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

Yaping Shou1*, Douglas M. Robinson1, Dereck D. Amakye2*, Kristine L. Rose2, Yoong-Jae Cho3, Keith L. Ligon4-6, Thad Sharp1*, Asifa S. Haider2*, Raj Bandaru1*, Yuichi Ando7, Birgit Georger8, François Doz9, David M. Ashley10, Darren R. Hargrave11, Michela Casanova12, Hussein A. Tawbi13, Jordi Rodon14, Anne L. Thomas15, Alain C. Mita16, Tobey J. MacDonald17, and Mark W. Kieran4

1Novartis Institutes for BioMedical Research, Inc, Cambridge, MA; 2Novartis Pharmaceuticals Corporation, East Hanover, NJ; 3Departments of Neurology and Neurosurgery, Stanford University School of Medicine, Stanford, CA; 4Pediatric Neuro-Oncology, Dana-Farber Cancer Institute and Boston Children’s Hospital and Harvard Medical School, Boston, MA; 5Department of Pathology, Children’s Hospital Boston, Brigham and Women’s Hospital, and Harvard Medical School, Boston MA; 6Department of Medical Oncology and Center for Molecular Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA; 7Nagoya University Hospital, Japan; 8Institut Gustave Roussy, University Paris-Sud, Villejuif, France; 9Institut Curie and University Paris Descartes, Sorbonne Paris Cité, France; 10Deakin University/Barwon Health, Melbourne, Australia; 11Great Ormond Street Hospital for Children, London; 12Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy; 13University of Pittsburgh Cancer Institute and University of Pittsburgh School of Medicine, Pittsburgh, PA; 14Vall d’Hebron Institut d’Oncologia, and Universitat Autonoma de Barcelona,
Barcelona, Spain; University of Leicester, Leicester, United Kingdom; Cancer Therapy and Research Center, University of Texas Health Science Center, San Antonio, TX; Children’s Healthcare of Atlanta, Aflac Cancer and Blood Disorders Center, Emory University School of Medicine, Atlanta, GA

*No longer a Novartis Employee

**Running title**

A five-gene hedgehog signature in medulloblastoma

**Keywords**

Gene expression profiling, molecular modeling, Phase I-III trials_Brain/central nervous system cancers, novel antitumor agents, five-gene Hh signature assay, preselection, sonidegib (LDE225)

**Financial support**

Supported by Novartis Pharmaceuticals Corporation for clinical studies and medical editorial support.

**Corresponding author**

Mark W. Kieran, MD, PhD
Dana-Farber Cancer Institute
450 Brookline Avenue
Conflicts of Interest

Douglas Robinson and Kristine Rose are employees of Novartis and have stock ownership in Novartis. Yaping Shou, Dereck Amakye, Thad Sharp, Asifa Hader, and Raj Bandaru are former employees of Novartis. Yoon-Jae Cho, Birgit Geoerger, François Doz, Darren Hargrave, Hussein Tawbi, Jordi Rodon, Tobey MacDonald, and Mark Kieran acted as consultants/advisors for Novartis; Michela Casanova acted as a consultant/advisor for F. Hoffmann-La Roche. Yoon-Jae Cho, Mark Kieran, and Yuichi Ando received speaker honoraria from Novartis. Yuichi Ando, Mark Kieran, and Keith Ligon received research funding from Novartis. François Doz, David Ashley, Anne Thomas, and Alain Mita have no conflicts of interest.
Statement of Translational Relevance

A significant proportion of patients with medulloblastoma (MB) experience relapse after primary treatment. Due to 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 MB, including one characterized by activated hedgehog (Hh) signaling. Inhibitors targeting the Hh pathway, including sonidegib (LDE225), have demonstrated activity in patients with MB. An RT-PCR-based five-gene signature assay has been developed to identify patients with Hh pathway-activated MB 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.
ABSTRACT

Purpose
Distinct molecular subgroups of medulloblastoma (MB), 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 MB.

Experimental Design
Genes characteristic of the Hh MB 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. Based on 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 (LDE225) was analyzed.

