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Original Investigation

Differential Expression of microRNAs in Medulloblastoma and the Potential Functional Consequences

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ABSTRACT

AIM: To identify differentially expressed microRNAs in medulloblastoma, and to investigate their biological function.

MATERIAL and METHODS: Differentially expressed microRNAs were identified using gene chips, and significantly different microRNAs were selected for verification using real time quantitative PCR. Potential target genes and their biological pathways were predicted by bioinformatics software.

RESULTS: Our analysis identified two microRNAs, hsa-miR-208a-3p and hsa-miR-1207-5p, which were significantly downregulated in medulloblastoma. Bioinformatics analysis identified potential target genes in the Wnt and MAPK signaling pathways, including NLK, RAPGEF2, CACNA2D1, DUSP3, MAPK8IP3.

CONCLUSION: Downregulation of hsa-miR-208a-3p and hsa-miR-1207-5p may be involved in the occurrence of medulloblastoma, through modulations of the Wnt and MAPK signaling pathways.

KEYWORDS: Medulloblastoma, hsa-miR-208a-3p, hsa-miR-1207-5p, Target gene, Wnt signaling pathway, MAPK signaling pathway

INTRODUCTION

Medulloblastoma is a brain tumor that occurs with high incidence, accounting for 10%-20% of all central nervous system tumors in children (5). MicroRNAs (miRNAs) are highly conserved noncoding RNA, which have been shown to inhibit translation, control mRNA shear, promote mRNA degradation and regulate the expression of target genes. miRNAs have been linked to the occurrence and development of tumors (2,8). In medulloblastoma, miRNA also plays an important role; previous research has shown that miR-124a, miR-9, and miR-125a were significantly downregulated in medulloblastoma (4). Because of the complex miRNA regulatory network, through collaboration among multiple miRNAs, high throughput chip hybridization technology seemed a suitable approach for screening tumors to identify differentially expressed miRNAs. At present, the available miRNA chip cover all the miRNA expressed in all species. This study used miRNA chip screening to identify differentially expressed miRNAs in medulloblastoma, and verified the interest of these miRNAs using real time quantitative PCR. We also explored the potential function of the miRNAs using bioinformatics methods, predicting the potential target genes and enriched biological pathways. This gave some insight into the possible biological function of the differentially expressed miRNAs in medulloblastoma.

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MATERIAL and METHODS

Samples

The specimens used for these studies included three cases of tumor and three samples of corresponding neighboring tissue; tissue adjacent to the tumor was used as the negative control. Tissues were collected after informed consent from patients with medulloblastoma being treated by the neurosurgery department of Children's Hospital, Chongqing Medical University. Samples were stored in liquid nitrogen before RNA extraction. The patients were aged 1 to 6 years old, and included two males and one female.

miRNA microarray

miRNA slides were prepared as described by Zhang et al. (19). The seventh generation miRCURYTM LNA Array (v.18.0, Exiqon) was used and contains 3100 capture probes that cover all human, mouse and rat microRNAs annotated in miRBase 18.0, all viral microRNAs related to these species, and capture probes for 25 miRPlus™ human microRNAs.

Total RNA was isolated using TRIzol (Invitrogen) and the miRNeasy Mini Kit (QIAGEN) per the manufacturers' instructions; we recovered miRNAs. RNA quantity and quality were determined using a NanoDrop ND-1000 spectrophotometer; integrity of RNA was determined by gel electrophoresis. Isolated miRNAs were labeled using the miRCURY™ Hy3™/Hy5™ Power Kit (Exiqon, Vedbaek, Denmark) per the manufacturer's instructions. Each sample (1 µg) was 3'-end-labeled with a fluorescent Hy3™ label using T4 RNA ligase: 2.0 µL of RNA solution was mixed with 1.0 µL of CIP buffer and CIP (Exiqon), incubated at 37°C for 30 minutes, and then the reaction was terminated by heating at 95°C for 5 minutes. Next, 3.0 µL of labeling buffer, 1.5 µL of Hy3, 2.0 µL of dimethylsulfoxide, and 2.0 µL of labeling enzyme were added and the mixture incubated at 16°C for 1 hour. The reaction was terminated by heating to 65°C for 15 minutes.

Next, the Hy3™-labeled samples were hybridized with the miRCURYTM LNA Array per the user manual. The 25-µL mixture from Hy3 labeling was mixed with hybridization buffer (25 µL) and denatured at 95°C for 2 minutes, then cooled on ice for 2 minutes. The labeled probe was incubated with the microarray at 56°C for 16–20 hours in a 12-Bay Hybridization System (Hybridization System-Nimblegen Systems, Inc., Madison, WI, USA). Afterwards, the slides were washed several times with wash buffer (Exiqon), dried by centrifugation at 400 rpm for 5 minutes, and scanned using an Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA).

