

# Real-time PCR assay based on the differential expression of microRNAs and protein-coding genes for molecular classification of formalin-fixed paraffin embedded medulloblastomas

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**Background.** Medulloblastoma has recently been found to consist of 4 molecularly and clinically distinct subgroups: WNT, Sonic hedgehog (SHH), Group 3, and Group 4. Deregulated microRNA expression is known to contribute to pathogenesis and has been shown to have diagnostic and prognostic potential in the classification of various cancers.

**Methods.** Molecular subgrouping and microRNA expression analysis of 44 frozen and 59 formalin-fixed paraffin embedded medulloblastomas from an Indian cohort were carried out by real-time RT-PCR assay.

**Results.** The differential expression of 9 microRNAs in the 4 molecular subgroups was validated in a set of 101 medulloblastomas. The tumors in the WNT subgroup showed significant ( $P < .0001$ ) overexpression of miR-193a-3p, miR-224, miR-148a, miR-23b, and miR-365. Reliable classification of medulloblastomas into the 4 molecular subgroups was obtained using a set of 12 protein-coding genes and 9 microRNAs as markers in a real-time RT-PCR assay with an accuracy of 97% as judged by the Prediction Analysis of Microarrays. Age at diagnosis, histology, gender-related incidence, and the relative survival rates of the 4 molecular subgroups in the present Indian cohort were found to be similar to those reported

for medulloblastomas from the American and European subcontinent. Non-WNT, non-SHH medulloblastomas underexpressing miR-592 or overexpressing miR-182 were found to have significantly inferior survival rates, indicating utility of these miRNAs as markers for risk stratification.

**Conclusions.** The microRNA based real-time PCR assay is rapid, simple, inexpensive, and useful for molecular classification and risk stratification of medulloblastomas, in particular formalin-fixed paraffin embedded tissues, wherein the expression profile of protein-coding genes is often less reliable due to RNA fragmentation.

**Keywords:** Indian cohort, medulloblastoma, miRNA, molecular classification, risk stratification.

Medulloblastoma is a common malignant brain tumor in children, accounting for 20% of all pediatric brain tumors.<sup>1</sup> All medulloblastomas belong to WHO grade IV, the highest histological grade of malignancy. Standard treatment includes surgical resection, followed by craniospinal radiation and chemotherapy. Advances in surgical and radiation techniques have improved the 5-year survival rate to about 80% for average-risk patients and 55%–76% for high-risk patients.<sup>2</sup> The risk stratification of medulloblastomas is based on clinical parameters like age at diagnosis, presence of metastasis, and extent of resection. Recently several investigators around the world have demonstrated that medulloblastoma is not a single disease but consists

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of molecularly distinct subgroups.<sup>3–5</sup> According to the current consensus, there are 4 core molecular subgroups of medulloblastomas: WNT, SHH, Group 3, and Group 4, which not only are distinct in their underlying biology but also vary in their clinical characteristics, like age-related incidence, presence of metastasis, and survival rates.<sup>6</sup> In addition to the clinical parameters, molecular classification of medulloblastomas is now necessary for better risk assessment and management of the disease.<sup>7</sup>

MicroRNAs (miRNAs) are 18- to 22-nucleotide-long noncoding RNA molecules that regulate expression of the protein-coding genes.<sup>8</sup> MiRNAs bind to complementary sequences in the 3' untranslated regions of multiple target genes, usually resulting in their silencing.<sup>9</sup> Each miRNA is believed to target several hundred genes. Altered miRNA expression has been reported in various cancers.<sup>10,11</sup> Accumulating evidence indicates that deregulated miRNA expression plays an important role in pathogenesis. MiRNA expression profile has been found to have diagnostic and prognostic potential in the classification of various cancers.<sup>12</sup> Besides, miRNAs, being small in size, are protected from fragmentation during the process of formalin fixation and hence can be reliably studied in formalin-fixed paraffin embedded (FFPE) tissues.<sup>13</sup> Several studies have shown an excellent correlation between miRNA expression in fresh frozen and FFPE tissues and have found the miRNA expression profile to be superior to that of the protein-coding genes in FFPE tissues.<sup>14,15</sup>

We earlier reported a genome-wide expression profile of protein-coding genes and miRNAs done in parallel on a set of 19 medulloblastomas and 4 normal cerebellar tissues using Gene 1.0 ST arrays (Affymetrix) and Taqman Low Density miRNA array version 1.0 (Applied Biosystems), respectively.<sup>16</sup> The protein-coding genes as well as the miRNA profile could segregate the medulloblastomas into the 4 molecular subgroups, with the WNT medulloblastomas having the most distinctive miRNA profile. In the present study, molecular subgrouping of 103 medulloblastomas that included 59 FFPE tissues was carried out using a set of 12 protein-coding genes as markers. Further, expression of a set of 11 miRNAs was studied in these medulloblastomas by real-time RT-PCR, validating the differential expression of these miRNAs in the 4 molecular subgroups. This study demonstrates miRNAs as useful markers for molecular subgrouping of medulloblastomas from archived FFPE tissues.