Results
Five differentially expressed genes in MB (GLI1, SPHK1, SHROOM2, PDLIM3, and OTX2) were found to associate with Hh pathway activation status. In an independent validation study, Hh activation status of 25 MB samples showed 100% concordance between the five-gene signature and Affymetrix profiling. Further, in MB samples from 50 patients treated with sonidegib, all six 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 MB and may be used to preselect patients who might benefit from sonidegib treatment.
INTRODUCTION

Medulloblastoma (MB), a malignant primitive neuroectodermal tumor arising in the cerebellum, is the most common brain tumor in children aged < 4 years (1). The current standard of care involves surgery followed by craniospinal radiation and chemotherapy, which can be concurrent 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 MB. Recently however, several studies have identified distinct molecular subgroups of MB 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 MBs (4, 6, 7, 9, 10); however, MBs 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 MB models (13, 14). In recent phase 1 studies testing single-agent sonidegib in adult and pediatric patients with advanced solid tumors, antitumor activity was demonstrated in several patients with MB (15, 16).

Genetic analyses of MBs have identified mutations in several Hh pathway genes; however, due to the low incidence of MB, 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 MB, 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 MB 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 MB who are most likely to derive benefit from Hh inhibitor therapy.

PATIENTS AND METHODS

Patient Samples

MB tumor specimens were obtained from the Dana-Farber Cancer Institute/Boston Children’s Hospital (Boston, MA; see Appendix for additional information) (n = 40) and Children’s Healthcare of Atlanta (CHOA; Atlanta, GA) (n = 25). MB samples (n = 37) were also obtained from patients enrolled in three separate phase 1 studies of oral sonidegib in patients with advanced solid tumors (Supplementary Table S1) (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. MB and normal cerebral tissue samples used for assay validation were obtained from commercial vendors, Asterand (Detroit, MI) (n = 1, MB) and Biochain (Newark, CA) (n = 1, MB; 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 data set from Thompson et al (4) included data obtained by the Affymetrix HG-U133Av2 microarray chip from 46 patients (aged < 21 years) with MB 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 data set from Kool et al (5) included 62 MB 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 data set, reported by Cho et al (7), included 194 MB tumor samples profiled on the Affymetrix HT-U133A. Six stable molecular subgroups of MB 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 U133Av2, 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 data sets, respectively, to enhance verification of the clustering below (4, 5, 7).

**Data normalization and unbiased filtering**—Normalization was performed independently for the three separate data sets using the robust multiarray averaging normalization algorithm in R Bioconductor package (http://www.r-project.org/). Without regard to Hh activation status, the data sets 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 data set (Thompson data set coefficient of variation [CV] < 5% and log$_2$ expression < 4; Kool data set CV < 5% and log$_2$ expression intensity < 5; Cho data set, CV < 5% and log$_2$ expression < 5) (4, 5, 7). Distinct thresholds were used for each of the three data sets 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 data set
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 data set 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 data sets.

