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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 microRNAs 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. 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. 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
of molecularly distinct subgroups.\textsuperscript{3–5} 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.\textsuperscript{6} In addition to the clinical parameters, molecular classification of medulloblastomas is now necessary for better risk assessment and management of the disease.\textsuperscript{7}

MicroRNAs (miRNAs) are 18- to 22-nucleotide-long noncoding RNA molecules that regulate expression of the protein-coding genes.\textsuperscript{8} MiRNAs bind to complementary sequences in the 3′ untranslated regions of multiple target genes, usually resulting in their silencing.\textsuperscript{9} Each miRNA is believed to target several hundred genes. Altered miRNA expression has been reported in various cancers.\textsuperscript{10,11} 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.\textsuperscript{12} 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.\textsuperscript{13} 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.\textsuperscript{14,15}

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.\textsuperscript{16} 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-μm sections were deparaffinized using xylene followed by absolute ethanol washes and subsequent digestion with proteinase K overnight at 55 °C in Tris–sodium dodecyl sulfate–NaCl–EDTA buffer as per the protocol described by Korbl et al,\textsuperscript{17} followed by acid phenol–chloroform or phenol-chloroform extraction and ethanol precipitation for isolation of RNA and DNA, respectively.\textsuperscript{18} 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 μg) 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 $RQ = 2^{(\text{Ct}_{\text{Gene}} - \text{Ct}_{\text{Ref}}) \times 100}$.

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). The expression levels of the marker genes obtained as RQs by real-time RT-PCR were log2 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. 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. 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 under-expression 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 NRP3, MYC, and IMP2 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. 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 groups was based on our data and other reports on the differential miRNA expression in medulloblastoma subgroups. 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-133b 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. 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 ~8 cycles was observed between the average Ct values of RNU48 (19 ± 1.7) and GAPDH (27 ± 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

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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 log2 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 medulloblastomas 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) 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 ≥0.9. Twenty-six of 29 Group 4 tumors and 14 of 18 Group 3 tumors had predicted posterior probabilities ≥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 (≥18 y) were SHH (65%) and WNT (35%; Fig. 3B). The ratio of male to female patients in the WNT subgroup was lowest, at ≏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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95% CI of ratio</th>
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<tbody>
<tr>
<td>Percent Survival - SHH subgroup MYCN high vs. low (n = 20)</td>
<td>14.89</td>
<td>1.573 to 140.9</td>
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<tr>
<td>Percent Survival - Group 3 and 4, (n = 37)</td>
<td></td>
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<tr>
<td>Group 3 vs. Group 4</td>
<td>5.318</td>
<td>1.424 to 19.86</td>
</tr>
<tr>
<td>miR-592 (low vs. high)</td>
<td>6.647</td>
<td>1.721 to 25.66</td>
</tr>
<tr>
<td>miR - 182 (high vs. low)</td>
<td>3.527</td>
<td>1.045 to 11.90</td>
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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. 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. 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 children (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. 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. 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 subgrouping 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. MYCN amplification has been shown to associate with relatively inferior survival in the SHH medulloblastomas. 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.
References

Original Article

Distinctive microRNA signature of medulloblastomas associated with the WNT signaling pathway

ABSTRACT

Aim: Medulloblastoma is a malignant brain tumor that occurs predominantly in children. Current risk stratification based on clinical parameters is inadequate for accurate prognostication. MicroRNA expression is known to be deregulated in various cancers and has been found to be useful in predicting tumor behavior. In order to get a better understanding of medulloblastoma biology, miRNA profiling of medulloblastomas was carried out in parallel with expression profiling of protein-coding genes.

Materials and Methods: miRNA profiling of medulloblastomas was carried out using Taqman Low Density Array v 1.0 having 365 human microRNAs. In parallel, genome-wide expression profiling of protein-coding genes was carried out using Affymetrix gene 1.0 ST arrays.

