RESULTS

Chapter 4
Deregulation of gene expression profiles and disruption of molecular networks is a hallmark of cancer, and knowledge of this has been vital towards better understanding of a particular cancer. Gene expression profiling is a useful method for identifying and studying deregulated gene expression in various cancers. It has proven to be valuable in more accurately classifying tumors, predicting clinical outcome and thereby deciding treatment strategies. Genome-wide expression profiling data from our group as well as reports by other investigators have indicated that medulloblastoma is not a single disease, but is comprised of molecularly distinct subgroups [15-16, 58-59]. There are four core molecular subgroups: WNT, SHH, Group 3 and Group 4 according to the current consensus [17].

MicroRNAs (miRNAs) are a class of non-coding RNAs, 18-22 nucleotides in length that regulate the expression of protein-coding genes. MiRNAs bind to complementary sequences in the 3’-UTR region of multiple target genes usually resulting in silencing [19]. Each miRNA is believed to target several hundred genes. Most miRNA expression analysis of human cancers have arrived at a common conclusion that miRNAs are deregulated in cancer and miRNA expression profile represents tumor biology better than the expression profile of protein-coding genes. MiRNA expression profile has been found to have diagnostic and prognostic potential in the classification of various cancers [21-22]. Hence, in addition to protein-coding genes, it is crucial to study the role played by miRNAs and their functional significance in the development of medulloblastoma, for further understanding the pathogenesis of this tumor. Therefore, in the present study miRNA expression profiling was carried out on a set of medulloblastomas that were molecularly subgrouped based on genome-wide expression profiling of protein-coding genes.
The expression profiling study reported using Affymetrix gene 1.0 ST arrays was performed on 19 medulloblastomas and 4 normal cerebellar tissues [15]. The expression profile identified the four core subgroups, which are distinguished by their gene expression profiles. The 19 tumors comprised of 6 WNT, 3 SHH, 2 Group 3, and 8 Group 4 tumors. The WNT subgroup is best distinguished by overexpression of WNT inhibitory factor 1 (WIF1), Dickkopf (DKK) family, Naked cuticle 1 (NKD1), Lymphoid enhancer-binding factor 1 (LEF1), AXIN2, MYC etc. and having CTNNB1 mutation and monosomy 6. SHH subgroup tumors overexpress Hedgehog interacting protein (HHIP), Atonal homolog 1 (ATOHI), Secreted frizzled-related protein 1 (SFRP1), GLI family zinc finger 2 (GLI2), Patched 1 (PTCH1), MYCN, Eyes absent homolog 1 (EYA1), etc. and show underexpression of Orthodenticle homeobox 2 (OTX2). Group 3 and 4 have an overlapping gene signature that includes overexpression of transcription factors involved in brain development like Eomesodermin (EOMES) and Forkhead box G1B (FOXG1B), RNA binding proteins like LEM domain containing 1 (LEMD1) and KH domain containing, RNA binding, signal transduction associated 2 (KHDRBS2). Group 3 tumors overexpress proliferation related genes like MYC, Cyclin D2 (CCND2), TGF-beta signaling components like Transforming growth factor-beta 1 (TGFB1), retina-specific genes like Interphotoreceptor matrix proteoglycan 2 (IMPG2), Neural retina leucine zipper (NRL), Cone-rod homeobox (CRX) and others like Natriuretic peptide receptor 3 (NPR3) while Group 4 tumors overexpress neuronal differentiation genes like Glutamate receptor, metabotropic 8 (GRM8), Potassium voltage-gated channel, shaker-related subfamily, member 1 (KCNA1), Unc-5 homolog D (UNC5D) etc. A heat map depicting the signature genes for each molecular subgroup is shown in Figure 4.1.
Figure 4.1: Heat map depicting expression of subgroup specific signature genes for each of the four subgroups. The dendrogram above the heat map clearly shows the four distinct clusters of medulloblastoma. Blue: WNT subgroup; Red: SHH subgroup; Yellow: Group 3 and Green: Group 4 [15].
MicroRNA profiling of medulloblastoma tissues so as to identify distinct molecular subgroups.

