A Five-Gene Hedgehog Signature Developed as a Patient Preselection Tool for Hedgehog Inhibitor Therapy in Medulloblastoma

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Abstract

**Purpose:** Distinct molecular subgroups of medulloblastoma, including hedgehog (Hh) pathway–activated disease, have been reported. We identified and clinically validated a five-gene Hh signature assay that can be used to preselect patients with Hh pathway–activated medulloblastoma.

**Experimental Design:** Gene characteristics of the Hh medulloblastoma subgroup were identified through published bioinformatic analyses. Thirty-two genes shown to be differentially expressed in fresh-frozen and formalin-fixed paraffin-embedded tumor samples and reproducibly analyzed by RT-PCR were measured in matched samples. These data formed the basis for building a multi-gene logistic regression model derived through elastic net methods from which the five-gene Hh signature emerged after multiple iterations. On the basis of signature gene expression levels, the model computed a propensity score to determine Hh activation using a threshold set a priori. The association between Hh activation status and tumor response to the Hh pathway inhibitor sonidegib (1DE225) was analyzed.

**Results:** Five differentially expressed genes in medulloblastoma (GLI1, SFRH1, SHROOM2, PDLIM3, and OTX2) were found to associate with Hh pathway activation status. In an independent validation study, Hh activation status of 25 medulloblastoma samples showed 100% concordance between the five-gene signature and Affymetrix profiling. Further, in medulloblastoma samples from 50 patients treated with sonidegib, all 6 patients who responded were found to have Hh-activated tumors. Three patients with Hh-activated tumors had stable or progressive disease. No patients with Hh-nonactivated tumors responded.

**Conclusions:** This five-gene Hh signature can robustly identify Hh-activated medulloblastoma and may be used to preselect patients who might benefit from sonidegib treatment. *Clin Cancer Res;* 21(3); 585–93. ©2014 AACR.

Introduction

Medulloblastoma, a malignant primary neuroectodermal tumor arising in the cerebellum, is the most common brain tumor in children ages 3–4 years (1). The current standard of care involves surgery followed by craniospinal radiation and chemotherapy, which can be given concurrently with or following radiation (2). Not infrequently, young children treated with chemotherapy alone will respond to second-line salvage therapy, including radiation; however, there is no standard, effective salvage treatment for recurrence following craniospinal radiation and the prognosis following recurrence is dismal (3). Moreover, especially for young children, radiation can lead to long-term toxicities, including neurocognitive damage (2). Therefore, targeted therapies, with improved efficacy and reduced toxicity, are greatly needed for young children and for patients with relapsed disease.

Currently, there is no gold-standard method for the identification of molecular subtypes of medulloblastoma. Recently...
Translational Relevance

A significant proportion of patients with medulloblastoma experience relapse after primary treatment. Because of the lack of standard salvage regimens and long-term toxicities associated with available therapies, especially for younger patients, novel targeted therapies are greatly needed for patients with relapsed disease. Gene expression profiling studies have identified 4 molecular subgroups of medulloblastoma, including one characterized by activated hedgehog (Hh) signaling. Inhibitors targeting the Hh pathway, including sonidegib (LDE225), have demonstrated activity in patients with medulloblastoma. An RT-PCR-based five-gene signature assay has been developed to identify patients with Hh pathway–activated medulloblastoma and is associated with response to sonidegib treatment. Given the potential for drug-induced premature growth plate closure in children who have not achieved skeletal maturity, the ability to preselect patients who are most likely to obtain clinical benefit is a valuable advance in the development of Hh inhibitor therapy.

however, several studies have identified distinct molecular subgroups of medulloblastoma through gene expression profiling (4–7). Four subtypes, wingless (WNT), sonic hedgehog (SHH), group 3, and group 4, with distinct gene expression profiles and molecular abnormalities, have been described (5–8). Mutations that activate the hedgehog (Hh) pathway have been identified and found exclusively in the SHH subclass, which constitutes approximately one third of medulloblastomas (4, 6, 7, 9, 10); however, medulloblastomas can be classified within the SHH subclass in the absence of Hh pathway mutations (4–7, 11).