Results: Both the profiling studies identified four molecular subtypes of medulloblastomas. Expression levels of select protein-coding genes and miRNAs could classify an independent set of medulloblastomas. Twelve of 31 medulloblastomas were found to overexpress genes belonging to the canonical WNT signaling pathway and carry a mutation in CTNNB1 gene. A number of miRNAs like miR-193a, miR-224/miR-452 cluster, miR-182/miR-183/miR-96 cluster, and miR-148a having potential tumor/metastasis suppressive activity were found to be overexpressed in the WNT signaling associated medulloblastomas. Exogenous expression of miR-193a and miR-224, two miRNAs that have the highest WNT pathway specific upregulation, was found to inhibit proliferation, increase radiation sensitivity and reduce anchorage-independent growth of medulloblastoma cells.

Conclusion: Expression level of tumor/metastasis suppressive miRNAs in the WNT signaling associated medulloblastomas is likely to determine their response to treatment, and thus, these miRNAs would be important biomarkers for risk stratification within the WNT signaling associated medulloblastomas.

KEY WORDS: Medulloblastoma, miRNA profile, molecular subtype, WNT signalling

INTRODUCTION

Medulloblastoma is a highly malignant brain tumor that occurs predominantly in children. Medulloblastomas are located in the cerebellar region of the brain and have a tendency to spread through the cerebrospinal fluid. Therefore, standard post-operative treatment not only includes local radiotherapy but also craniospinal radiation and chemotherapy. One-third of the patients are incurable, while the long term survivors suffer from permanent neurological deficits resulting from the intensive therapies administered to the developing child brain. All medulloblastomas are classified pathologically as grade IV tumors. Molecular markers for risk stratification are required, so that standard risk patients can be spared from excessive treatment and survival of high risk patients can be improved. Understanding of the molecular mechanism/s underlying the pathogenesis of medulloblastomas is crucial for designing novel targeted therapies, which could be more effective and free of undesirable side effects.

Most microRNA expression analyses of human cancers have arrived at the common conclusions that miRNAs are deregulated in cancer, and miRNA expression profile represents tumor biology better than the expression profile of protein-coding genes. In order to get a better understanding of medulloblastoma biology, miRNA profiling of medulloblastomas was carried out using Taqman Low Density array v 1.0 having 365 human microRNAs. In parallel, genome-wide expression profiling of protein-coding genes was carried out using Affymetrix gene 1.0 ST arrays. Both the profiling studies segregate medulloblastoma tumor tissues into almost identical molecular subtypes.

MATERIALS AND METHODS

Tumor tissue specimens of sporadic medulloblastomas and normal cerebellar tissues were procured with the approval of the Institutional Review Board of Tata Memorial Centre, Kharghar, Navi Mumbai 410210, Maharashtra, India.
miRNA signature of WNT signaling associated medulloblastomas

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Review Board after getting informed consent from the patients. Immediately following surgery, tumor tissues were snap-frozen in liquid nitrogen and stored at −70°C. Normal cerebellum tissues were obtained from the Brain Tissue Repository, National Institute of Mental Health and Neurosciences, Bangalore, India.

Total RNA was extracted from tumor tissues using mirVANA kit Ambion, Austin, TX, USA, as per the manufacturer’s protocol after ensuring at least 80% tumor cell content. Normal cerebellar tissues labeled NC01 and NC02 were from 6-month and 2-month old infants, while NC03 and NC04 were from 4-year and 35-year old males, respectively. Total RNA (100 ng) was reverse transcribed using stem-loop RT primer pools from Applied Biosystems (Foster City, CA, USA). Polymerase chain reactions (PCRs) were carried out using the Taqman Low Density Arrays v 1.0 on ABI 7900HT Fast real time RT-PCR system. Relative quantities (RQ) of each miRNA in each of the tissue samples as compared to the endogenous control small RNA RNU48 were computed by comparative Ct method.

Total RNA extracted, as described before, was further purified using RNeasy columns (Qiagen, Valencia, CA, USA), as per the manufacturer’s instructions. RNAs having more than 7.0 RIN value and no detectable genomic DNA contamination were used for the analysis (Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA). RNA (100 ng) was reverse transcribed, amplified, and labeled with biotin using the whole transcript sense target labeling kit and hybridized to gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA), as per the manufacturer’s instructions.