4.1. Identification of differentially expressed miRNAs by miRNA profiling

MiRNA profiling was carried out on the aforementioned 19 sporadic human medulloblastomas and 4 normal human cerebellar tissues using TaqMan Low Density Array v 1.0. Of the 365 miRNAs studied, 216 were found to be expressed in the medulloblastomas and/or normal cerebellar tissues. Significance analysis of Microarrays (SAM) at FDR 0 % was performed in order to identify the miRNAs significantly differentially expressed in each subgroup (Appendix I). Figure 4.2 shows a heat map depicting the expression of 54 miRNAs that are significantly differentially expressed in the four medulloblastoma subgroups and normal cerebellar tissues. The fold change of the miRNAs significantly differentially expressed in each medulloblastoma subgroup as compared to both normal as well as other subgroups is listed in Table 4.1.

Unsupervised hierarchical clustering using the expression data of the 216 miRNAs segregated the tumors into 4 clusters/subgroups with the normal samples segregating as a distinct cluster [Figure 4.3 (B)]. Bootstrap analysis was performed to determine the strength of each cluster. All four normal cerebellar tissues clustered together with a bootstrap support of 95 %. All 6 WNT subgroup tumors segregated as one distinct cluster with the highest bootstrap support of 100 %. Group 4 tumors were split as two sub-clusters on either side of the normal cerebellar tissues, with 5 tumors forming one cluster of support 43 % and 3 tumors forming another with a support of 38 %.
Figure 4.2: Heat map showing expression of 54 miRNAs significantly differentially expressed in the molecular subgroups of medulloblastomas and normal cerebellar tissues as judged by SAM analysis with a False Discovery Rate of 0%. Grey: Normal; Blue: WNT; Red: SHH; Yellow: Group 3; Green: Group 4. The miRNAs in the yellow box within the heat map represent the most upregulated/ downregulated miRNAs in each of the four subgroups. MiRNAs forming a part of a cluster have been shown on the right. The expression levels are represented as log10 transformed RQ values.
Table 4.1: MiRNAs significantly differentially expressed in each medulloblastoma subgroup as compared to both normal cerebellar tissues as well as other subgroups as obtained by SAM analysis at FDR 0 %. Ratio of the mean expression level of each miRNA in a specific subgroup with its mean expression level in other subgroups is indicated as fold change (FC). Red denotes activated/upregulated while green denotes downregulated.

The two tumors of Group 3 were clustered together with a bootstrap support of 42 %. Two of the three tumors of SHH subgroup having MYCN amplification formed one cluster with 63 % bootstrap support. Thus hierarchical cluster analysis on miRNA profile data clustered all but one SHH subgroup tumor similar to the clusters obtained using expression profile data of protein-coding genes, although, the overall bootstrap support was not as strong [Figure 4.3 (A)].
Chapter 4: Results

Figure 4.3: Support tree analysis. (A) Bootstrap analysis of microarray profiling data of medulloblastomas using 1000 high SD genes. Samples were clustered using Pearson correlation and average linkage [15]. (B) Bootstrap analysis of miRNA profiling data of 19 medulloblastomas and 4 normal cerebellar tissues done using 216 miRNAs expressed in medulloblastoma tumor tissues. Analysis was done on log10 transformed miRNA RQ values. The number on each node denotes the percentage of times a given node was supported following 100 iterations. Grey: Normal, Blue: WNT subgroup, Red: SHH subgroup, Yellow: Group 3, Green: Group 4.

The specific miRNA profiles of each of the four medulloblastoma subgroups are described below and depicted in Figure 4.2.

4.1.1. WNT subgroup

The WNT subgroup tumors showed the most robust miRNA signature with 16 miRNAs differentially expressed as compared to normal cerebellar tissues as well as all other subgroups. A number of miRNAs like miR-193a, miR-224/452 cluster, miR-182/183/96 cluster, miR-365, miR-135a, miR-148a, miR-23b/27b/24, miR-204, miR-146b, miR-449/449b cluster, miR-335, and miR-328 were found to be overexpressed by 3-70 fold almost exclusively in the tumors associated with the WNT signaling pathway [Figure 4.2 and Appendix I]. Most of the miRNAs belonging to a cluster (group) of miRNAs located next to each other on a chromosome were found to be co-expressed. MiR-224 and miR-
452 belong to a single miRNA cluster located in the intron of *GABRE* gene coding for GABA receptor, which is known to be overexpressed in the WNT tumors. Similarly, miR-23b/27b/24 cluster is located in the intron of *C9ORF3* gene, which was found to be overexpressed in all WNT tumors. These miRNAs therefore appear to be co-expressed with their host genes.