Several small-molecule inhibitors of smoothened (SMO), the G protein–coupled receptor–like transducer of Hh signaling, are being explored as novel, targeted therapies designed to treat cancers associated with aberrant Hh signaling (12). Sonidegib (LDE225) is a potent and selective SMO inhibitor that has demonstrated dose-dependent tumor regression in patched−/− (Ptch−/−) p53−/−, and Ptch−/− hypermethylated in cancer 1(Hic1−/−) mouse medulloblastoma models (13, 14). In recent phase I studies testing single-agent sonidegib in adult and pediatric patients with advanced solid tumors, antitumor activity was demonstrated in several patients with medulloblastoma (15, 16).

Genetic analyses of medulloblastomas have identified mutations in several Hh pathway genes; however, due to the low incidence of medulloblastoma, reports of recurrent mutations are limited (4–7). In addition, because few of these are hot-spot mutations, targeted genotyping cannot be used and mutational screening of large Hh pathway genes such as PTCH1 and SMO would be required (17). Furthermore, several studies have demonstrated that in medulloblastoma, the incidence of Hh pathway activation is more prevalent than Hh pathway mutations (4–7, 11).

For these reasons, preselection of patients by direct gene sequence analysis is not suitable for use in clinical studies, especially considering the importance of a short turnaround time to determine patient eligibility. Recent genomic-based molecular subclassification of medulloblastoma suggests that identification of an Hh gene signature through gene expression profiling could be used for treatment decisions (18). However, the current expression profiling methods require a large quantity of tumor and are time intensive, both of which are not amenable for translation to the clinic (18). In addition, these methodologies do not work efficiently with the formalin-fixed and paraffin-embedded (FFPE) tumor specimens that are widely available in clinical practice. In this study, we developed a five-gene Hh signature assay as a clinically applicable tool for preselecting patients with Hh-activated medulloblastoma who are most likely to derive benefit from Hh inhibitor therapy.

Patients and Methods

Patient samples

Medulloblastoma tumor specimens were obtained from the Dana-Farber Cancer Institute/Boston Children’s Hospital (Boston, MA; see Supplementary Methods for additional information; n = 40) and Children’s Healthcare of Atlanta (CHOA; Atlanta, GA; n = 25).

Medulloblastoma samples (n = 50) were also obtained from patients enrolled in three separate phase I studies of oral sonidegib in patients with advanced solid tumors (Supplementary Table S1; refs. 19–21). The protocols and amendments of each study were approved by the institutional review board, independent ethics committee, or research ethics board at each center. Medulloblastoma and normal cerebral tissue samples used for assay validation were obtained from commercial vendors, Asterand (n = 1, medulloblastoma), and Biochain (n = 1, medulloblastoma; n = 3, normal cerebral tissue).

Candidate gene selection

For selection of the initial list of candidate genes for further development of the signature, gene expression data from three independent external studies were reanalyzed. The first dataset from Thompson and colleagues (4) included data obtained by the Affymetrix HG-U133A microarray chip from 46 patients (ages < 21 years) with medulloblastoma tumors. Nine of the samples were defined as group D with target genes of the SHH pathway significantly overrepresented among upregulated genes in their profiles. The second dataset from Kool and colleagues (5) included 62 medulloblastoma tumor samples profiled on the Affymetrix U133 Plus 2.0 chip. The 62 samples were classified into five subgroups, with subgroup B comprising 15 cases defined mostly by SHH-activated pathway genes. The third dataset, reported by Cho and colleagues (7), included 194 medulloblastoma tumor samples profiled on the Affymetrix HT-U133A. Six stable molecular subgroups of medulloblastoma were classified (c1 to c6), including the c3 subgroup that showed enrichment of gene sets associated with SHH signaling. Data from five, 20, and 11 normal cerebellum samples (profiled on Affymetrix U133A, Affymetrix U133 Plus 2.0, and HT-133A, respectively) obtained from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) database were combined with the Thompson, Kool, and Cho datasets, respectively, to enhance verification of the clustering below (4, 5, 7).