Data normalization was done using GCRMA algorithm in the Bioconductor package of the R statistical environment (http://www.bioconductor.org). Protein-coding genes and miRNAs significantly differentially expressed in each cluster were identified by Significance Analysis of Microarrays (SAM) analysis and t-test, respectively (http://www.TM4.org). Hierarchical clustering and bootstrap analysis steps were implemented using MeV module of TM4 package (http://www.TM4.org), miRNA target prediction common to at least two adjacent exons, and wherever possible, were located at exon boundaries to avoid amplification of genomic DNA. Expression was analyzed by SYBR Green assay using GAPDH as a housekeeping gene control. The differential expression of a select set of protein-coding genes and miRNAs was confirmed by real time RT-PCR analysis. Total RNA (500 ng) was treated with amplification grade RNase-free DNase (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using MMLV-RT (MBI Fermentas, Burlington, Canada). Primers were designed such that they correspond to two adjacent exons, and wherever possible, were located at exon boundaries to avoid amplification of genomic DNA. Expression was analyzed by SYBR Green assay using GAPDH as a housekeeping gene control. Expression of each miRNA was analyzed using specific Taqman assay. Each assay was validated using RNA expressing specific miRNA as a positive control and RNA (no RT) as a negative control. RNU48 was used as an endogenous control RNA. Relative expression levels were quantified by comparative Ct method. Analysis was also done using RNU44 as an endogenous control for confirmation (data not shown). Supplementary Table 1 lists sequences of the primers used.

Human medulloblastoma cell line Daoy (ATCC, Manassas, VA, USA) was grown in Dulbecco’s Modified Eagle Medium DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO2. Daoy cells were transfected with 100 nM of miR-193a mimic, miR-224 mimic, or miR-23b mimic using Dharmafect 2 reagent, as per the manufacturer’s protocol (Dharmacon, Lafayette, CO, USA) for a period of 48 h. miRNA levels in transfected cells were estimated by real time RT-PCR analysis using RNU48 as an endogenous control RNA. As a negative control, Daoy cells were transfected with 100 nM of siGLO (a RISC-free control siRNA) or siRNA negative control (Dharmacon, Lafayette, CO, USA). microRNA mimic negative controls from Dharmacon were found to affect proliferation of Daoy cells. The transfected cells were allowed to recover for a period of 24 h before analyzing their growth characteristics. For MTT reduction assay, miRNA transfected Daoy cells were plated at a density of 500 cells/well of a 96-well microtiter plate. Growth of these cells was followed over a period of 8 days with replenishment of the medium every 3rd day. 20 μl of MTT (5 mg/ml) was added to each well at the end of the incubation period and the cells were incubated further for a period of 4 h. 100 μl of 10% sodium dodecyl sulfate (SDS) in 0.1 N HCl was added per well to dissolve the dark blue formazan crystals. Optical density was read on an Enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 540 nm with a reference wavelength of 690 nm.

For thymidine incorporation assay, 2500 miRNA transfected cells were plated per well of a 96-well microtiter plate. The cells were incubated in the presence of 1 μCi of tritiated thymidine (specific activity 240 Gbq/mmole, Board of Radiation and Isotope Technology, Navi Mumbai, India) per well for a period of 20 h before harvesting by trypsinization. Tritiated thymidine incorporated was estimated by scintillation counting. Radiation sensitivity of the miRNA transfected cells was studied by clonogenic assay. 1000 miRNA transfected cells were plated per 55 mm plate and then irradiated at a dose of 6 Gy (Cobalt-60 gamma irradiator, developed by Bhabha Atomic Research Centre, India). The medium was changed 24 h later and the cells were allowed to grow for 6-8 days until microscopically visible colonies formed. The cells were fixed by incubation in chilled methanol/acetic acid and the colonies were visualized by staining with 0.5% crystal violet.

Anchorage-independent growth of the miRNA transfected cells was studied by their potential to form colonies in soft agar. 10,000 cells were seeded in DMEM/10% FBS medium containing 0.3% agarose. The cells were seeded onto a basal layer containing 1% agarose. The cells were incubated for
about 3–4 weeks and the colonies formed were counted. All the experiments were performed in triplicates. Student’s t-test was performed to evaluate statistical significance of the difference in the performance of miRNA-transfected cells as compared to siGLO or siRNA negative control transfected cells.