The second approach combined all 3 data sets, necessitating the use of distinct hierarchical clustering (performed using the Pearson dissimilarity distance metric and complete linkage) (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 data set, 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 two-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 [AUC] ≥ 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 was 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
MB tumors were also selected from the normalized data set of 302 fresh frozen MB
samples from three published sources (4, 5, 7). Criteria for selection included low
variability in expression across all three MB data sets (CV ≤ 4%), and a target gene–
specific Affymetrix probe set with robust expression in MB tumors (log2 > 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 reverse transcriptase–polymerase chain reaction (RT-PCR) assays
(Applied Biosystems Inc, Foster City, CA) 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+ MB, one Hh− MB, 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 \( \leq 30 \) 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 TaqMan low-density array (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, Hilden, Germany) and reverse transcribed to complementary DNA (cDNA) using random hexamers and a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Real-time polymerase chain reaction 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, Santa Clara, CA) 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 MB tumor samples using the Trizol protocol (15596-018; Invitrogen, Grand Island, NY) and was profiled by AROS Biosciences (Aarhus, Denmark) 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, while 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 $\alpha$ and $\lambda$, 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 $\alpha$s and 91 $\lambda$s were generated. For each $\alpha$ and $\lambda$ pair, a model was fit in a five-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 $\alpha$ and $\lambda$, were selected in approximately 80% of the iterations. In each case, the same eight genes (glioma-associated oncogene homolog 1 [GLI1], orthodenticle homeobox 2 [OTX2], shroom family member 2 [SHROOM2], PDZ and LIM domain 3
[PDLIM3], 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, PDLIM3, 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 data set. 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 vs 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+ vs Hh− tumors and another 22 potential normalization genes were selected from data derived from 313
MBs 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 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 MB 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 MB fresh frozen samples as described by Cho et al (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 five-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) (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 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 MB 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 MB 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 to be 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, while 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 MB who were enrolled in three phase 1 clinical studies of sonidegib were profiled for the five-gene Hh signature by RT-PCR (19-21). Six patients who achieved partial (PR) or complete response were determined to have Hh+ tumors. One patient with an Hh+ tumor had stable disease for 112 days and two 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 PRs on sonidegib (at doses of 200, 800, and 1500 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/m\(^2\), 425 mg/m\(^2\), 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% 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 MB from the three studies did not have tumors evaluated with the five-gene signature assay. Of particular interest, one patient with confirmed PR underwent five independent surgeries over a ten-year period, from initial diagnosis to the latest relapse just prior to 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 SD, 3 PD) were determined to have Hh- tumors over the course of their disease, one patient (CR) had a Hh+ tumor throughout the disease course, and another patient (CR) 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.

**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 MB treated with the SMO inhibitor, sonidegib, in three phase 1 studies demonstrated a strong association between Hh-positivity and tumor response (Table 3). Tumors from all six patients who achieved either a partial or complete tumor response were classified as Hh activated according to the five-gene Hh signature, while 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) (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 MB.
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 ten years prior to 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) (28). Sonidegib exhibited exposure-dependent inhibition of GLI1 mRNA expression in normal skin, which correlated with changes in tumor samples in the phase 1 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 contractile network formation in endothelial cells, PDLIM3, which plays a role in muscle differentiation, and SPHK1, which catalyzes the phosphorylation of
sphingosine to form sphingosine 1 phosphate (29, 29-31). None of these genes has been previously associated with MB, and their role, if any, in the pathogenesis of MB 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 MBs (21%) (32). *OTX2* is differentially expressed in different MB subsets and is highly expressed in MBs with amplification (21%) (6, 32). However, it appears that *OTX2* expression is low in Hh-activated MBs (6, 32).

In conclusion, the five-gene Hh signature is a robust tool for identifying patients with Hh pathway-activated MB 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 MB (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 MB. 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.
Acknowledgments

We thank John Heath from Royal Children’s Hospital in Melbourne, Australia, Eric Bouffet from the Division of Haematology/Oncology at the Hospital for Sick Children in Toronto, Canada, Alba A. Brandes from the Medical Oncology Department from Azienda USL in Bologna, Italy, and Julia Chisholm from The Royal Marsden Hospital in Surrey, UK who provided patient samples. We would also like to thank Sha-Sha Wang and Feng Yang from Novartis Pharmaceuticals Corporation for technical support. Medical editorial assistance was provided by Jillian Brechbiel, PhD, and Karen Miller-Moslin, PhD. Financial support for medical editorial assistance was provided by Novartis Pharmaceuticals Corporation.
REFERENCES


### Tables

**Table 1.** Five-Gene Hh Signature and Control Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Uni-Gene Accession No.</th>
<th>Expression Level&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLI1</td>
<td>2139596</td>
<td>High</td>
</tr>
<tr>
<td>SHROOM2</td>
<td>1782725</td>
<td>High</td>
</tr>
<tr>
<td>SPHK1</td>
<td>139253</td>
<td>High</td>
</tr>
<tr>
<td>PDLIM3</td>
<td>5795643</td>
<td>High</td>
</tr>
<tr>
<td>OTX2</td>
<td>178376</td>
<td>Low</td>
</tr>
<tr>
<td>HUWE1</td>
<td>150675</td>
<td>Control</td>
</tr>
<tr>
<td>YME1L1</td>
<td>714304</td>
<td>Control</td>
</tr>
<tr>
<td>SOD1</td>
<td>238951</td>
<td>Control</td>
</tr>
<tr>
<td>LARP1</td>
<td>179374</td>
<td>Control</td>
</tr>
</tbody>
</table>