Data normalization and unbiased filtering. Normalization was performed independently for the three separate datasets using the robust multiarray averaging normalization algorithm in R Bioconductor package (http://www.r-project.org/).
Without regard to Hh activation status, the datasets were reduced by filtering out probe sets that showed variability and \( \log_2 \) expression intensities below a defined threshold in all of the samples for that specific dataset [Thompson dataset coefficient of variation (CV) < 5% and \( \log_2 \) expression < 4; Kool dataset \( \text{CV} < 5\% \) and \( \log_2 \) expression intensity < 5; Cho dataset \( \text{CV} < 5\% \) and \( \log_2 \) expression < 5; refs. 4, 5, 7]. Distinct thresholds were used for each of the three datasets to account for the inherently different background noise caused by the use of different versions of the Affymetrix platform in different laboratories.

**Candidate gene selection.** Two parallel methods to determine candidate genes were initiated and later compared for consistency. One approach assessed each dataset independently and for each, collapsed the clusters defined previously into Hh active or Hh inactive. A univariate logistic regression analysis approach was employed on each dataset to identify genes that were potentially predictive of Hh activation status. The probe sets were then ranked by their significance and area under the receiver operating characteristic curve, and compared for consistency across datasets.

The second approach combined all 3 datasets, necessitating the use of distinct hierarchical clustering (performed using the Pearson dissimilarity distance metric and complete linkage; ref. 4). Distinct clusters were selected to define samples as Hh active (\( n = 63 \)) or Hh inactive (\( n = 236 \)). Samples that clustered less distinctly with either subgroup (\( n = 3 \)) were excluded from further analysis. The resulting dataset, with gene expression intensity for 10,995 probe sets, was used to run both a parametric \( t \) test and Wilcoxon rank-sum test to compare the two classes.

**Identification of candidate genes and control genes.** Candidate genes that showed at least a 2-fold difference in mean expression levels between the Hh-active and Hh-inactive groups were identified. Initially, a false discovery rate \( P \) value of 0.01 was employed. This rate, however, resulted in a large pool of candidate genes, leading to a more complex selection strategy, in which statistical significance \( (P < 0.00001 \) and average area under the curve \( > 95\% \)) in at least two of the three independent logistic regression fits and significance \( (P < 0.001) \) by Wilcoxon rank-sum or parametric \( t \) test were required for selection. In some rare instances, a candidate gene was selected if it showed high predictivity in a single experiment because different versions of the Affymetrix chip that were used contained their own unique gene candidates. Considering the potential for bias, the use of \( P \) values should be interpreted as a statistical filtering mechanism for identifying a reasonable number of potential candidate genes for the next stage of the analysis. Thirty-two genes were identified, including 21 genes upregulated and 11 genes downregulated in Hh pathway–activated samples.

Candidate control genes that were expressed nondifferentially across all subclasses of medulloblastoma tumors were also selected from the normalized dataset of 307 fresh-frozen medulloblastoma samples from three published sources (4, 5, 7). Criteria for selection included low variability in expression across all three medulloblastoma datasets (CV < 4%) and a target gene–specific Affymetrix probe set with robust expression in medulloblastoma tumors (\( \log_2 > 8 \)). Twenty-two potential control genes were selected that fit these criteria. Zona pellucida glycoprotein 2, identified as distinctly upregulated in the set of 25 normal cerebellum samples, was used to analyze the nontumor contamination present in the FFPE samples and was therefore included on the TaqMan low-density array (TLDA) array (data not shown).

**Assay design and optimization for FFPE**

TaqMan-based RT-PCR assays (Applied Biosystems Inc) designed with small amplicon size, and targeted at different regions of the gene, were screened for each of the 54 candidate genes (32 differentially expressed genes and 22 nondifferentially expressed or control genes) to assess their feasibility, utility, and robustness in FFPE samples. On average, three assays per gene were evaluated using a titration of complementary DNA (cDNA) from three FFPE tissue specimens (one Hh+ medulloblastoma, one Hh– medulloblastoma, and one normal cerebellum). Eighteen of 32 differentially expressed candidate genes and four of 22 candidate control genes that had at least one assay that showed robust expression (raw cycle threshold value \( < 30 \) using 50 ng of cDNA input) in at least one of the three FFPE samples and high assay efficiency across all three samples (90% to 110%) were selected. A custom TLDA (Applied Biosystems) was built using these 22 selected genes to further the signature identification process.