## Materials and Methods

### *Tumor Samples and RNA/DNA Extraction*

All tumor tissues were obtained with the approval of the institutional review board. Fresh tumor tissues were collected following surgery, snap frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . All the medulloblastoma cases studied were treated per standard practices, with surgery followed by radiation (with the exception of children  $<3$  y old) and chemotherapy. Forty-four fresh frozen medulloblastoma tissues (including a set of 30 tissues reported earlier) and 59 medulloblastomas available as FFPE blocks were included in this

study. Hematoxylin and eosin staining were done to ensure at least 80% tumor content, after which the tissues were used for RNA and DNA extraction. For FFPE tissues, 10- $\mu\text{m}$  sections were deparaffinized using xylene followed by absolute ethanol washes and subsequent digestion with proteinase K overnight at  $55^{\circ}\text{C}$  in Tris–sodium dodecyl sulfate–NaCl–EDTA buffer as per the protocol described by Korbler et al,<sup>17</sup> followed by acid phenol–chloroform or phenol–chloroform extraction and ethanol precipitation for isolation of RNA and DNA, respectively.<sup>18</sup> DNA and RNA quantity and quality were evaluated using a spectrophotometer (Nanodrop ND-1000, Thermo Scientific) and agarose gel electrophoresis, respectively. Validation of the assay was done on total RNA from 34 medulloblastoma FFPE tissues obtained from the German Cancer Research Centre (DKFZ).

### *Reverse Transcription and Real-time PCR*

The differential expression of the protein-coding genes and miRNAs was analyzed by real-time RT-PCR. Total RNA (1–2  $\mu\text{g}$ ) was reverse transcribed using random hexameric primers and M-MLV reverse transcriptase (Invitrogen). The primers for real-time PCR analysis were designed such that they corresponded to 2 adjacent exons and, wherever possible, were located at exon boundaries to avoid amplification of genomic DNA. Supplementary Table S1 lists the sequences of the primers used. The amplicon size was maintained below 75–80 bp, so as to enable amplification of the fragmented RNA from FFPE tissues. The expression was analyzed by SYBR Green PCR amplification assay on an Applied Biosystems 7900HT real-time PCR system using 10 ng cDNA per reaction for frozen tissues and 10–100 ng cDNA per reaction for FFPE tissues. For miRNA expression analysis, 50 ng RNA from fresh tissues and 50–200 ng RNA from FFPE tissues were reverse transcribed using multiplex RT primer pools and the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The expression of each miRNA was analyzed by TaqMan real-time miRNA assay (Applied Biosystems) on the ABI 7900HT real-time PCR system using 10 ng cDNA from frozen tissues and 10–40 ng cDNA from FFPE tissues. The relative quantity (RQ) of each protein-coding gene/miRNA compared with *GAPDH/RNU48* was determined by the comparative cycle threshold (Ct) method, where  $\text{RQ} = 2^{-(\text{Ct}_{\text{Gene}} - \text{Ct}_{\text{Ref}})} \times 100$ .

### *Mutation Analysis*

Exon 3 of the *CTNNB1* gene was amplified from the WNT subgroup tumor tissues and sequenced to identify mutations, if any, using a 3100 Avant Genetic Analyzer (Applied Biosystems).

### *Statistical Analysis*

Descriptive statistics were used for the subgroup assignment of each tumor tissue based on the expression levels

of the marker genes evaluated by real-time RT-PCR analysis. The nearest shrunken centroid classifier implemented in the Prediction Analysis of Microarray (PAM) for Excel package was used for class prediction analysis (pamr\_1.54 package at <http://cran.r-project.org>).<sup>19</sup> The expression levels of the marker genes obtained as RQs by real-time RT-PCR were  $\log_2$  transformed for PAM analysis. Robustness of the training set was assessed by cross-validation (random 10% left out at each cycle). The cross-validation was performed by selecting various thresholds associated with the lowest error rate on the training set and then used for class prediction of the test set at the threshold having the least cross-validation error rate. Analysis of receiver operating characteristic (ROC) curves was performed using SPSS 15.0 software. Descriptive statistics were used to describe the demographic and histological data of the 4 subgroups. Event for overall survival was calculated from the date of surgery until death or last follow-up date. Survival percentages were estimated by the Kaplan–Meier method, and statistical significance between the groups was estimated by the log-rank test using GraphPad Prism v5.0.