### 4.1.2. SHH subgroup

The SHH subgroup tumors were distinctive in their marked underexpression of miR-135b, miR-204, miR-153, and miR-182. One of the SHH tumors reported to have *C9ORF3* overexpression was found to have upregulation of the miR-23b cluster.

### 4.1.3. Group 3 and 4

MiR-135b, a miRNA located in an intron of the *LEMD1* gene, was found to be over-expressed in both Group 3 and Group 4 tumors. *LEMD1* gene is also overexpressed in these tumors. MiR-204 and miR-153, underexpressed in SHH subgroup tumors, were also found to be underexpressed in Group 3 tumors. MiR-204 is located in an intron of *TRPM3*, a gene which has been reported to be downregulated in SHH and Group 3 tumors [15]. While miR-182 was found to be over-expressed in some Group 3 / Group 4 tumors, miR-204 was seen to be over-expressed in most Group 4 medulloblastomas.

### 4.1.4. MiRNAs common to multiple subgroups of medulloblastomas

MiR-17/92 cluster miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a) were overexpressed in WNT, SHH and Group 3 medulloblastomas. Normal adult cerebellar tissues had the least expression of miR-17/92 cluster miRNAs. MiR-106b and miR-25, which belong to miR-17/92 paralog cluster, were also overexpressed in all
medulloblastomas relative to normal cerebellar tissues. MiR-214 and miR-199a were found to be overexpressed in all the tumors having overexpression of wound healing pathway genes (*TGFB1, ITGA1, COL3A1, PDGFRB, IGFBP4*, etc.). MiR-379/miR-656 cluster miRNAs, located within an imprinted region on chromosome 14, were found to be underexpressed in WNT, SHH, and Group 3 tumors as compared to normal cerebellar tissues and Group 4 tumors. MiR-127/miR-432/miR-433 miRNA cluster on chromosome 14 was also similarly underexpressed in WNT, SHH, and Group 3 tumors. Another miRNA, considerably down-regulated in WNT, SHH and Group 3 medulloblastomas was miR-124a.

Besides this, several miRNAs were highly expressed in normal cerebellum relative to medulloblastomas. Group 4 tumors appeared to have the most overlapping miRNA expression profile with the normal cerebellar tissues consistent with the expression of neuronal differentiation related genes in this subgroup.

### 4.2. Validation of the differential miRNA expression in the molecular subgroups of medulloblastomas

#### 4.2.1. Molecular sub-grouping based on the expression profile of protein-coding genes

Differential miRNA expression was validated on a set of 44 fresh frozen (inclusive of 18 profiled tumors) and 59 FFPE tissues. Molecular sub-grouping of 103 medulloblastomas was done by a real-time RT-PCR based evaluation of expression levels of a set of protein-coding genes as markers. Twelve genes (*WIF1, DKK2, MYC, HHIP, EYA1, MYCN, IMPG2, NPR3, GRM8, UNC5D EOMES, OTX2*) were selected as markers based on the standardized fold change in the expression of the gene in a particular subgroup from our profiling data (Appendix II) as well as that in other published reports [16, 58].
Overexpression or under-expression of a gene was defined as expression above or below a cut-off level, based on the mean ± confidence interval as well as the inter-quartile range of the expression levels of that gene in the entire cohort and its known level of over or underexpression in its target subgroup.

Overexpression of *WIF1*, *DKK2*, and *MYC* identified WNT subgroup medulloblastomas. Over-expression of *HHIP*, *EYA1*, *MYCN* and under-expression of *OTX2* served as markers for the SHH subgroup. Over-expression of *EOMES* helped to identify Group 3 and Group 4 tumors while higher expression of *NPR3*, *MYC*, and *IMPG2* and lower expression of *GRM8*, *UNC5D* helped in distinguishing Group 3 tumors from Group 4 tumors. 98/103 cases were reliably classified based on the expression of the 12 marker genes. Five of 59 FFPE medulloblastomas were unclassifiable due to poor RNA quality. The set of 44 fresh frozen medulloblastoma tissues comprised of 12 WNT, 8 SHH, 11 Group 3 and 13 Group 4 cases while the set of 54 FFPE medulloblastomas consisted of 10 WNT, 20 SHH, 9 Group 3 and 15 Group 4 medulloblastomas. The scatter dot plot [Figure 4.4] shows the differential expression of the 12 marker genes in 103 medulloblastomas classified in the four subgroups.