**RNA extraction, RT-PCR, and gene expression profiling**

RNA was extracted from FFPE sections using the QiagenRNeasy FFPE extraction Kit (Qiagen) and reverse transcribed to cDNA using random hexamers and a high-capacity cDNA archive kit (Applied Biosystems). Real-time PCR on the TLDA array was performed using the PRISM 7900HT sequence detection system (Applied Biosystems) with a universal human reference RNA (740000; Agilent Technologies, Inc) included as a technical control in every array. The delta cycle threshold (\( Ct \)) method was used to compute the expression levels of individual genes after data normalization as described below.

RNA was extracted from 25 frozen medulloblastoma tumor samples using the Trizol protocol (15596-018; Invitrogen) and was profiled by AROS Biosciences on the Affymetrix human genome U133 Plus 2.0 array with the 3’ IVT express labeling Kit (Affymetrix).

**Data normalization.** The 18 differentially expressed genes and high assay efficiency in FFPE samples were normalized using the average expression of four control genes (HECT, UBA, and WWE domain containing 1; YME1-like 1; superoxide dismutase 1; and La ribonucleoprotein domain family member 1). The common control gene glyceraldehyde-3-phosphate dehydrogenase showed high variability across the sample sets and therefore was not used in the analysis (data not shown). With the delta \( Ct \) method, the raw expression level of each candidate gene was subtracted from the average of the four control genes. Following normalization, the 18 differentially expressed genes formed the basis of the model-building exercise described below.

**Computational methods/model building**

**Model building.** The elastic net is a regularized regression method that uses a weighted sum of the L1 and L2 penalty terms used in the lasso (22) and ridge (23) regression models, respectively. This method was selected due to the potential for correlation among the genes. In practice, the ridge portion of the penalty tended to group correlated genes together, whereas
the lasso portion of the penalty then included or excluded these groups as a set in the final model. This algorithm can be used to calculate optimal values for α and λ, which control the weight applied to each of the penalty terms and the amount of shrinkage exerted on the data, respectively. For optimization of the model, a series of 100 αs and 91 λs were generated. For each α and λ pair, a model was fit in a 5-fold cross-validation framework, resulting in an estimate of deviance or model error, the lowest of which was deemed optimal. In addition to model error, the optimal model was also defined by the exact number and identity of genes, as well as model coefficients.

Optimal model selection. To account for potential cross-validation error, the cross-validation procedure was iterated 10,000 times. Eight- and five-gene models, defined by selection of α and λ, were selected in approximately 80% of the iterations. In each case, the same eight genes [glioma-associated oncogene homolog 1 (GLI1), orthodontic homebox 2 (OTX2), shroom family member 2 (SHROOM2), PDZ and LIM domain 3 (PDLM3), sphingosine kinase 1 (SPHK1), secreted frizzled-related protein 1, amyloid β A4 precursor protein-binding family A member 2, and spermatogenesis-associated 20; Table 1], or a subset of five genes (GLI1, OTX2, SHROOM2, PDLM3, and SPHK1) were selected, with slight variations in the precise model coefficients. For each model size, the most frequently selected α and λ pair was selected as the optimal model and used for further evaluation in the independent validation dataset. Ultimately, the five-gene model was selected for further development.

Model thresholds. The elastic net method generates a multigene logistic regression model, which produces a propensity score in the range of 0 to 1 that estimates the probability of being Hh activated for a given sample. A threshold was then selected to separate Hh-activated and non-Hh-activated classes. During the model generation/discovery phase of this exercise, the separation between tumors categorized as Hh activated versus those categorized as Hh nonactivated was relatively large; thus, any threshold selected within that range was indistinguishable in terms of predictive performance. A threshold of 0.500 was selected as the score threshold because it partitions the range of scores into equal parts and may also be interpreted as the least biased choice. With the threshold set at 0.500, a value of ≥0.500 was classified as Hh activated, whereas a value of <0.500 was classified as non-Hh activated.

### Results

Development of the five-gene Hh signature model

Briefly, a panel of 32 candidate genes differentially expressed in Hh+ versus Hh− tumors and another 22 potential normalization genes were selected from data derived from 313 medulloblastomas in three independently published profiling studies (4, 5, 7). RT-PCR assays for these candidate genes were developed and optimized for use in FFPE specimens. Assays with robust performance for 18 differentially expressed genes and four control genes were further selected. This panel of genes was assayed in the 40 FFPE medulloblastoma specimens with established Hh activation status (7) and subjected to model building by the elastic net method for signature identification. FFPE medulloblastoma tumor samples were obtained from the Dana-Farber Cancer Institute/Boston Children’s Hospital. The 40 matching fresh-frozen samples were a subset of the 194 MB samples previously profiled (7).