## Results

### *Molecular Subgrouping Based on the Expression Profile of Protein-coding Genes*

Molecular subgrouping of 103 medulloblastomas was carried out using real-time RT-PCR based on the evaluation of expression of a set of protein-coding genes as markers. The 103 medulloblastomas consisted of 44 fresh frozen and 59 FFPE tissues. The genes significantly differentially expressed in the 4 molecular subgroups were identified by Significance Analysis of Microarray (MeV, <http://www.TM4.com>) of our expression profiling data on 19 medulloblastoma tissues obtained using the Affymetrix Gene 1.0 ST array.<sup>16</sup> The selection of the marker genes for classification from these significantly differentially expressed genes was based on the standardized fold change in the expression of the gene in the particular subgroup from our data (Supplementary Table S2) as well as that in other published reports.<sup>3,4</sup> The heat map (Fig. 1A) and the scatter dot plot (Supplementary Fig. S1) show the differential expression of the marker genes in the 103 medulloblastomas that could be accurately classified. Concomitant overexpression of *WIF1*, *DKK2*, and *MYC* identified WNT medulloblastomas. Overexpression of *HHIP*, *EYA1*, and *MYCN* and underexpression of *OTX2* served as markers for the SHH subgroup. The overexpression of *EOMES* helped to identify Group 3 and Group 4 tumors, while higher expression of *NPR3*, *MYC*, and *IMPG2* and lower expression of *GRM8* and *UNC5D* helped to distinguish Group 3 from Group 4 tumors. Five of the 103 medulloblastomas were classified primarily based on their miRNA profile due to poor RNA quality (to be discussed). Seven out of 8 FFPE WNT medulloblastomas, which could be analyzed for *CTNNB1* exon 3 sequence, were found to harbor a single point mutation that altered D32, S33, or

S37 amino acid, validating their subgroup identification (Supplementary Fig. S2). Mutations in the *CTNNB1* gene in the 12 frozen WNT medulloblastomas have been previously reported.<sup>16</sup> Thus, the presence of the *CTNNB1* mutation in 19 out of 20 WNT tumors analyzed confirmed its known prevalence in WNT medulloblastomas.

### *Differential MiRNA Expression in the Molecular Subgroups of Medulloblastomas*

The expression of a select set of miRNAs differentially expressed in the 4 molecular subgroups was studied in parallel by real-time RT-PCR analysis. Total RNA was not available for 2 fresh frozen tumor tissues for miRNA expression analysis. The selection of miRNAs differentially expressed in the 4 subgroups was based on our data and other reports on the differential miRNA expression in medulloblastoma subgroups.<sup>5,16,20</sup> WNT tumors showed significant ( $P < .0001$ ) overexpression of miR-193a-3p, miR-224, miR-148a, miR-23b, miR-365, and miR-10b compared with other subgroup medulloblastomas (Fig. 1 and Supplementary Fig. S1). MiR-182 was found to be overexpressed in all WNT medulloblastomas and in many (16/21) Group 3 and some (7/29) Group 4 medulloblastomas, while miR-204 was overexpressed in all WNT medulloblastomas and in most (25/29) Group 4 medulloblastomas. MiR-182, miR-135b, and miR-204 were found to be underexpressed in SHH medulloblastomas. MiR-135b was found to be overexpressed in Group 3 and Group 4 tumors. MiR-592, a miRNA located within the *GRM8* gene, was overexpressed in Group 4 medulloblastomas. MiR-10b was expressed at the highest level in WNT medulloblastomas, followed by Group 3 medulloblastomas. MiR-376a belongs to the miR-379/miR-656 cluster of miRNAs located within an imprinted region on chromosome 14.<sup>21</sup> MiR-376a expression was found to be significantly higher in Group 4 medulloblastomas compared with Group 3 medulloblastomas.

### *Molecular Subgrouping Using Both Protein-coding Genes and MiRNAs by Prediction Analysis of Microarrays*

A difference of  $\sim 8$  cycles was observed between the average Ct values of *RNU48* ( $19 \pm 1.7$ ) and *GAPDH* ( $27 \pm 2.1$ ), wherein the amount of cDNA used for *GAPDH* evaluation was 2.5 times higher than that used for *RNU48* evaluation, indicating integrity of small RNAs (miRNAs) being about 600-fold higher than that of protein-coding gene RNAs. Therefore, the evaluation of miRNA expression was reliable, reproducible, and sensitive even in 7- to 8-year-old FFPE tumor tissues (Supplementary Fig. S3).