Abbreviations: GLI1, glioma-associated oncogene homolog 1; Hh, hedgehog; HUWE1, HECT, UBA, and WWE domain containing 1; LARP1, La ribonucleoprotein domain family member 1; OTX2, orthodenticle homeobox 2; PDLIM3, PDZ and LIM domain 3; SHROOM2, Shroom family member 2; SOD1, superoxide dismutase 1, soluble; SPHK1, sphingosine kinase 1; YME1L1, YME1-like 1.

<sup>a</sup> Relative to Hh-negative MB samples.
**Table 2.** Observed Responses to Sonidegib Treatment Correlated With Hh Activation Status as Determined by the Five-Gene Signature in Individual Patients With MB

<table>
<thead>
<tr>
<th>Study</th>
<th>Dose (once daily)</th>
<th>Best Overall Response&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Five-Gene Signature–Predicted Hh Status</th>
<th>Propensity Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT00880308</td>
<td>200 mg</td>
<td>PR</td>
<td>Activated</td>
<td>0.983</td>
</tr>
<tr>
<td></td>
<td>1500 mg</td>
<td>PR</td>
<td>Activated</td>
<td>0.967</td>
</tr>
<tr>
<td></td>
<td>800 mg</td>
<td>PR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Activated</td>
<td>0.929, 0.607&lt;sup&gt;c&lt;/sup&gt;, 0.931, 0.880, 0.873</td>
</tr>
<tr>
<td></td>
<td>250 mg&lt;sup&gt;d&lt;/sup&gt;</td>
<td>PD</td>
<td>Activated</td>
<td>0.806</td>
</tr>
<tr>
<td></td>
<td>800 mg</td>
<td>PD</td>
<td>Nonactivated</td>
<td>0.384</td>
</tr>
<tr>
<td></td>
<td>1500 mg</td>
<td>PD</td>
<td>Nonactivated</td>
<td>0.092</td>
</tr>
<tr>
<td>NCT01208831</td>
<td>400 mg</td>
<td>PD</td>
<td>Nonactivated</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>600 mg</td>
<td>PD</td>
<td>Nonactivated</td>
<td>0.003</td>
</tr>
<tr>
<td>NCT01125800</td>
<td>372 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>CR&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Activated</td>
<td>0.327, 0.874</td>
</tr>
<tr>
<td></td>
<td>425 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>CR</td>
<td>Activated</td>
<td>0.949</td>
</tr>
<tr>
<td></td>
<td>800 mg</td>
<td>CR&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Activated</td>
<td>0.906, 0.729</td>
</tr>
<tr>
<td></td>
<td>800 mg</td>
<td>SD</td>
<td>Activated</td>
<td>0.866</td>
</tr>
<tr>
<td></td>
<td>680 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>PD</td>
<td>Activated</td>
<td>0.603</td>
</tr>
<tr>
<td></td>
<td>800 mg</td>
<td>PD</td>
<td>Nonactivated</td>
<td>0.246</td>
</tr>
<tr>
<td></td>
<td>800 mg</td>
<td>PD</td>
<td>Nonactivated</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>800 mg</td>
<td>SD</td>
<td>Nonactivated</td>
<td>0.306</td>
</tr>
<tr>
<td></td>
<td>233 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>SD&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Nonactivated</td>
<td>0.001, 0.002</td>
</tr>
<tr>
<td></td>
<td>233 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>PD</td>
<td>Nonactivated</td>
<td>range, 0.001–0.007&lt;sup&gt;n&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>233 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>unknown</td>
<td>Nonactivated</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>372 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>PD&lt;sup&gt;l&lt;/sup&gt;</td>
<td>Nonactivated</td>
<td>range, 0.001–0.012&lt;sup&gt;n&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>372 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>unknown</td>
<td>Nonactivated</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>425 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>SD</td>
<td>Nonactivated</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>425 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>PD</td>
<td>Nonactivated</td>
<td>range, 0.001–0.014&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>425 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>unknown</td>
<td>Nonactivated</td>
<td>0.003, 0.011&lt;sup&gt;k&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>680 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>SD</td>
<td>Nonactivated</td>
<td>0.004, 0.304&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>680 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>PD</td>
<td>Nonactivated</td>
<td>range, 0.011, 0.403&lt;sup&gt;m&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>680 mg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>unknown</td>
<td>Nonactivated</td>
<td>0.022, 0.445&lt;sup&gt;n&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations: CR, complete response; Hh, hedgehog; MB, medulloblastoma; PD, progressive disease; PR, partial response; SD, stable disease.
a Tumor response was determined by RECIST 1.0 (33) (NCT01208831) and the Neuro-Oncology Criteria of Tumor Response (34) (NCT00880308, NCT01125800).
b Patient provided five blocks of tumor tissues collected throughout the disease course; all samples were Hh activated as per the five-gene signature assay.
c Low sample quantity.
d This patient was treated on a twice daily schedule.
e Patient provided two blocks of tumor tissues collected throughout the disease course; first sample was Hh nonactivated and the second sample was Hh activated as per the five-gene signature assay.
f Patient provided two blocks of tumor tissues collected throughout the disease course; both samples were Hh activated as per the five-gene signature assay.
g Patient provided two blocks of tumor tissues collected throughout the disease course; both samples were Hh nonactivated as per the five-gene signature assay.
h Data from six patients treated at 233 mg/m², each with progressive disease.
i Data from eight patients treated at 372 mg/m², each with progressive disease. Three patients provided two (n = 2) or three (n =1) blocks of tumor tissues throughout the disease course; all samples were Hh nonactivated as per the five-gene signature assay.
j Data from four patients treated at 425 mg/m², each with progressive disease.
k Data from two patients treated at 425 mg/m², each with unknown response.
l Data from two patients treated at 680 mg/m², each with stable disease.
m Data from six patients treated at 680 mg/m², each with progressive disease.
n Data from two patients treated at 680 mg/m², each with an unknown response.
**Table 3.** Summary of Sonidegib Activity in Hh Pathway-Activated MB Tumors From Patients Evaluated in Three Phase 1 Trials