Figure 1.

Development of the five-gene Hh signature. A panel of 32 candidate genes differentially expressed in Hh+ vs. Hh− tumors and 22 potential normalization genes were selected from a combined dataset of 313 medulloblastoma (MB) samples in three independently published profiling studies (4, 5, 7). Data from normal cerebellum samples obtained from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) database was also included in the analysis. RT-PCR assays for these candidate genes were developed and optimized for use in 5 FFPE specimens (1 Hh+, 1 Hh−, 1 normal cerebellum) obtained from Biochain. Assays with robust performance for 18 differentially expressed genes and four control genes were further selected. This panel of genes was assayed in the 40 FFPE medulloblastoma specimens with established Hh activation status (7) and subjected to model building by the elastic net method for signature identification. FFPE medulloblastoma tumor samples were obtained from the Dana-Farber Cancer Institute/Boston Children’s Hospital. The 40 matching fresh-frozen samples were a subset of the 194 medulloblastoma samples previously profiled (7). The five-gene Hh signature was externally validated in a set of 25 matching FFPE and fresh-frozen medulloblastoma samples obtained from Children’s Healthcare of Atlanta.
performance in FFPE for 18 differentially expressed genes (ten upregulated and eight downregulated in Hh⁺ vs. Hh⁻ tumors) plus four control genes were further selected and assembled onto a TLDA card. The expression of this panel of genes was assayed in the 40 FFPE medulloblastoma specimens and formed the basis of the multigene model–building exercise (Fig. 1). The matching fresh-frozen specimens of these 40 cases were previously profiled as part of a larger group of 194 medulloblastoma fresh-frozen samples as described by Cho and colleagues (7), and each case was classified as Hh⁺ or Hh⁻ based on its Affymetrix gene expression profile.

The 18 differentially regulated candidate genes normalized by the average of the control genes were subjected to a model-building exercise using the elastic net algorithm (24) in a 5-fold cross-validation framework. The elastic net method selects the optimal model that uses the least number of genes to identify Hh activation status with minimal error (Fig. 2; ref. 25). To understand the impact of variation in the cross-validation procedure, the entire model-building exercise was iterated 10,000 times, resulting in a distribution of optimal models. Models with five genes were strongly represented in this distribution. The most frequently selected five-gene model was deemed optimal (Fig. 2B). The five-gene signature includes four upregulated genes, GLI1, SPHK1, SHROOM2, and PDLIM3, and one downregulated gene, OTX2.

As described above, the model computes a propensity score of being Hh⁺ (0 to 1) for a given sample based on the expression levels of the five genes. A cutoff of 0.5 was set a priori and used to determine the Hh activation status (Hh⁺ vs. Hh⁻; Fig. 3).

Detailed methods are provided (see Patients and Methods section).

Validation of the five-gene model

The five-gene model was fixed and an analysis plan was written and approved in our validated clinical repository. The model was then validated with a sample set of 25 matched FFPE and fresh-frozen medulloblastoma tumors (CHOA) that were not used during the model-building exercise (Fig. 1). Tissue specimens from 25 patients with a median age of 3 years (range, 0.5–16 years) were collected at diagnosis (n = 24) or following chemotherapy (n = 1). The fresh-frozen samples were analyzed by Affymetrix gene expression profiling, and Hh activation status was determined as previously described (7). The matching FFPE medulloblastoma samples were processed and subjected to gene expression analysis using the custom TLDA. The five-gene model was used to calculate the propensity score and predict Hh activation status for each tumor in this validation set.