The 12 protein-coding genes and 11 microRNAs differentially expressed in the 4 molecular subgroups of medulloblastomas were tested as markers for molecular classification of medulloblastomas by PAM analysis. MiR-376a and miR-10b expression levels were found to be less consistent within a subgroup and considerably

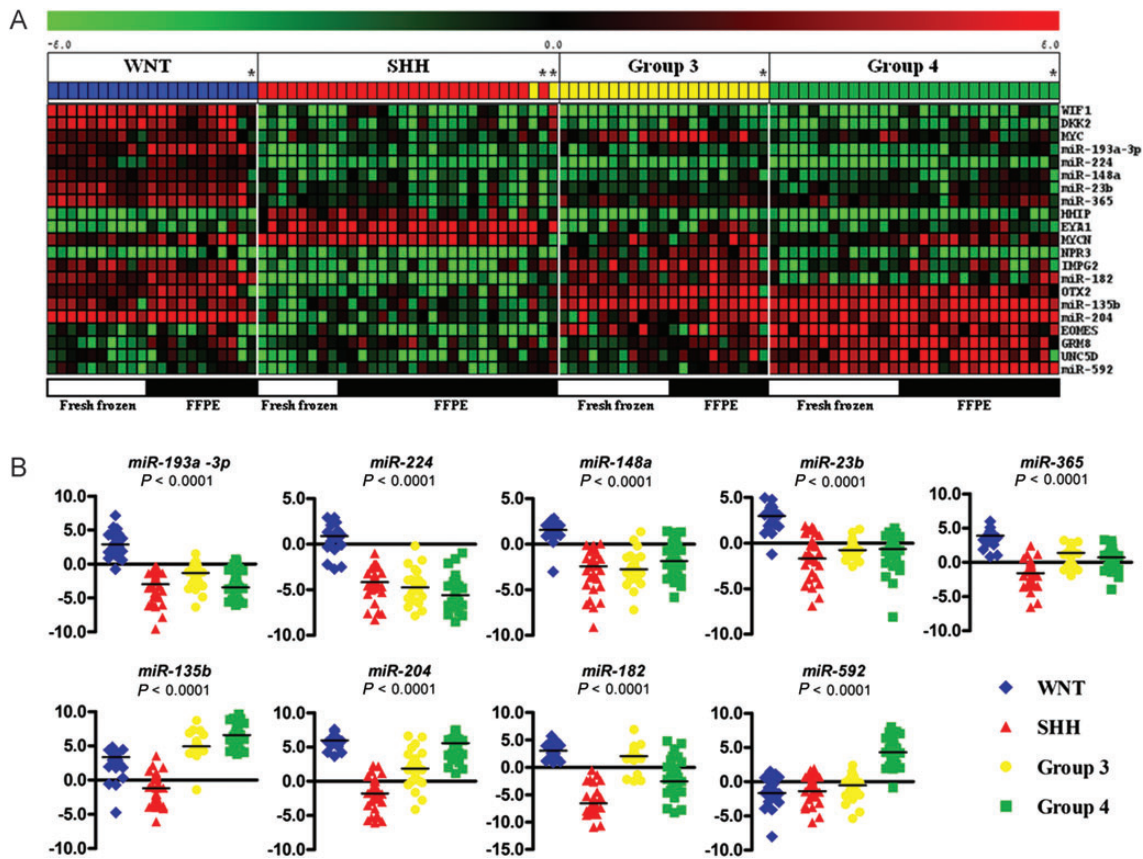


Fig. 1. (A) Heat map showing differential expression of 12 protein-coding genes and 9 miRNAs in the 101 tumor tissues (2 tumors lacking miRNA profile excluded). \*indicates the tumor tissues classified primarily based on miRNA expression profile. Subgroup assignment based on PAM analysis using 42 fresh frozen tumor tissues as a training set is indicated above the heat map. (B) The scatter dot plot shows log<sub>2</sub> transformed RQs of the indicated miRNA in the 101 medulloblastomas assigned to the 4 molecular subgroups. The P values given on the top of each scatter indicates the significance of the differential expression of the marker gene in the 4 subgroups as determined by ANOVA tests.