WNT pathway activation in the WNT subgroup tumors was further confirmed by sequencing exon 3 of *CTNNB1* gene that codes for the N-terminal region of β-catenin protein. Seven out of eight FFPE WNT subgroup medulloblastomas, which could be analyzed for *CTNNB1* exon 3 sequence, were found to harbor a single point mutation that altered the serine residues S33 or S37 (whose phosphorylation leads to *CTNNB1* protein degradation) or the neighboring aspartate, D32, residue in the N-terminal region of the β-catenin protein, validating their subgroup identification [Figure 4.5]. Mutations in *CTNNB1* gene in the twelve fresh WNT subgroup medulloblastomas have been previously reported [15].
Figure 4.4: Molecular subgrouping of medulloblastomas by real-time RT-PCR using 12 protein-coding genes. The scatter dot plot shows log 2 transformed relative expression levels (Y-axis) of the indicated protein-coding genes in the 103 medulloblastomas assigned to the four molecular subgroups (X-axis). The horizontal black line within each cluster denotes the median level of expression. The p-value given on the top of each scatter indicates the significance of the differential expression of the marker gene across the four subgroups as determined by ANOVA.

4.2.2. Differential miRNA expression in the molecular subgroups of medulloblastomas

The expression of a select set of eleven miRNAs (miR-193a-3p, miR-224, miR-148a, miR-23b, miR-365, miR-182, miR-135b, miR-204, miR-592, miR-10b, and miR-376a) most significantly differentially expressed was validated by real-time RT-PCR. The selection of miRNAs differentially expressed in the four subgroups was based on the profiling data using SAM analysis (Appendix I) as described earlier. MiR-592 was not present on the TLDA array and was selected for validation based on published reports of its differential expression in Group 3 and Group 4 tumors [59, 83].
Figure 4.5: Mutation analysis of exon 3 of β-catenin (CTNNB1) in the WNT subgroup FFPE medulloblastomas: Nucleotide Sequence of exon 3 of the CTNNB1 gene in 7 FFPE WNT subgroup medulloblastomas. Arrow indicates the altered nucleotide and (*) indicates the altered amino acid.
Validation was done on a total of 101 medulloblastomas. Total RNA was not available for 2 fresh frozen tumor tissues for miRNA expression analysis. WNT subgroup tumors showed significant overexpression \((p < 0.0001)\) of miR-193a-3p, miR-224, miR-148a, miR-23b, miR-365 and miR-10b as compared to other subgroup medulloblastomas thereby confirming the distinctive miRNA signature of the WNT subgroup medulloblastomas. MiR-182 over-expression \((p < 0.0001)\) was confirmed in all WNT subgroup medulloblastomas. In addition, many \((17/21)\) Group 3 \((p < 0.0001)\) and some \((7/29)\) Group 4 medulloblastomas \((p < 0.0462)\) also showed significant miR-182 over expression. MiR-204 was overexpressed \((p < 0.0001)\) in all WNT subgroup medulloblastomas and in most \((25/29)\) Group 4 medulloblastomas \((p < 0.0001)\). Underexpression of miR-182 \((p < 0.0001)\), miR-135b \((p < 0.0001)\), and miR-204 \((p < 0.0001)\) was confirmed in SHH subgroup medulloblastomas. Group 3 and Group 4 tumors showed significant overexpression of miR-135b \((p < 0.0001)\) as compared to WNT and SHH subgroup. MiR-592, a miRNA located within the \(GRM8\) gene, was significantly overexpressed \((p < 0.0001)\) in Group 4 medulloblastomas. MiR-10b was expressed at the highest level in WNT subgroup medulloblastomas \((p < 0.0001)\) while it was significantly underexpressed \((p < 0.0001)\) in SHH tumors. MiR-376a, which belongs to the miR-379/miR-656 cluster miRNAs, was found to be significantly higher \((p = 0.0082)\) in Group 4 medulloblastomas as compared to Group 3 medulloblastomas.