Eight patients were classified as Hh⁺ and 17 as Hh⁻ based on Affymetrix profiling. The identification of Hh activation status from the five-gene signature showed 100% agreement with the determination made by Affymetrix profiling. With the five-gene model, the eight tumors identified as Hh⁺ had a median propensity score of 0.879 (range, 0.691–0.976), and the remaining 17 tumor samples were considered to be Hh⁻, with a median propensity score of 0.007 (range, 0.001–0.03; Fig. 3). The considerable difference in propensity scores between the positive and negative cases reflects a robustness of Hh status determination by the five-gene model.
Predictive value of five-gene Hh signature for tumor response

Classification of tumors by the five-gene signature and Affymetrix profiling both rely on measures of gene expression; therefore, a strong agreement between the two methods may be somewhat expected. A more rigorous assessment of the five-gene signature may be determined using patient samples with accompanying data showing clinical response to treatment with the potent Hh pathway inhibitor sonidegib. If the signature is accurate, only those patients who are classified as Hh activated should respond to sonidegib therapy, whereas those who are Hh non-activated should not receive benefit.

To test this hypothesis, the same locked model used in the validation above was then applied to different samples from patients enrolled in clinical trials; these data were independent from those used during the model-building exercise. Pretreatment archival FFPE tumor specimens from 50 patients with relapsed medulloblastoma who were enrolled in three phase I clinical studies of sonidegib were profiled for the five-gene Hh signature by RT-PCR (19–21). Six patients who achieved partial or complete response were determined to have Hh− tumors. One patient with an Hh+ tumor had stable disease for 112 days and 2 patients progressed after 36 and 65 days. All 41 remaining patients were predicted to have Hh− tumors and had disease progression (n = 30), stable disease (n = 5), or were not evaluable for tumor response (n = 6). Duration on treatment for patients with Hh+ tumors ranged from 36 to 288 days, whereas duration on treatment for patients with Hh− tumors was lower, ranging from 10 to 169 days. In retrospect, Hh+ tumors appeared to be particularly sensitive to sonidegib, with tumor responses observed after just 2 months of treatment. For the three adult patients who obtained partial responses on sonidegib (at doses of 200, 800, and 1,500 mg once daily), the duration of tumor responses ranged from four to eight months before documented disease progression (15).

Two pediatric and one adult patient (dosed at 372 mg/m², 425 mg/m², and 800 mg sonidegib once daily, respectively) also responded and were in complete remission for 22, >18, and >2 months, respectively (16).

The association between Hh activation status and tumor response to sonidegib treatment is shown in Tables 2 and 3. The positive and negative predictive values of the signature for tumor response are estimated to be 0.67 [95% confidence interval (CI), 0.30–0.93] and 1.00 (95% CI, 0.91–1.00), respectively. All model scores were derived using patient samples with blinded response data to remove any potential bias. Thirty-one patients with medulloblastoma from the three studies did not have tumors evaluated with the five-gene signature assay.

Of particular interest, one patient with confirmed partial response underwent five independent surgeries over a 10-year period, from initial diagnosis to the latest relapse just before treatment with sonidegib. Tissue from all five specimens was determined to be Hh activated by the five-gene signature, indicating that Hh pathway activation status can be stably maintained over an extended period of time and following recurrence. Several other patients had multiple tumor samples tested—four patients (1 stable disease, 3 progressive disease) were determined to have Hh+ tumors over the course of their disease, one patient (complete response) had a Hh+ tumor throughout the disease course, and another patient (complete response) had two tumor samples analyzed with different results (1 Hh+, 1 Hh−; Table 2). The reason for this discrepancy is being investigated; however, it could be due in part to a large difference in tumor content between the two samples.
In this study, we have developed and validated a five-gene signature that robustly identifies Hh pathway activation and patients most likely to respond to Hh pathway–targeted therapy. Other studies identifying and analyzing gene signatures have shown impressive results; however, these studies often lacked rigorous data analysis and validation (26). Conversely, the gene signature, based on data from previous gene expression profiling studies, was independently selected and validated in separate settings. The five-gene Hh signature assay was optimized for FFPE tumor samples. The accuracy of the Hh signature model was demonstrated by a perfect concordance with the Hh status determined by standard Affymetrix profiling. Furthermore, tumor specimens from patients with relapsed medulloblastoma treated with the SMO inhibitor, sonidegib, in three phase I studies demonstrated a strong association between Hh positivity and tumor response (Table 3). Tumors from all 6 patients who achieved either a partial or complete tumor response were classified as Hh activated according to the five-gene Hh signature, whereas tumors from 35 nonresponders were Hh nonactivated. The underlying reason for lack of response in three patients with Hh-activated tumors is unknown but may be due to pathway activation downstream of SMO. Data from this study are consistent with profiling studies, suggesting that Hh-activated tumors are more prevalent in adults and young children (≤3 years of age) than in older children (Table 3; refs. 6, 8). The low preponderance of Hh-activated tumors observed in children evaluated in this study may be attributed to their age as most children were older than 3 years of age. This observation may have a significant impact on studies designed to preselect children with Hh-activated medulloblastoma.