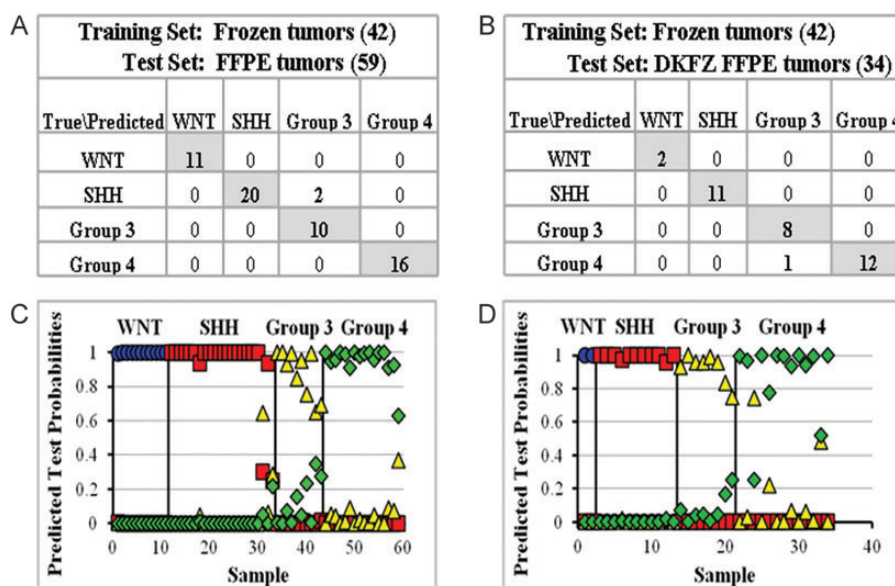


Fig. 2. The results of PAM analysis showing the subgroup prediction matrix and the predicted test probabilities of the test set based on the expression profile of 12 protein-coding genes and 9 miRNAs.

low compared with other miRNAs and hence were not included as markers for molecular classification. PAM analysis using the set of 101 medulloblastomas as a training set showed a cross-validation accuracy of 99%. Supplementary Fig. S4 shows a centroid plot of all the marker genes used in the PAM analysis. The set of 42 fresh frozen medulloblastoma tissues consisted of 10 WNT, 8 SHH, 11 Group 3, and 13 Group 4 cases, while the set of 59 FFPE medulloblastomas consisted of 11 WNT, 22 SHH, 10 Group 3, and 16 Group 4 medulloblastomas. Using a training set of 42 fresh frozen medulloblastomas, all FFPE tumors were accurately classified, with the exception of 2 SHH tumors (Fig. 2A and C and Fig. 1A). Four out of 5 tumors classified primarily based on their miRNA profiles due to poor RNA quality were accurately classified by PAM analysis using both protein-coding genes and miRNAs. One of these 5 tumors belonging to the WNT subgroup was found to possess a mutation in the *CTNNB1* gene, confirming its classification.

The assay was validated on a set of 34 well-annotated FFPE medulloblastoma tumor tissues (subgroup assignment based on NanoString assay<sup>22</sup> from DKFZ. The RNAs of this set of tumor tissues were analyzed for expression of the 12 protein-coding genes and 9 miRNAs by the present real-time PCR assay (Supplementary Fig. S5). PAM analysis using the training set of 42 fresh frozen tumor tissues accurately classified all DKFZ FFPE tissues, with the exception of a single Group 4 tumor misclassified as a Group 3 tumor (Fig. 2B and D). The 2 SHH medulloblastomas that were misclassified using our fresh frozen tumor tissues as a training set

were correctly classified using the DKFZ tumor set for training (data not shown). This misclassification is therefore likely to be due to the insufficient number of SHH tumors in our training set of fresh frozen tumors. The overall predicted posterior probabilities for all WNT and 31 of 33 SHH tumors were  $\geq 0.9$ . Twenty-six of 29 Group 4 tumors and 14 of 18 Group 3 tumors had predicted posterior probabilities  $\geq 0.8$  (Fig. 2C and D). The present real-time RT-PCR assay thus had an overall accuracy of 97% with an area under the ROC curve of 1.00 for all 4 subgroups.