RESULTS

Gene expression profiling of 19 medulloblastoma tumor tissues was done using Affymetrix gene 1.0 ST array that contains probe sets for 28,869 genes. Unsupervised hierarchical clustering using 1000 most differentially expressed genes segregates tumor tissues into four clusters, viz. ‘A’, ‘B’, ‘C’, and ‘D’ with a bootstrap support of 100% for each cluster [Figure 1A]. Hierarchical clustering of medulloblastomas using the most significantly differentially expressed genes (FDR < 1%) is shown in Supplementary Figure 1.

Of the 365 miRNAs studied, 216 were found to be expressed in medulloblastomas. Hierarchical unsupervised clustering using these 216 miRNAs segregates tumors into clusters/subtypes similar to those identified by expression profiling of protein-coding genes [Figure 1B]. Figure 2 is a heat map depicting the expression of selected miRNAs that are significantly differentially expressed in the four medulloblastoma subtypes and normal cerebellar tissues. The protein-coding genes and the miRNAs significantly differentially expressed in each subtype are listed in Supplementary Tables 2 and 3, respectively. Real-time PCR analysis confirmed the expression of selected set of protein-coding genes and miRNAs in the four molecular subtypes. Molecular subtyping of an independent set of 12 medulloblastomas was done by analyzing the expression of these selected marker genes and miRNAs [Figure 3].

Subtype A (six tumors) is characterized by the overexpression of a number of genes involved in the canonical WNT signaling pathway like WIFI, DKK1, DKK2, DKK4, AXIN2, LEF1, NKF1, and MYC. Based on the overexpression of WIFI and GABRE, six out of 12 tumors from an independent set of medulloblastomas were found to belong to subtype A [Figure 3]. WNT pathway activation in these tumors was confirmed by sequencing exon 3 of CTNB1 gene that codes for the N-terminal region of β-catenin protein. A point mutation was found in all 12 subtype A tumors that modified either the serine residues 533 or 537 which get phosphorylated or the neighboring D32 or I35 residue in the N-terminal region of the β-catenin protein [Supplementary Figure 2].

Subtype A has the most robust miRNA signature with 16 miRNAs differentially expressed as compared to the normal cerebellar tissues as well as all other subtypes [Table 1]. A number of miRNAs like miR-193a, miR-224/miR-452 cluster, miR-182/miR-183/miR-96 cluster, miR-365, miR-135a, miR-148a, miR-23b/miR-24/miR-27b cluster, miR-204, miR-146b, miR-449/miR-449b cluster, miR-335, and miR-328 are overexpressed by 3-100 fold almost exclusively in tumors associated with the WNT signaling pathway [Figure 2 and Supplementary Table 3]. Real time RT-PCR analysis confirmed significant overexpression of miR-224, miR-193a, miR-365, miR-148a, miR-182, and miR-23b in the WNT signaling associated medulloblastomas [Figure 3]. miR-224 and miR-452 belong to a single cluster located in the intron of GABRE gene coding for GABA receptor. The gene GABRE and miR-224/miR-452 are specifically expressed in subtype A tumors. miR-224 cluster, therefore, appears to be co-expressed with GABRE gene. GABRE is overexpressed exclusively in all nine tumors having WNT pathway activation of Kool et al., data set as well.

Three tumors show overexpression of SHH signaling components that include HHIP, ATOH1, MYCN, PTCH1, and GLI2. One tumor from the additional set belongs to subtype B as it shows overexpression of MYCN and ATOH1. All four subtype B tumors underexpress TRPM3 gene and miR-204 as well as miR-135b [Figure 3, Table 1]. Two of the B subtype tumors, HMED13
and HMED22, show overexpression of MYCNOS. MYCNOS is a MYCN related gene that is expressed from the DNA strand complementary to the MYCN coding strand. MYCNOS and MYCN are known to be co-expressed in MYCN amplified tissues. A 30-60 fold increase in copy number of MYCN gene was confirmed by real-time PCR analysis of genomic DNA from these two tumors (data not shown). miR-23b, miR-27b and miR-24 belong to a single miRNA cluster that is located in an intron of C9orf3 gene. Two subtype B tumors lacking MYCN amplification overexpress C9orf3 and miR-23b, while the two having MYCN amplification underexpress miR-153. C9orf3 and miR-23b cluster miRNAs are overexpressed in all subtype A tumors of our data set as well as Kool et al., data set. C9orf3 is overexpressed in 8 out of 15 subtype B tumors of Kool et al., data set.[7]