The clinical utility of a gene signature is largely dependent on the assay used to analyze it. Our procedure overcomes the limitations of standard profiling techniques, including insufficient sample

### Table 2. Observed responses to sonidegib treatment correlated with Hh activation status as determined by the five-gene signature in individual patients with medulloblastoma

<table>
<thead>
<tr>
<th>Study</th>
<th>Dose (once daily)</th>
<th>Best overall responsea</th>
<th>Five-gene signature–predicted Hh status</th>
<th>Propensity score</th>
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<tbody>
<tr>
<td>NCT00880308</td>
<td>200 mg</td>
<td>PR</td>
<td>Activated</td>
<td>0.983</td>
</tr>
<tr>
<td>800 mg</td>
<td>PRb</td>
<td>Activated</td>
<td>0.929, 0.607^t, 0.931, 0.880, 0.873</td>
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<tr>
<td>1,500 mg</td>
<td>PR</td>
<td>Activated</td>
<td>0.967</td>
<td></td>
</tr>
<tr>
<td>250 mgd</td>
<td>PD</td>
<td>Activated</td>
<td>0.806</td>
<td></td>
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<td>PD</td>
<td>Nonactivated</td>
<td>0.384</td>
<td></td>
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<td>PD</td>
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<tr>
<td>NCT0125800</td>
<td>372 mg/m²</td>
<td>CR^a</td>
<td>Activated</td>
<td>0.327, 0.874</td>
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<td>CR</td>
<td>Activated</td>
<td>0.949</td>
<td></td>
</tr>
<tr>
<td>800 mg</td>
<td>CR^d</td>
<td>Activated</td>
<td>0.906, 0.729</td>
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<td>SD</td>
<td>Activated</td>
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<td>800 mg</td>
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<td>SD^g</td>
<td>Nonactivated</td>
<td>0.001, 0.002</td>
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<td>233 mg/m²</td>
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<td>Nonactivated</td>
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<td>SD</td>
<td>Nonactivated</td>
<td>0.004, 0.30^d</td>
<td></td>
</tr>
<tr>
<td>680 mg/m²</td>
<td>PD</td>
<td>Nonactivated</td>
<td>range, 0.011–0.403^m</td>
<td></td>
</tr>
<tr>
<td>680 mg/m²</td>
<td>Unknown</td>
<td>Nonactivated</td>
<td>0.022, 0.445^s</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

*Data from 6 patients treated at 233 mg/m², each with progressive disease.
*Data from 6 patients treated at 425 mg/m², each with progressive disease.
*Data from 6 patients treated at 233 mg/m², each with unknown response.
*Data from 6 patients treated at 680 mg/m², each with unknown response.

**Discussion**

In this study, we have developed and validated a five-gene signature that robustly identifies Hh pathway activation and patients most likely to respond to Hh pathway–targeted therapy. Other studies identifying and analyzing gene signatures have shown impressive results; however, these studies often lacked rigorous data analysis and validation (26). Conversely, the gene signature, based on data from previous gene expression profiling studies, was independently selected and validated in separate settings. The five-gene Hh signature assay was optimized for FFPE tumor samples. The accuracy of the Hh signature model was demonstrated by a perfect concordance with the Hh status determined by standard Affymetrix profiling. Furthermore, tumor specimens from patients with relapsed medulloblastoma treated with the SMO inhibitor, sonidegib, in three phase I studies demonstrated a strong association between Hh positivity and tumor response (Table 3). Tumors from all 6 patients who achieved either a partial or complete tumor response were classified as Hh activated according to the five-gene Hh signature, whereas tumors from 35 nonresponders were Hh nonactivated. The underlying reason for lack of response in three patients with Hh-activated tumors is unknown but may be due to pathway activation downstream of SMO. Data from this study are consistent with profiling studies, suggesting that Hh-activated tumors are more prevalent in adults and young children (≤3 years of age) than in older children (Table 3; refs. 6, 8). The low preponderance of Hh-activated tumors observed in children evaluated in this study may be attributed to their age as most children were older than 3 years of age. This observation may have a significant impact on studies designed to preselect children with Hh-activated medulloblastoma.