### Demographic Analysis

Of the 103 medulloblastomas studied, 23 belonged to the WNT subgroup, 30 to the SHH subgroup, 21 to Group 3, and 29 to Group 4 (Fig. 3A). The overall median age of the cohort was 9 years (range, <1–45 y). Tumors in children <3 years of age were SHH (67%) and Group 3 (33%). Those in older children (>8 y) were primarily Group 4 (40%) and WNT (40%). Tumors in adult patients ( $\geq 18$  y) were SHH (65%) and WNT (35%; Fig. 3B). The ratio of male to female patients in the WNT subgroup was lowest, at  $\sim 1:1$ , while 40 of 50 cases in Group 3 and Group 4 were male patients (Fig. 3C). Most of the tumors studied were of classical histology (79%), followed by tumors having large cell/anaplastic (10.6%) and desmoplastic (10.6%) histology. While all the desmoplastic tumors belonged to the SHH subgroup, 64% of tumors with large cell/anaplastic histology were Group 3 (Fig. 3D).

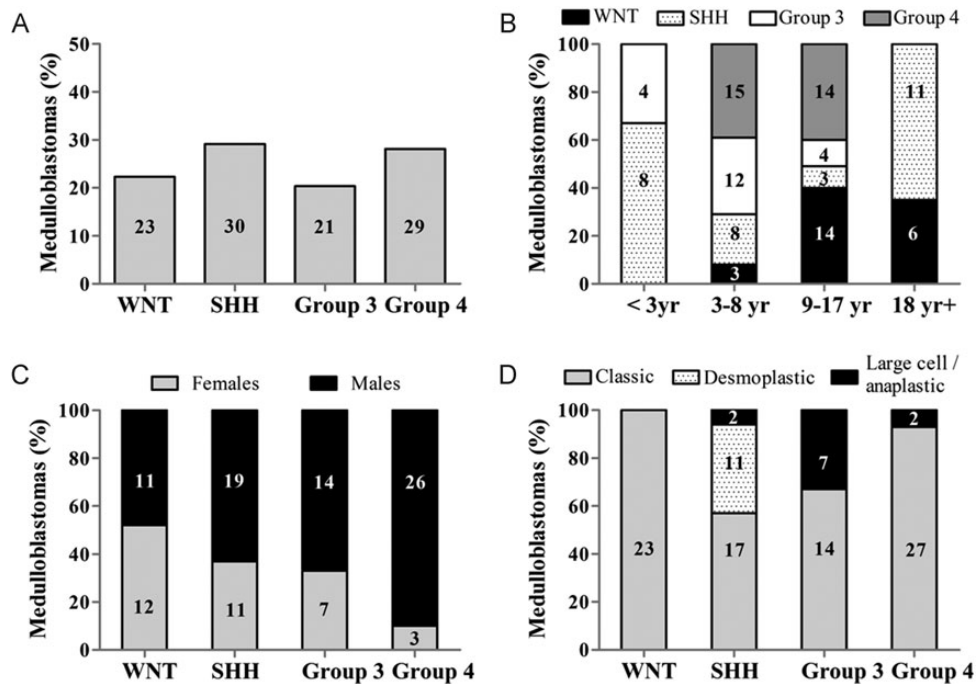


Fig. 3. (A) The demographic distribution of the 4 molecular subgroups in the present cohort; (B) subgroup distribution with respect to the age at diagnosis; (C) gender; (D) histological variants. The numbers indicate the number of tumors in each category.

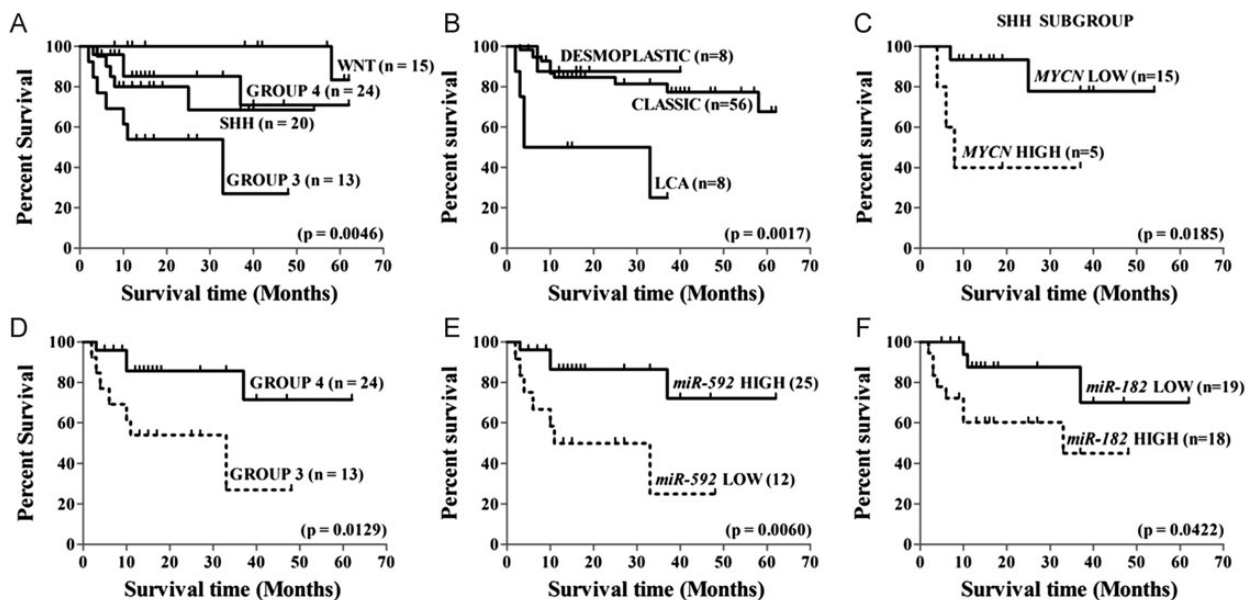
### Correlation of the Molecular Subgroups With Overall Survival