Remaining 10 medulloblastomas segregate into cluster C (2 tumors) and cluster D (8 tumors). Non-WNT, non-SHH subtype medulloblastomas in Kool et al., study segregate into three subtypes, viz. C, D and E, with overlapping gene signature.[7] The genes specifically expressed in tumors belonging to C, D and E subtypes of Kool et al., data set as well as those belonging to C and D subtypes of our data set include transcription factors involved in brain development, viz. EOMES and FOXG1B, a testes specific gene LEMD1, and genes involved in neuronal migration like UNCSD and EPHA8. Expression of neuronal differentiation related genes in subtypes C and D and the expression of retinal differentiation related genes in subtypes D and E distinguish the three subtypes C, D and E of Kool et al., data set. Subtype C tumors from our data set overexpress retina-specific genes like CRX, NRL, TULP1, PDE6H and underexpress most neuronal differentiation genes like GRM1, GRM8, MYRIP, thus resembling subtype E of Kool et al., data set. Equivalence of our subtype C to subtype E data set is supported further by the expression of subtype E specific genes like GABRB5, SMARCD3 as well as the overexpression of cell cycle genes and a number of ribosomal protein coding genes in subtype C tumors of our data set.

Subtype D tumors of our data set express a number of neuronal differentiation genes including those encoding synaptic proteins like MYRIP, SYN2, SYT6, SYT13, those involved in transmission of nerve impulse like GRM1, GRM8, GABARAPL1, GABBR2, GABRG2, as well as genes involved in axon guidance like EPHA6, EPHB1, EFNB1, RND1, RND2, and SEMA3A. None of the subtype D medulloblastomas from our data set overexpress retinal differentiation genes like CRX, NRL, TULP1, and PDE6H. Therefore, subtype D of our data set is equivalent to subtype C of Kool et al., data set.

Table 1: Representative miRNAs significantly differentially expressed in each molecular subtype of medulloblastomas as compared to all the other subtypes as well as the normal cerebellar tissues

<table>
<thead>
<tr>
<th>Clusters</th>
<th>Markers</th>
<th>miRNAs up-regulated / down-regulated</th>
<th>WNT Signaling</th>
<th>SHH Signaling</th>
<th>Proliferation Differentiation</th>
<th>Differentiation Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>mir-193a 71.68 mir-199a 3.10</td>
<td></td>
<td></td>
<td>mir-135b 21.48</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>mir-183 58.40 mir-92 2.81</td>
<td></td>
<td></td>
<td>mir-193b 4.81</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>mir-224 50.30 mir-565 2.29</td>
<td></td>
<td></td>
<td>mir-19a 4.58</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>mir-182 45.60 mir-135b 0.01</td>
<td></td>
<td></td>
<td>mir-32 2.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mir-452 39.13 mir-204 0.03</td>
<td></td>
<td></td>
<td>mir-204 0.01</td>
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<td></td>
<td></td>
<td>mir-204 14.13 mir-163 0.09</td>
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<td></td>
<td>mir-153 0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mir-365 10.43 mir-135a 0.27</td>
<td></td>
<td></td>
<td>mir-410 0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mir-23b 9.93 mir-148a 9.31</td>
<td></td>
<td></td>
<td>mir-487d 0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mir-27b 7.34 mir-24 5.97</td>
<td></td>
<td></td>
<td>mir-433 0.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mir-146b 4.58 mir-335 3.25</td>
<td></td>
<td></td>
<td>mir-127 0.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mir-134 0.18 mir-181d 0.20</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>mir-9 0.27 mir-181c 0.35</td>
<td></td>
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</tr>
</tbody>
</table>