<table>
<thead>
<tr>
<th></th>
<th>Responders/Hh Activated</th>
<th>Responders/Non-Hh Activated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults</td>
<td>4/6&lt;sup&gt;b&lt;/sup&gt; (1 CR, 3 PR)</td>
<td>0/7</td>
<td>13</td>
</tr>
<tr>
<td>Children&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2/3 (2 CR, 1 PD)</td>
<td>0/34</td>
<td>37</td>
</tr>
<tr>
<td>PPV&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.67 (0.30, 0.93)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPV&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.00 (0.91, 1.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>1.00 (0.54, 1.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>0.93 (0.81, 0.99)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CR, complete response; Hh, hedgehog; MB, medulloblastoma; NPV, negative predictive value; PD, progressive disease; PR, partial response; PPV, positive predictive value.

<sup>a</sup> This data represents a convenience sample. Thirty-one patients with MB from the three studies did not have tumors evaluated with the five-gene signature assay.

<sup>b</sup> Includes one patient with stable disease.

<sup>c</sup> Most of the children evaluated were older than 3 years of age.

<sup>d</sup> Values are shown as estimate (Clopper Pearson exact 95% confidence intervals) (35).
FIGURE LEGENDS

**Fig. 1.** Development of the five-gene hedgehog (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 data set of 313 medulloblastomas (MBs) 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. Reverse transcriptase–polymerase chain reaction (RT-PCR) assays for these candidate genes were developed and optimized for use in 3 formalin-fixed paraffin-embedded (FFPE) specimens (1 Hh+, 1 Hh−, 1 normal cerebellum) obtained from Biochain (Newark, CA). 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 MB specimens with established Hh activation status (7) and subjected to model building by the elastic net method for signature identification. FFPE MB 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). The five-gene Hh signature was externally validated in a set of 25 matching FFPE and fresh frozen MB samples obtained from the Children’s Healthcare of Atlanta.