Real time RT-PCR, thus confirmed the differential miRNA expression, as seen by miRNA profiling, of a select set of miRNAs in the four molecular subgroups of an independent set of medulloblastomas. The scatter dot plot representing the differential miRNA expression across the four molecular subgroups in the 101 medulloblastoma samples is shown in Figure 4.6. The five FFPE medulloblastomas that were unclassifiable
due to poor RNA quality using protein-coding genes alone as markers were classified primarily based on their miRNA profile as discussed later.

Figure 4.6: Validation of differential miRNA expression in medulloblastomas by real time RT-PCR. The scatter dot plot shows log 2 transformed relative expression levels (Y-axis) of the indicated miRNA in 101 medulloblastomas assigned to the four molecular subgroups (X-axis). The horizontal black line within each cluster denotes the median level of expression. The p-value given on the top of each scatter indicates the significance of the differential expression of the marker gene across the four subgroups as determined by ANOVA.

A difference of ~ 8-cycles was observed between the average Ct values of RNU48 (19 ± 1.7) and GAPDH (27 ± 2.1) wherein 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,
Chapter 4: Results

the evaluation of miRNA expression was reliable, reproducible, and sensitive even in 7 to 8 yr old FFPE tumor tissues [Figure 4.7].

![Image](image.png)

**Figure 4.7:** House-keeping gene (*GAPDH*) and small RNA control (RNU48) Ct value correlation with age of the block. The Ct values for *GAPDH* and RNU48 were obtained by real time RT-PCR for each of the 59 FFPE medulloblastomas studied.

**Delineate the role of specific miRNAs in medulloblastoma pathogenesis.**

**4.3. Functional Significance of WNT- subgroup specific miRNAs on the growth and malignant potential of medulloblastoma cells**

MiRNA expression profiling indicated that the WNT subgroup tumors had the most robust miRNA signature. Medulloblastomas with activated WNT signaling pathway have been reported to have a lower metastatic potential and better prognosis [56]. In order to understand the functional significance of the 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 a human sporadic medulloblastoma. The effect of expression of these miRNAs on proliferation, clonogenic potential, radiation sensitivity, and anchorage-independent growth of Daoy cells was investigated.
4.3.1. Exogenous expression of WNT subgroup miRNAs in Daoy medulloblastoma cell line

MiR-193a and miR-224 were the most highly and specifically upregulated miRNAs in the WNT subgroup tumors, while miR-23b was overexpressed in both WNT and some SHH subgroup tumors. MiR-193a and miR-224 expression in Daoy cells was found to be comparable to that in normal developing cerebellar tissues (RQ: 0.05-0.19 and 0.009-0.11 respectively). MiR-23b expression in Daoy cells was higher than that of miR-193a or miR-224, while it was still about four-fold lower than that in normal developing cerebellar tissues (RQ: 0.38-0.51). Transient transfection of 100 nM of each of the three miRNA mimics in Daoy cells resulted in 10-100 fold increase in miRNA expression, as assessed by real time RT-PCR. MiR-193a mimic transfection resulted in 50-100 fold overexpression, while 10-15 fold overexpression was obtained with miR-23b and miR-224 in Daoy cells as compared to control siGLO transfected cells.

