time RT-PCR to measure the expression levels of WNT and SHH pathway signature genes among a separate and nonoverlapping cohort of 31 primary medulloblastomas. We identified two tumors that displayed clear evidence of WNT pathway activation (data not shown). Subsequent gene sequence analysis identified activating mutations in *CTNNB1* (D32Y and S33Y) in both of these tumors. An additional tumor was identified that expressed the SHH pathway signature (data not shown); this tumor contained a truncating mutation in *SUFU*. Thus, real-time RT-PCR analysis provides a rapid and accurate method of selecting medulloblastomas that contain activating mutations in the WNT and SHH pathways.

DISCUSSION

Despite an aggressive combination of surgery, radiation, and chemotherapy, medulloblastoma remains incurable in a significant proportion of patients.[35-37] Thus, similar to most human cancers, there is a great need to identify alternative therapeutic approaches to treat medulloblastoma. Small-molecule inhibitors that target cell signaling pathways that are activated aberrantly in medulloblastoma could have significant utility in the future management of this disease.[32] For example, SHHAntag, a drug that binds to and inhibits SMOOTHENED, has shown dramatic activity in *Ptch1*+/− mouse models of medulloblastoma.[25] Although molecular targeted therapies are promising agents for the future treatment of cancer, their successful translation into routine clinical practice will depend on our ability to test these drugs in appropriate patients. Our study of medulloblastoma demonstrates that gene mutations in cell signaling pathways are associated tightly with specific gene expression signatures. We further show that these gene expression signatures can be used to identify patient subgroups that are enriched for the presence of underlying gene mutations. Such mutation-specific gene expression signatures could prove extremely useful in the selection of patients for clinical trials of molecular targeted therapies. In this regard, patient tumor samples could be rapidly screened for mutation-specific gene expression signatures using relatively simple and widely available techniques such as real-time RT-PCR or IHC. Gene sequencing efforts could then be focused to confirm the presence of the underlying mutation in tumors in these patients before their enrollment onto a clinical trial of an appropriate molecular targeted therapy.

As well as predicting known gene mutations, we provide proof of principle that expression profiling can guide more focused gene sequencing and chromosome mapping (eg, FISH) efforts to identify unknown genetic alterations in cancer. In the current study, we annotated the U133av2 Affymetrix microarray to hunt for evidence of aberrant signaling within the WNT, SHH, BMP, and NOTCH pathways. Furthermore, because expression array analysis profiles the whole genome, we were able to detect previously unidentified concurrent genetic alterations in medulloblastoma (eg, mutation in *CTNNB1* and monosomy 6). Clearly, this approach could be used to screen other types of cancer for mutations in cell signaling pathways or alterations in specific chromosomal regions. The success of this approach will depend on the identification of genes that report the activity of specific signal pathways with a high degree of sensitivity. Such signature genes might by identified by expression profile analysis of cancer cell cultures, xenografts, and fresh tumor samples in which the activation and mutation status of the appropriate cell signal system is known. This approach provides an alternative and potentially more cost-effective method to identify new mutations in cancer than the unbiased genome-wide sequencing approaches recently proposed by the Human Cancer Genome Project.[5,6,8-10]

Finally, our data provide important new insight into the biology of medulloblastoma. In particular, they demonstrate, for the first time, that activating mutations within the WNT and SHH pathways affect distinct subsets of medulloblastoma. Thus, drugs that target the WNT and SHH pathways are likely to be effective in distinct populations of patients with medulloblastoma. These data underline further the need for a test that can distinguish these tumor subtypes. The finding that activating mutations in *CTNNB1* are associated significantly with monosomy of chromosome 6 is a further interesting new finding in the current study. Although monosomy chromosome 6 has been reported previously in a variety of tumor types including medulloblastoma,[38,39] to our knowledge, the relationship of this alteration with activating mutations in *CTNNB1* has not been reported. We are currently conducting high-density single nucleotide polymorphism mapping array studies to define further the region(s) of chromosome 6 that might harbor genes that suppress the development of *CTNNB1* mutant medulloblastoma.

Our data demonstrate that genome-wide expression profiles can partition large tumor cohorts into subgroups that are enriched for specific mutations. Furthermore, mutation-specific gene expression signatures that are identified from expression profiles provide a rapid, accurate, and inexpensive tool to select appropriate patients for clinical trials of molecular targeted therapies.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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