The number displayed against each miRNA indicates the ratio of the mean expression level of the miRNA in a specific subtype with its mean expression level in all the other subtypes. The underlined text indicates underexpression or downregulation, while the rest indicates overexpression or upregulation.
miR-135b is upregulated in tumors belonging to subtypes C and D. miR-135b is located in an intron of LEMD1 gene that is overexpressed in the C and D subtype medulloblastomas. miR-204 and miR-153 are underexpressed in subtype B and C medulloblastomas. miR-204 is located in an intron of TRPM3 gene that is downregulated in B and C subtype tumors. TRPM3 gene is underexpressed in all subtype B and 9 out of 11 subtype E medulloblastomas of Kool et al., data set.[7]

HMED16 and HMED18 form a sub-cluster of cluster D on hierarchical cluster analysis of microarray profiling data of the protein-coding genes. These tumors segregate into a distinct cluster (D2) on hierarchical cluster analysis of miRNA data. Sub-cluster D2 (two tumors) differs from sub-cluster D1 (six tumors) based only on the overexpression of genes encoding various extracellular matrix proteins and TGF-β signaling components. Gene expression profile of cluster D2 is a characteristic wound healing signature.[8] This wound healing signature is also evident in all cluster B tumors and some cluster A tumors of our data set as well as those of Kool et al., data set. miR-214 and miR-199a are overexpressed in all these tumors having overexpression of wound healing pathway genes.

Five tumors from the additional set belong to subtype C or D as they overexpress LEMD1, KHDRBS2 and miR-135b. Three out of the five tumors most likely belong to subtype C as they underexpress TRPM3, miR-204 and GRM8 (a neuronal differentiation marker). miR-153 is underexpressed in two out of these three tumors. Thus, overexpression of LEMD1, KHDRBS2 and miR-135b is specific for subtypes C and D, while underexpression of TRPM3, GRM8 and miR-204 further distinguishes subtype C from subtype D [Figure 3 and Table 1].

miR-17-92, a polycistronic miRNA cluster, has been reported to be overexpressed in a wide variety of human cancers. Overexpression of miR-17-92 cluster miRNAs in A, B and C subtype medulloblastomas is consistent with their reported upregulation by MYC, MYCN and E2F transcription factors.[9, 10] Normal adult cerebellums have the least expression of miR-17-92 cluster miRNAs. miR-106b and miR-25, which belong to miR-17-92 paralog cluster, are also overexpressed in all medulloblastomas as compared to the normal cerebellar tissues.

miR-379/miR-656 cluster miRNAs located within an imprinted region on chromosome 14 are underexpressed in subtype A, B and C tumors as compared to normal cerebellar tissues and subtype D tumors.[11] miR-379/miR-656 cluster miRNAs may play a role in neural differentiation as suggested by their predominant expression in the brain. miR-127/miR-432/miR-433 miRNA cluster on chromosome 14 is also similarly underexpressed in subtype A, B and C tumors. miR-127 has been reported to be underexpressed in various other cancers as a result of promoter hypermethylation.[12] miR-124a is also considerably downregulated in A, B and C subtype medulloblastomas and has been shown to promote neural differentiation by triggering brain-specific pre-mRNA alternate splicing.[13]
To understand the functional significance of miRNAs overexpressed in medulloblastomas associated with WNT signaling activation, three miRNAs, viz. miR-193a, miR-224 and miR-23b, were exogenously expressed in Daoy cell line established from human sporadic medulloblastoma. miR-193a and miR-224 are the most highly and specifically upregulated miRNAs in cluster A tumors, while miR-23b is overexpressed in both A and B subtype tumors. miR-193a and miR-224 expression in Daoy cells is comparable to normal developing cerebellar tissues. miR-23b expression in Daoy cells is higher than that of miR-193a or miR-224, while it is still about four-fold lower than that in normal developing cerebellar tissues. Transfection of 100 nM of miRNA mimics in Daoy cells resulted in 10-100 fold increase in miRNA expression. A 50-100 fold overexpression of miR-193a in Daoy cells resulted in 50-60% growth inhibition, while 10-15 fold overexpression of miR-23b resulted in 1.6-1.8 fold increased proliferation of Daoy cells as judged by thymidine incorporation assay [Figure 4A]. miR-193a induced growth inhibition and miR-23b mediated proliferation stimulation of Daoy cells was also evident on analysis by MTT assay [Figure 4B]. A 10-15 fold miR-224 overexpression, on the other hand, showed a marginal difference on proliferation of Daoy cells by the thymidine incorporation assay, while the MTT assay demonstrated growth inhibitory effect. Five hundred cells were plated per well for the MTT assay, while 2500 cells/well were plated for the thymidine incorporation assay. It, therefore, appears that the difference in the behavior of miR-224 transfected Daoy cells is likely to be due to the difference in plating density, indicating increased growth factor requirement of miR-224 transfected Daoy cells. This observation is further supported by the fact that plating efficiency of miR-224 transfected Daoy cells was found to be reduced by 50% in clonogenic assay [Figure 5A]. Thus, miR-224 appears to reduce proliferation of Daoy cells in a density-dependent manner. The plating efficiency of miR-193a transfected Daoy cells was found to be reduced by almost 80%, while the plating efficiency of miR-23b transfected Daoy cells did not change significantly from control cells. Irradiation at a dose of 6 Gy resulted in about 70% reduction in the number of colonies formed by control siGLO or siRNA transfected Daoy cells in clonogenic assay. miR-193a overexpressing Daoy cells on irradiation at a dose of 6 Gy failed to form any colonies, while irradiation of miR-224 overexpressing Daoy cells resulted in more...
than 90% reduction in colony formation. No significant change was observed in radiation sensitivity of miR-23b overexpressing Daoy cells [Figure 5A]. miR-224 and miR-193a overexpression in Daoy cells was found to bring about 60 to 90% reduction in soft agar colony formation [Figure 5B]. There was no significant difference in the number of soft agar colonies formed by miR-23b overexpressing cells as compared to siGLO or control siRNA transfected cells.