**Fig. 2.** Selection of the five-gene model. An optimal model with minimal error (red arrow) was determined using the elastic net algorithm (24) in a five-fold cross-validation framework. (A) Cross-validation was run for each model parameter pair, shown by the x and y axes, in which each model provided an estimate of model error (z axis). The
optimal model was the one that produced minimal model error in cross-validation (red arrow). (B) To better understand the potential cross-validation error, the procedure was iterated 10,000 times. The frequency distribution of the models with five genes was plotted in a heat-map format (B, left) and in three dimensions (B, right) for each model parameter pair, in which the most frequently selected model was chosen as optimal (red arrow).

**Fig. 3.** External validation data for the five-gene hedgehog (Hh) signature. The five-gene Hh signature was validated in an independent set of 25 medulloblastoma specimens. (A) The prediction of Hh activation status from the five-gene signature showed 100% agreement with the determination made by Affymetrix profiling for all tumor specimens. (B) The eight tumors predicted to be Hh activated had a median propensity score of 0.879 (range, 0.691–0.976), and the remaining 17 tumor samples were predicted to be non–Hh activated, with a median propensity score of 0.007 (range, 0.001–0.03). Model-predicted propensity score threshold for Hh activation call was 0.5 (dotted line).
Candidate genes (32 differentially expressed and 22 control genes) selected via reanalysis of a combined profiling data set from 3 external studies (4, 5, 7) in 313 MB samples

Candidate genes (18 differentially expressed and 4 control genes) selected via RT-PCR optimization in 3 FFPE samples (1 Hh+, 1 Hh-, 1 normal cerebellum)

Candidate gene analysis via RT-PCR in FFPE MB tumor samples (n = 40) with matching fresh frozen tissue previously profiled (7) AND Hh status predictions made

Hh+ (n = 15)  Hh– (n = 25)

Elastic net method used to define optimal model using five-fold cross-validation

External validation of the optimal model and performance characterization in 25 matching FFPE and fresh frozen MB samples

---

*a* 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.

*b* FFPE samples were obtained from BioChain Institute, Inc.

*c* FFPE MB tumor samples were obtained from the Dana-Farber Cancer Institute/Boston Children's Hospital. The 25 matching fresh frozen samples were a subset of the 194 MB samples previously profiled (7).

*d* MB tumor samples were obtained from Children's Healthcare of Atlanta.
Figure 2

A

B

Number of Times Selected During Iterations

Lambda

Alpha

0.02 0.04 0.06 0.08 0.10

0.02 0.04 0.06 0.08 0.10

0.40 0.35 0.30

0.35 0.30

Error

Lambda

Alpha

0.02 0.04 0.06 0.08 0.10

0.02 0.04 0.06 0.08 0.10

0.40 0.35 0.30

0.35 0.30
## Table: Predicted Hh Status (Affymetrix profiling)

<table>
<thead>
<tr>
<th>Predicted Hh Status (Five-Gene Signature)</th>
<th>Hh Activated</th>
<th>Non-Hh Activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hh positive</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Hh negative</td>
<td>0</td>
<td>17</td>
</tr>
</tbody>
</table>

## Figure 3

### A

Predicted Hh Status (Affymetrix profiling)

<table>
<thead>
<tr>
<th>Predicted Hh Status (Five-Gene Signature)</th>
<th>Hh Activated</th>
<th>Non-Hh Activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hh positive</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Hh negative</td>
<td>0</td>
<td>17</td>
</tr>
</tbody>
</table>

### B

Predicted Negative  Predicted Positive

<table>
<thead>
<tr>
<th>Propensity of Being Hh Positive (Five-Gene Model)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>0.8</td>
</tr>
<tr>
<td>0.6</td>
</tr>
<tr>
<td>0.4</td>
</tr>
<tr>
<td>0.2</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>-0.2</td>
</tr>
</tbody>
</table>

Propensity score of 0.5 as threshold

<table>
<thead>
<tr>
<th>n = 17</th>
<th>n = 0</th>
</tr>
</thead>
</table>

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

Yaping Shou, Douglas M. Robinson, Dereck D Amakye, et al.

Clin Cancer Res  Published OnlineFirst December 3, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-1711

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2014/12/06/1078-0432.CCR-13-1711.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.