Overall survival data were available for 72 of 103 medulloblastomas, which were adequately treated per standard practice. The patients who expired within the first month after surgery were excluded from the analysis. Kaplan–Meier analysis showed the best survival rate for the WNT subgroup patients, followed by Group 4 and SHH patients, with the worst survival rate for Group 3 patients (Fig. 4A). The log-rank test showed survival curves to be significantly different ( $P = .0046$ ) for the 4 subgroups. The survival analysis of the histological variants showed significantly ( $P = .0017$ ) worse survival rates for the tumors with large cell/anaplastic histology compared with those with classic or desmoplastic histology (Fig. 4B). The survival curve of patients <3 years of age was not found to be significantly different from that of patients older than 3 years of age, possibly due to the lack of sufficient number of cases (data not shown). Within the SHH subgroup, tumors with *MYCN* overexpression comparable to *MYCN* amplification levels were found to have significantly ( $P = .0185$ ) poorer survival rates (Fig. 4C). In the combined cohort of Group 3 and Group 4 medulloblastomas, tumors with *miR-592* overexpression were found to have significantly

( $P = .0060$ ) better survival rates, while those with *miR-182* overexpression were found to have significantly ( $P = .0422$ ) worse survival rates (Fig. 4E and F). The difference in the survival rates of non-WNT, non-SHH tumors having *miR-592* overexpression from those lacking the overexpression is comparable to the difference in the survival rates of Group 3 versus Group 4 medulloblastomas (Fig. 4D).

## Discussion

In the present study, differential expression of 11 miRNAs in the 4 molecular subgroups was validated in a set of 101 medulloblastomas that confirmed the distinctive miRNA signature of WNT medulloblastomas. Reliable classification of medulloblastomas into the 4 molecular subgroups was demonstrated using a set of 12 protein-coding genes and 9 miRNAs as markers by a real-time RT-PCR based assay with an overall accuracy of 97%. Molecular classification based on the 9 miRNAs alone was found to have accuracies of 100%, 93.3%, 85.7%, and 100% for WNT, SHH, Group 3, and Group 4, respectively, in cross-validation analysis by PAM using the set of 101 medulloblastomas (data not shown). MicroRNAs therefore served as useful markers for the molecular subgrouping



Univariate Analysis		
Variable	Hazard Ratio	95% CI of ratio
Percent Survival - SHH subgroup <i>MYCN</i> high vs. low (n = 20)	14.89	1.573 to 140.9
<b>Percent Survival - Group 3 and 4, (n = 37)</b>		
Group 3 vs. Group 4	5.318	1.424 to 19.86
<i>miR-592</i> (low vs. high)	6.647	1.721 to 25.66
<i>miR-182</i> (high vs. low)	3.527	1.045 to 11.90

Fig. 4. Overall survival analysis of (A) 4 molecular subgroups; (B) histological variants; (C) SHH subgroup tumors with and without *MYCN* overexpression; (D) Group 3 vs Group 4 tumors; (E) non-SHH, non-WNT tumors with or without *miR-592* overexpression; (F) non-SHH, non-WNT tumors with or without *miR-182* overexpression.  $P$  value indicates level of significant difference in the Kaplan–Meier survival curves estimated by the log-rank test.

of FFPE tumor tissues, wherein RNA is fragmented, resulting in less reliable evaluation of the expression of protein-coding genes. MiRNA expression levels helped particularly in the classification of the tumor tissues for which *GAPDH* Ct values were closer to 30, wherein the expression levels of protein-coding genes could not be completely relied upon. In the case of tumor tissues for which Ct values were in the range of 32 and higher for *GAPDH*, miRNA expression levels also became unreliable (data not shown).