**DISCUSSION**

Genome-wide expression profiling of protein-coding and miRNA coding genes identified almost identical four molecular subtypes of medulloblastomas. 38% (12 out of 31) medulloblastomas in our study were found to carry a mutation in CTNNB1 gene and thereby WNT pathway activation. Median age at diagnosis for WNT signaling associated medulloblastomas is reported to be higher (10.4 years) than that reported for medulloblastomas (6 years).[7] Median age at diagnosis of subtype A tumors in our data set is also high (12 years). Four out of 12 medulloblastoma patients belonging to the WNT subtype in our study are adults. Prevalence of SHH signaling associated medulloblastomas is reported in children less than 3 years of age.[7] Lack of medulloblastomas from children less than 3 years of age can explain lower number of medulloblastomas with SHH signaling activation in our data set. Even if SHH signaling associated tumors are not taken into account, medulloblastomas associated with WNT signaling appear to be more common in the Indian subcontinent (38% incidence vs. reported incidence of 10-15%), which needs to be confirmed on a larger data set. Six out of 12 (50%) subtype A tumors belong to female patients, while 5 out of the rest 19 (20%) patients in our data set are females. In Kool et al., data set as well, 44% (4/9) of subtype A tumors as compared to 32% (12/37) of the rest of the medulloblastomas (excluding subtype B tumors) belong to female patients.[7] Prevalence of medulloblastomas resulting from deregulated WNT pathway activation in females over 3 years of age probably explains better survival of female patients in this age group as reported from the retrospective analysis of 1226 medulloblastoma cases.[15]

Medulloblastomas having WNT pathway activation have been reported to have lower metastatic potential and better survival rates.[7,14] One out of 10 informative subtype A patients of our data set had metastasis at the time of diagnosis as compared to 4 out of 13 subtype C and D patients [Supplementary Table 4]. Higher incidence of metastasis at diagnosis in subtype C and D tumors has also been reported by Kool et al.[7] Expression profile of subtype A tumors seems paradoxical to this observation. Subtype A tumors have higher expression of genes encoding ribosomal proteins, cell cycle regulators and genes encoding components of RAS-MAPK, TGF-β and NOTCH signaling pathways as compared to the subtype D tumors. Robust overexpression of a number of miRNAs in subtype A tumors is similar to the robust expression of negative regulators of WNT signaling like WIFI, DKK family genes, AXIN2, NKD1, NKD2. Many of these miRNAs are likely to be direct/indirect transcriptional targets of mutant β-catenin protein and may target components of WNT signaling machinery. Predicted targets of subtype A specific miRNAs include the WNT signaling components. miR-135a has been shown to target APC and its levels correlate with APC levels in colorectal cancer.[16] APC gene is downregulated in subtype A medulloblastomas. WNT1-inducible signaling pathway protein 2 (WISP2) has been shown to be a direct target of mir-449, another miRNA overexpressed in subtype A tumors.[17]