4.3.2. Effect of expression of WNT- subgroup miRNAs on proliferation of Daoy cells

The effect of miR-193a, miR-224, and miR-23b on the proliferation of Daoy cells was studied by thymidine incorporation assay. Exogenous expression of miR-193a in Daoy cells resulted in 50-60 % growth inhibition (p < 0.0001); while that of miR-23b resulted in 1.6-1.8 fold increase in proliferation of Daoy cells (p < 0.0001) when compared to the cells transfected with control siGLO on Day 3 [Figure 4.8 (A)]. Mir-224 overexpression, on the other hand, did not show a significant difference in the proliferation of Daoy cells.
Figure 4.8: Functional analysis of WNT – specific miRNAs on the growth and malignant potential of medulloblastoma cells. (A) Growth kinetics of miRNA mimic transfected Daoy cells by thymidine incorporation assay. Histogram shows the counts per million of thymidine incorporated in proliferating miRNA or control siGLO transfected Daoy cells over a period of 5 days obtained using a beta- scintillation counter. (B) Plating efficiency and radiation sensitivity of miRNA transfected Daoy cells by clonogenic assay. Histogram indicates number of colonies formed by control/ miRNA transfected Daoy cells with or without irradiation. Colonies were visualized and counted by staining with 0.5 % crystal violet after about a week of incubation (C) Anchorage- independent growth of miRNA transfected Daoy cells by soft agar colony formation assay. Histogram indicates number of colonies formed in soft agar medium by control/ miRNA transfected Daoy cells. Colony count was done using an inverted microscope after about a month of incubation. All data points are presented as Mean ± S.E. p value indicates significance with control siGLO using Students t-test.
4.3.3. Effect of expression of WNT- subgroup miRNAs on plating efficiency and radiation sensitivity of Daoy cells

Clonogenic assay was performed to study the effect of expression of miR-193a, miR-224, and miR-23b on the plating efficiency and radiation sensitivity of Daoy cells. Transient expression of miR-193a was found to reduce the plating efficiency of Daoy cells by almost 80 % (p < 0.0001). The plating efficiency of miR-224 transfected Daoy cells was found to be reduced by 50 % (p < 0.0006), while that of miR-23b transfected Daoy cells did not change significantly from control cells [Figure 4.8 (B)].

To evaluate the radiation sensitivity of these miRNA- transfected Daoy cells, effect of irradiation at a dose of 6Gy on the clonogenic potential of Daoy cells was studied. Irradiation at a dose of 6 Gy resulted in about 70 % reduction in the number of colonies formed by control siGLO transfected Daoy cells. 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 the radiation sensitivity of miR-23b overexpressing Daoy cells [Figure 4.8 (B)].

4.3.4. Effect of expression of WNT- subgroup miRNAs on anchorage – independent growth

The effect of expression of miR-193a, miR-224, and miR-23b on the anchorage independent growth of Daoy cells was investigated by soft agar assay. Daoy cells transiently expressing miR-193a showed a 90 % reduction (p < 0.0001) in the number of soft agar colonies formed as compared to siGLO transfected cells. MiR-224 overexpression in Daoy cells was found to bring about 60 % reduction (p < 0.0006) in soft agar colony formation. There was no significant difference in the number of soft agar
colonies formed by miR-23b overexpressing cells as compared to siGLO transfected cells [Figure 4.15 (C)].

4.4. Development of an assay based on real time RT-PCR for molecular classification of medulloblastomas

Based on the differential expression of miRNAs in the four molecular subgroups an assay was developed for molecular classification of medulloblastomas. Due to the lack of sufficient number of significantly differentially expressed miRNAs in the non-WNT subgroups, a combination of 12 protein-coding genes and 9 miRNAs were tested as markers for molecular classification by PAM analysis. MiR-376a and miR-10b were excluded as markers from the analysis as their expression levels were found to be less consistent within a subgroup and considerably low as compared to other miRNAs. The set of 42 fresh frozen medulloblastoma tissues comprised 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. Heat map depicting the differential expression of the 21 markers across 101 medulloblastomas is shown in Figure 4.9.

PAM analysis using the 101 medulloblastomas as a training set showed a cross-validation accuracy of 99%. Figure 4.10 shows a centroid plot of all the marker genes and miRNAs used in the PAM analysis. Using a training set of 42 fresh frozen medulloblastomas, all FFPE tumors were accurately classified with the exception of two SHH subgroup tumors [Figure 4.11 and 4 (A) and (D)]. Four out of five tumors, which were 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 [Figure 4.9]. One of these five tumors belonging to the WNT subgroup was found to possess mutation in CTNNBI gene further confirming its classification [Figure 4.5].
Figure 4.9: Heat map showing differential expression of 12 protein-coding genes and 9 miRNAs in the 101 tumor tissues. * 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. The clinical profile of each medulloblastoma case is indicated below the heat map. Age: <3yr- blue, 3-8yr-brown, 9-17yr-green, ≥18yr-yellow; Gender: Male-blue, Female-pink; Histology: Classic-grey, Desmoplastic-yellow, Large cell/anaplastic-orange. The number of fresh and FFPE tumor tissues within each subgroup is denoted below the clinical profile by the white and black bars respectively.
Chapter 4: Results

Figure 4.10: Centroid plot. Shrunken centroids for the 21 markers (12 genes + 9 miRNAs) employed as the PAM classifier are depicted for each of the 4 molecular subgroups using the training set of all 101 medulloblastomas.