The clinical utility of a gene signature is largely dependent on the assay used to analyze it. Our procedure overcomes the limitations of standard profiling techniques, including insufficient sample
quality and/or quantity, high cost, and inefficiency. Furthermore, because nucleic acids are degraded or modified during FFPE tissue processing, transcript analysis becomes difficult. To compensate, we developed a rigorous screening process to identify a group of genes that were still present and can be robustly analyzed in FFPE samples. Of note, analyses of serial tumor samples collected from several patients up to 10 years before treatment with sonidegib indicate that Hh pathway activation status is maintained from the initial diagnosis to the time of recurrence. However, in one patient with multiple tumor samples, Hh pathway activation status changed during the course of the disease, from Hh− to Hh+. Additional studies using the five-gene signature are necessary to confirm the ability to use archival tumor samples to preselect patients for future clinical studies of sonidegib.

The five-gene Hh signature identified in this study is composed primarily of genes not previously associated with canonical Hh signaling. One exception is the zinc finger transcription factor GLI1, which mediates transcriptional responses to Hh signaling (27). Upregulation of GLI1 has been observed in numerous malignancies, including basal cell carcinoma (BCC; ref. 28). Sonidegib exhibited exposure-dependent inhibition of GLI1 mRNA expression in normal skin, which correlated with changes in tumor samples in the phase I study described above and further validates the robustness of this assay (15). Other genes in the Hh signature that were upregulated include SHROOM2, which facilitates contrac tile network formation in endothelial cells, PDLM3, which plays a role in muscle differentiation, and SPPH1, which catalyzes the phosphorylation of sphingosine to form sphingosine 1 phosphate (29–31). None of these genes has been previously associated with medulloblastoma, and their role, if any, in the pathogenesis of medulloblastoma is unclear. Conversely, OTX2, a transcription factor involved in early development of the central nervous system, is amplified and critical for the maintenance and progression of a subset of medulloblastomas (21%; ref. 32). OTX2 is differentially expressed in different medulloblastoma subsets and is highly expressed in medulloblastomas with amplification (21%; refs. 6, 32). However, it appears that OTX2 expression is low in Hh-activated medulloblastomas (6, 32).

In conclusion, the five-gene Hh signature is a robust tool for identifying patients with Hh pathway–activated medulloblastoma using FFPE tumor samples and can be used to optimize the risk-benefit of treatment with Hh pathway inhibitors. The techniques used in this study could be adapted for large-scale analysis of patient samples. Indeed, the five-gene Hh signature assay received an investigation device exemption from the United States Food and Drug Administration for use in a clinical trial (Novartis Pharmaceuticals Corporation, documentation on file) and is currently being used to determine eligibility in a trial testing the efficacy of sonidegib in patients with Hh pathway–activated relapsed medulloblastoma (NCT01708174). This and future trials testing sonidegib in cancers with evidence of activated Hh signaling may serve to further validate the positive predictive value of the Hh signature and provide a clinical tool to guide treatment selection in patients with medulloblastoma. The five-gene Hh signature is also being tested in additional tumor types, including BCC; however, the results thus far are inconclusive as the clinical data are not yet mature.

**Disclosure of Potential Conflicts of Interest**

Y. Shou and D.D. Amakye were employees of Novartis. D.N. Robinson is an employee of and has ownership interest (including patents) in Novartis. K.L. Rose has ownership interest (including patents) in Novartis. Y. Ando reports receiving a commercial research grant and speakers bureau honoraria from Novartis. B. Georger is a consultant/advisory board member for Novartis. D.R. Hargrave is a consultant/advisory board member for Novartis. H.A. Tawbi, T.J. MacDonald, and M.W. Kieran are consultants/advisory board members for Novartis. No potential conflicts of interest were disclosed by the other authors.

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