Overexpression of miR-193a and miR-224, the two most upregulated miRNAs in subtype A tumors, was found to inhibit proliferation, increase radiation sensitivity and inhibit anchorage-independent growth of medulloblastoma cells. Overexpression of miR-224 has been shown to promote apoptosis of hepatocarcinoma cells and API5 has been shown to be a target of miR-224.[18] miR-193a expression has been found to be downregulated in oral squamous cell carcinoma cell lines as a result of tumor-specific hypermethylation of CpG islands and its ectopic expression has been found to be growth inhibitory to these cell lines.[19] miR-193a has been reported to be downregulated in various types of solid tumors in a study that was done on 2532 tumor tissues.[20] mir-23b cluster miRNAs have been shown to inhibit migration of hepatocellular carcinoma cells and inhibit TGF-β signaling by targeting SMAD proteins.[21,22]

Among other miRNAs overexpressed in subtype A tumors, miR-148a has been shown to be downregulated as a result of promoter hypermethylation in cancer cell lines established from lymph node metastasis and further shown to inhibit motility, tumor growth and metastasis on overexpression.[23] Overexpression of mir-183 in lung cancer cells has recently been shown to inhibit migration and invasion of lung cancer cells and Ezrin has been identified as a bonafide target of mir-183.[24] Thus, while the expression of proliferation stimulating and apoptosis inhibitory genes like MYC, CCND1, BIRC5 drives tumorigenesis resulting from activated WNT signaling, expression of miRNAs like miR-193a, miR-224, miR-148a, miR-183 appears to contribute to lower metastatic potential and better response to radiation therapy and thereby better survival rate of these medulloblastomas.

Subtype C medulloblastomas have been reported to have the highest metastatic potential followed by subtype D tumors.[7] miR-135b overexpressed in subtype C and D medulloblastomas has been shown to be upregulated in relapsed prostate cancer patients, indicating the oncogenic nature of this miRNA.[25] miR-124a and mir-137 are known to induce differentiation of glioma stem cells.[26] Relatively higher expression of these miRNAs in subtype D medulloblastomas is consistent with the expression of various differentiation related genes in these tumors. Subtype B and C tumors have lower expression of miRNAs like miR-204 and miR-153 whose predicted targets include components of TGF-β signaling pathway. Ferreti et
al., have reported underexpression of miR-153 in high risk medulloblastomas which are either metastatic or belong to children less than 3 years of age. [27] These high risk tumors are likely to belong to the subtypes B and C. Thus, potential oncogenic miRNA miR-135b is overexpressed while the potential tumor-suppressive miRNAs like miR-204 and miR-153 are underexpressed in C and D subtype tumors having higher metastatic potential.

In summary, genome-wide expression profiling of both protein-coding genes and miRNAs segregates medulloblastomas into four molecular subtypes. These four molecular subtypes closely match the four molecular variants reported in a recent study of genome wide expression profiling coupled with DNA copy number alterations in medulloblastomas. [28] Relative expression levels of a select set of protein-coding genes and miRNAs could successfully identify these molecular subtypes in an independent set of medulloblastomas and thus they can serve as markers for molecular subtyping. A number of miRNAs having potential tumor/metastasis suppressive role were found to be overexpressed in WNT signaling associated medulloblastomas. Exogenous expression of miR-193a and miR-224, two miRNAs that have the highest WNT pathway specific upregulation, was found to inhibit proliferation, increase radiation sensitivity and reduce anchorage-independent growth of medulloblastoma cells. Detailed functional studies on miRNAs differentially expressed in WNT signalling associated medulloblastomas and correlation of their expression with clinical outcome on a larger sample size would indicate if these miRNAs could serve as important biomarkers for risk stratification.

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REFERENCES