Data Analysis

For grid alignment and data extraction, scanned images were imported into GenePix Pro 6.0 software (Axon). Expression data were normalized using median normalization. Replicated miRNAs were averaged and miRNAs with intensity ≥30 in all samples were used for calculating the normalization factor. MiRNAs with significantly different expression were identified by volcano plot. R Script was used for hierarchical clustering.

Bioinformatics Analysis

We used the Targetscan, microRNA.ORG and miRDBA databases to identify target genes shared across the three databases for pathway analysis, using all pathways in the genetic information of the KEGG and BIOCARTA. Depending on the known function of the target genes, we used Gene Ontology Analysis to calculate how many target genes were identified, obtain the target genes GO annotation clustering and investigate enrichment of the biological pathways.

Real time Quantitative PCR

We reverse transcribed RNA into cDNA by real time PCR to detect the expression of target genes, using expression of the U6 gene as an internal reference. The PCR reaction mix included 1 µl cDNA, 10 µl SYBR premix Taq, 0.5 µl each of upstream and downstream primers, and 8 µl RNase-free H_2O. Cycle parameters were as follows: 95°C for 30 seconds, followed by 45 cycles of 95°C for 5 seconds, 60°C for 30 seconds. This was followed by a melt curve reaction: 95°C for 15 seconds, then slowly heating from 55°C to 95°C, for a total of 40 cycles. After the reactions were finished, the melting curves were generated.

RESULTS

RNA Quality Control

For pure RNA, the absorbance ratio 260 nm/280 nm should be 1.8–2.1, and OD 260 nm/230 nm should be >1.8 (Table I). When RNA samples are analyzed by gel electrophoresis, the 28S and 18S ribosomal RNA bands should be sharp and intense, with the upper band having about twice the intensity of the lower. Smaller, relatively diffuse bands may be present, arising from low molecular weight RNAs (tRNA and 5S ribosomal RNA). A diffuse smear is commonly seen between the 18S and 28S ribosomal bands and largely consists of mRNA and other heterogeneous RNA species. DNA contamination is evident as a high-molecular-weight smear, or a band migrating above the 28S ribosomal RNA, as described by Zhang et al. (19). RNA degradation is indicated by smearing of the ribosomal RNA bands (Figure 1).

Chip data analysis and quantitative PCR found 48 miRNAs upregulated more than two fold in medulloblastoma, and 57 miRNAs downregulated more than two fold. The significant altered miRNAs are shown in Figure 2 and Table II.

Largest Differences in Multiples of 20 miRNAs Bioinformatics Analysis

Since the main function of miRNA is to regulate gene expression, we investigated the target genes of our identified miRNA in the Targetscan, microRNA.ORG and miRDBA databases. Enriched biological pathways were predicted according to the pathways in KEGG and BIOCARTA. The top ten pathways (sorted according to p values) are shown in Table III. We found that the predicted target genes of hsa-miR-647, hsa-miR-318, hsa-miR-208a-3p, hsa-miR-1207-5p, hsa-miR-1283 and hsa-miR-3202 were enriched in the Wnt signaling pathway, the MAPK signaling pathway and other pathways in cancer.

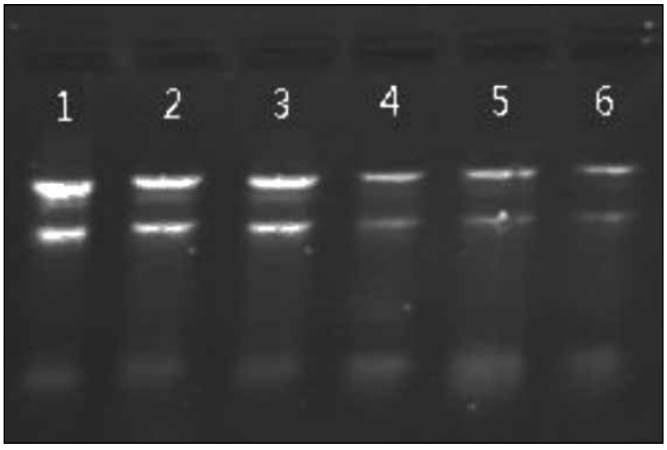

Figure 1: RNA Integrity and DNA contamination test by Denaturing Agarose Gel Electrophoresis.

Real time Quantitative PCR

In order to verify the reliability of the chip, we choose hsa-miR-647, hsa-miR-3183, hsa-miR-208a-3P, hsa-miR-1207-5P, and hsa-miR-3202 for real time quantitative PCR validation; a sample amplification curve as shown in Figures 3A-E. Hsa-miR-208a-3p and hsa-miR-1207-5p were downregulated in cancer tissue, consistent with the results of the chip.