The real-time RT-PCR assay based on the expression of 12 protein-coding genes and 9 miRNAs is comparable to the reported 98% accuracy of the NanoString assay using 22 subgroup-specific protein-coding genes as markers.<sup>22</sup> The PCR technology, being highly sensitive, allows analysis of the expression levels of protein-coding genes and miRNAs from FFPE tissues having considerable RNA degradation. The present assay is rapid and inexpensive and is based on real-time PCR technology that is now commonly available in molecular pathology labs around the world.

The study was performed on medulloblastomas from an Indian cohort. WNT, SHH, Group 3, and Group 4 accounted for 22.3%, 29.13%, 20.39%, and 28.16%, respectively, of the tumor tissues as against the reported incidences of 11%, 28%, 27%, and 34% based on the meta-analysis of the medulloblastoma data from the American and European subcontinents.<sup>23</sup> The WNT subgroup was found to be prevalent in older children (61%) and adults (26%). The SHH cases occurred across all age groups, with predominance in infants (27%), younger children (27%), and adults (37%). Group 3 cases were found predominantly in younger children (60%) and infants (20%), with none among adults. Group 4 cases were distributed almost equally in younger (52%) and older (48%) children, with no cases in infants and adults. The age-related incidences of the 4 subgroups are similar to the data reported.<sup>23</sup> Male ( $n = 70$ ) to female ( $n = 33$ ) ratio in the present cohort was 2.12, consistent with the known preferential occurrence of medulloblastoma in males. The WNT subgroup had almost equal male to female ratio, while the ratio was 1.7 : 1 and 2 : 1 for SHH and Group 3, respectively, which is consistent with the reported gender representation in these groups. Male to female ratio of Group 4 was 9 : 1, which is substantially higher than the reported ratio of 2 : 1.<sup>23</sup> Age at diagnosis, histology, and gender-related incidence and the relative survival rates of the 4 molecular subgroups in the present Indian cohort were found to be similar to those reported for the medulloblastomas from the American and European subcontinents, suggesting uniform mechanisms of medulloblastoma pathogenesis. The higher incidence of the WNT subgroup and relatively lower incidence of Group 3 tumors are therefore partly explained by the higher representation of older children and adults, who together accounted for 51% of the tumors in the present cohort. Nonetheless, frequency of WNT tumors is much higher than reported so far, even for these age groups, with as many as 40% of older children

and 35% of adults in the present Indian cohort belonging to the WNT subgroup.

Molecular markers in addition to the molecular sub-grouping are required for further improvement in risk stratification, particularly of the 3 non-WNT subgroups. As reported by several other studies, tumors with large cell/anaplastic histology were found to have significantly poor survival in the present study, indicating the importance of histology for risk stratification.<sup>23</sup> *MYCN* amplification has been shown to associate with relatively inferior survival in the SHH medulloblastomas.<sup>23</sup> Accordingly, SHH tumors with *MYCN* overexpression (*MYCN* levels comparable to the tumors having *MYCN* amplification; data not shown) were found to have poor survival rates in the present cohort as well. Group 3 and Group 4 tumors have an overlapping gene expression profile but strikingly distinct survival rates. MiR-182 was found to be overexpressed in the majority of Group 3 tumors, while miR-592 was found to be overexpressed in the majority of Group 4 tumors. Group 3/Group 4 medulloblastomas overexpressing miR-182 or underexpressing miR-592 were found to have significantly poor overall survival rates. MiR-592 and miR-182 could therefore act as surrogate markers for Group 3/Group 4 classification and as markers for risk stratification of non-WNT, non-SHH FFPE medulloblastomas.

In summary, a real-time RT-PCR based expression analysis of 12 protein-coding genes and 9 miRNAs accurately classified medulloblastomas into 4 molecular subgroups. The miRNA based classification was found to be particularly useful for FFPE tumor tissues, as miRNAs are known to be relatively resistant to fragmentation during formalin fixation. Further, the inclusion of oncogenes like *MYCN* and miRNAs like miR-182 and miR-592 in the assay not only helps in classification but can also help in risk stratification. Further study on a larger dataset would be necessary to confirm the role of miRNAs in prognostication.

## Supplementary Material

Supplementary material is available online at *Neuro-Oncology* (<http://neuro-oncology.oxfordjournals.org/>).

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*Conflict of interest statement.* None declared.

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