The assay was validated on a set of well-annotated 34 FFPE medulloblastoma tumor tissues from DKFZ, Germany. The tumor tissue RNAs from this cohort were analyzed for expression of the 12 protein-coding genes and 9 miRNAs by the present real time RT-PCR assay [Figure 4.12]. PAM analysis using the training set of 42 fresh frozen tumor tissues accurately classified all DKFZ FFPE tissues with the exception of one Group 4 (pMB 47) tumor misclassified as Group 3 tumor [Figure 4.11 (B) and (E)]. The present real-time RT-PCR assay was thus found to have an overall accuracy of 97%. The two SHH subgroup medulloblastomas that were misclassified using our fresh frozen tumor...
tissues as a training set were correctly classified using both the DKFZ FFPE and our profiled fresh frozen tumor sets for training [Figure 4.11 (C) and (F)]. This misclassification was therefore likely to be due to the insufficient number of SHH subgroup tumors in our training set of fresh frozen tumors. The overall predicted posterior probabilities for all WNT subgroup and 31 out of 33 SHH subgroup tumors were ≥ 0.9. Twenty six out of 29 Group 4 tumors and 14 out of 18 Group 3 tumors had predicted posterior probabilities ≥ 0.8 [Figure 4.11 (D) and (E)].

**Figure 4.11: Classification of medulloblastoma samples using PAM.** The subgroup prediction matrix showing the performance of the classifier (12 protein-coding genes and 9 miRNAs) on (A) our FFPE dataset (B) the DKFZ FFPE tumors and (C) our FFPE and non-profiled fresh tissues, as the test set (D), (E) and (F) depict the predicted test probabilities for (A), (B) and (C) respectively for each of the four subgroups. RQ values obtained by real time RT-PCR were log 2 transformed for PAM analysis.
Figure 4.12: Heat Map showing expression profile of 12 protein-coding genes and 9 miRNAs in the DKFZ FFPE medulloblastoma set. A and B indicate subgroup assignment by nanoString assay and PAM prediction using the present real time RT-PCR assay respectively.

ROC curve analysis was done to assess the predictive power of the classifier using the predicted posterior probabilities of the test set obtained by PAM analysis for each of the four subgroups. The Area under Receiver Operating Curve (AUC) of 1.00 was obtained for all the four subgroups, indicating a high (near perfect) predictive power of the classifier [Figure 4.13]
Figure 4.13: ROC curve Analysis to determine the strength of the classifier: (A), (B), (C) and (D) represent the ROC curves for the four subgroups (Test variables) with the Area Under the Curve (AUC) specified in each plot. Predicted posterior probabilities of the 59 FFPE medulloblastomas for each subgroup obtained using the 42 fresh tissues as the training set and the 21-marker classifier for PAM analysis, served as the input for the ROC curve analysis.
4.5. Correlation of the molecular subgrouping and miRNA expression with clinical parameters

Further, the correlation between the clinico-pathologic variables and the molecular subgrouping of the patients used in the study was investigated. Demographic comparison revealed notable differences between the subgroups, as described below:

4.5.1. Correlation with age, gender and histology

Of the 103 medulloblastomas studied, 23 belonged to WNT subgroup, 30 to SHH subgroup, 21 to Group 3 and 29 belonged to Group 4 [Figure 4.14 (A)].

a) Age: The median age at diagnosis for the entire cohort was 9 yr (< 1 yr – 45 yr), but varied across the four subgroups. Median age at diagnosis was 12 yr (7 – 45 yr) for WNT subgroup, 8 yr (< 1 – 36 yr) for SHH subgroup, 4.5 yr (1 – 13 yr) for Group 3 and 8 yr (4– 16 yr) for Group 4. Children of age < 3 yr belonged to SHH (67 %) or Group 3 (33 %) whereas; older children (> 8 yr) belonged primarily to Group 4 (40 %) or WNT subgroup (40 %). Adult patients (≥ 18 yr) belonged to either SHH (65 %) or WNT (35 %) subgroup [Figure 4.14 (B)].

b) Gender: The male-to-female ratio in the present cohort was 2.12:1. SHH subgroup comprised of 63 % males and 37 % females. Notably, while there were almost equal number of male and female patients in the WNT subgroup, 80 % (40/50) of patients in Group 3 and Group 4 were only males. Specifically, males were predominant in Group 4 (37 %) as compared to WNT (16 %), SHH (27 %) and Group 3 (20 %), whereas females were more common in the WNT subgroup (36 %) as compared to SHH (33 %), Group 3 (21 %) and Group 4 (9 %) [Figure 4.14 (C)].
c) **Histology:** Most of the tumors studied were of classical histology (79%) followed by tumors having large cell/anaplastic (10.6%) and desmoplastic (10.6%) histology. Tumors with classic histology were distributed among the four subgroups with the WNT subgroup showing predominance. Desmoplastic tumors were restricted to the SHH subgroup. Large cell/anaplastic variant which is a known indicator of poor prognosis, although present in SHH, Group 3 and Group 4 tumors, was more predominant in Group 3 tumors (64%) [Figure 4.14 (D)].

**Figure 4.14: Demographic profile of the four molecular subgroups.** (A) Distribution of the 103 medulloblastomas based on subgroups (B) Age at diagnosis distribution for the four subgroups, (C) Gender based distribution and (D) Distribution of histological variants. The numbers indicate the number of tumors in each category.
4.5.2. Correlation with overall survival

The overall survival data was available for 72 medulloblastoma cases with a median follow-up of 16.7 months. The patients who expired within the first month after surgery were excluded from the analysis as the death in peri-surgery period could be due to surgery related causes. Kaplan Meier analysis showed survival curves to be significantly different ($p = 0.0046$) for the four subgroups [Figure 4.15 (A)] with the best survival for the WNT subgroup patients (83 %) and the worst survival for Group 3 patients (27 %), while Group 4 and SHH subgroup patients had intermediate survival of close to 70 %.

Among the histological variants patients having large cell / anaplastic histology tumors had significantly ($p=0.0017$) worse survival (25 %) as compared to those having classic (68 %) or desmoplastic histology (88 %) [Figure 4.15 (B)].

Within the SHH subgroup, patients having tumors with $MYCN$ over-expression levels comparable to $MYCN$ amplification levels were found to have significantly ($p = 0.0185$) poorer survival (40 % vs. 70 %) [Figure 4.15 (C)].

In the combined cohort of Group 3 and Group 4 medulloblastomas cases, those with miR-592 overexpression were found to have significantly ($p = 0.0060$) better survival (72 % vs. 25 %) while those with miR-182 overexpression were found to have significantly ($p = 0.0422$) worst survival (45 % vs. 70 %) [Figure 4.15 (E) and (F)].

The difference in the survival rates of non-WNT, non-SHH subgroup medulloblastoma cases having miR-592 underexpression with those having overexpression (25 % vs.72 %) was comparable to the difference in the survival rates of Group 3 vs. Group 4 patients (27 % vs.72 %) [Figure 4.15 (D)]. The hazard ratios for the Group 3/4 combined cohort indicated that tumors with underexpression of miR-592 have a significantly higher risk
(6.65) than those with overexpression of miR-182 (3.53), but marginally higher than the risk of patients belonging to Group 3 (5.32).

Figure 4.15: Kaplan-Meier survival analysis: Overall survival of 72 patients in the present cohort separated on the basis of (A) subgroup, (B) histological variants, (C) SHH subgroup tumors with and without MYCN over-expression (D), Group 3 vs. Group 4 tumors (E), non-SHH, non-WNT tumors with or without miR-592 over-expression and (F) with or without miR-182 over-expression. p value indicates level of significant difference in the Kaplan Meier survival curves estimated by Log Rank Test. The table below shows the results of univariate survival analysis using the variables listed. The hazard ratios for the variables tested are indicated with the corresponding confidence interval for that group.