The ΔCt of hsa-miR-647 was significantly different between tumor samples and control samples ($t=3.068$, $p<0.05$), an average of $2^{-\Delta\Delta Ct} = 2^{-1.483} = 0.36$, was downregulated in cancer tissue, and was not consistent with the result of chip. The ΔCt of hsa-miR-3183 was significantly different between tumor samples and control samples ($t=6.361$, $p<0.05$), an average of $2^{-\Delta\Delta Ct} = 2^{-3.01} = 0.12$, was downregulated in cancer tissue, and was not consistent with the result of chip. The ΔCt of hsa-miR-208a-3p was significantly different between tumor

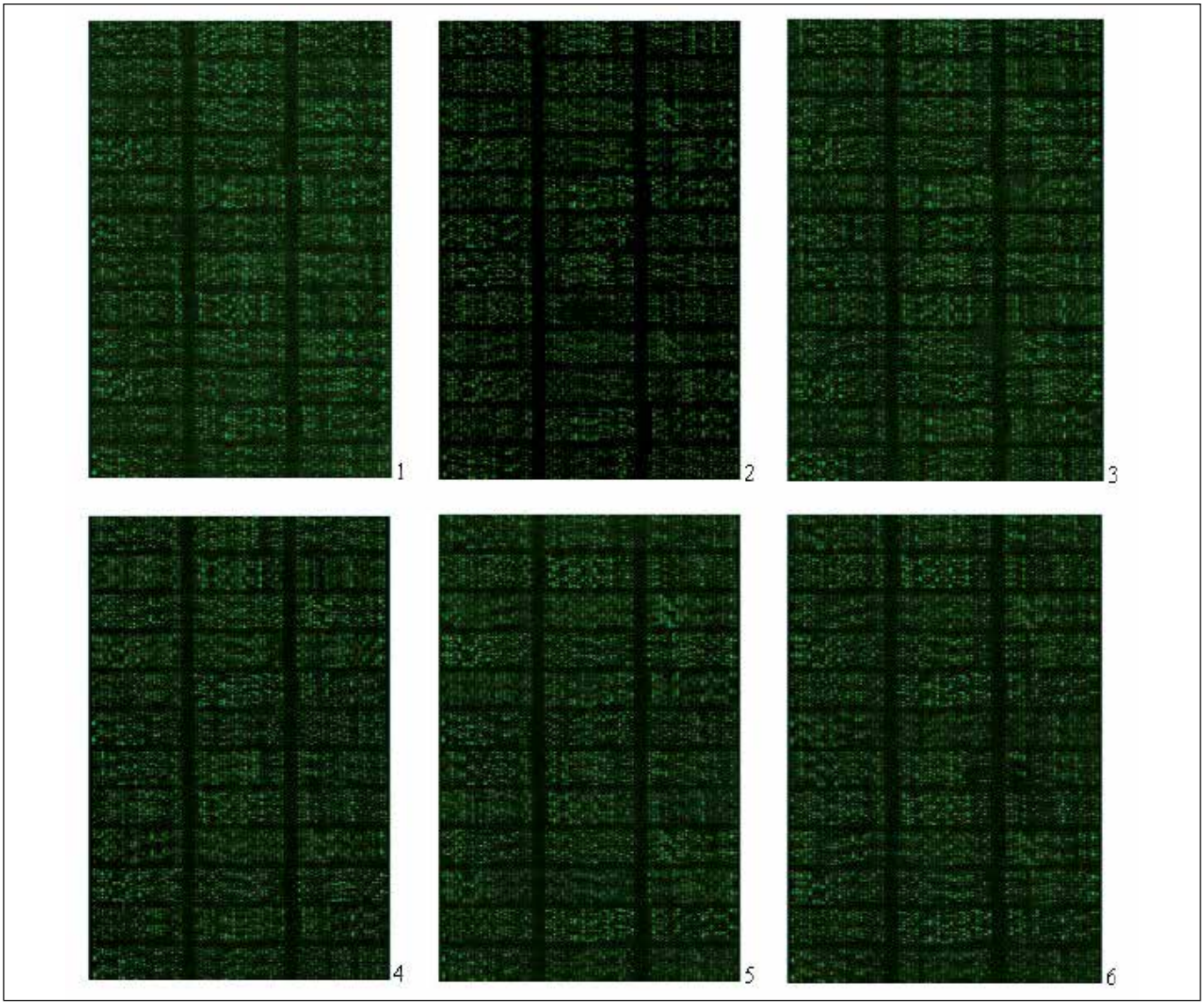

Figure 2: miRNA chip figures. Samples 1-3 were tumor tissue and Samples 4-6 were the corresponding negative controls.

Table I: RNA Quantification and Quality Assurance by NanoDrop ND-1000

Sample ID	OD260/280 Ratio	OD260/230 Ratio	Conc. (ng/µl)	Volume (µl)	Quantity (ng)	QC result pass or fail
1	2.01	2.36	2007.93	30	60237.90	pass
2	1.97	1.95	974.77	10	9747.70	pass
3	2.01	2.27	1713.17	40	68526.80	pass
4	1.98	2.29	827.37	20	16547.40	pass
5	1.97	2.06	1481.90	10	14819.00	pass
6	1.87	2.22	331.06	10	3310.60	pass

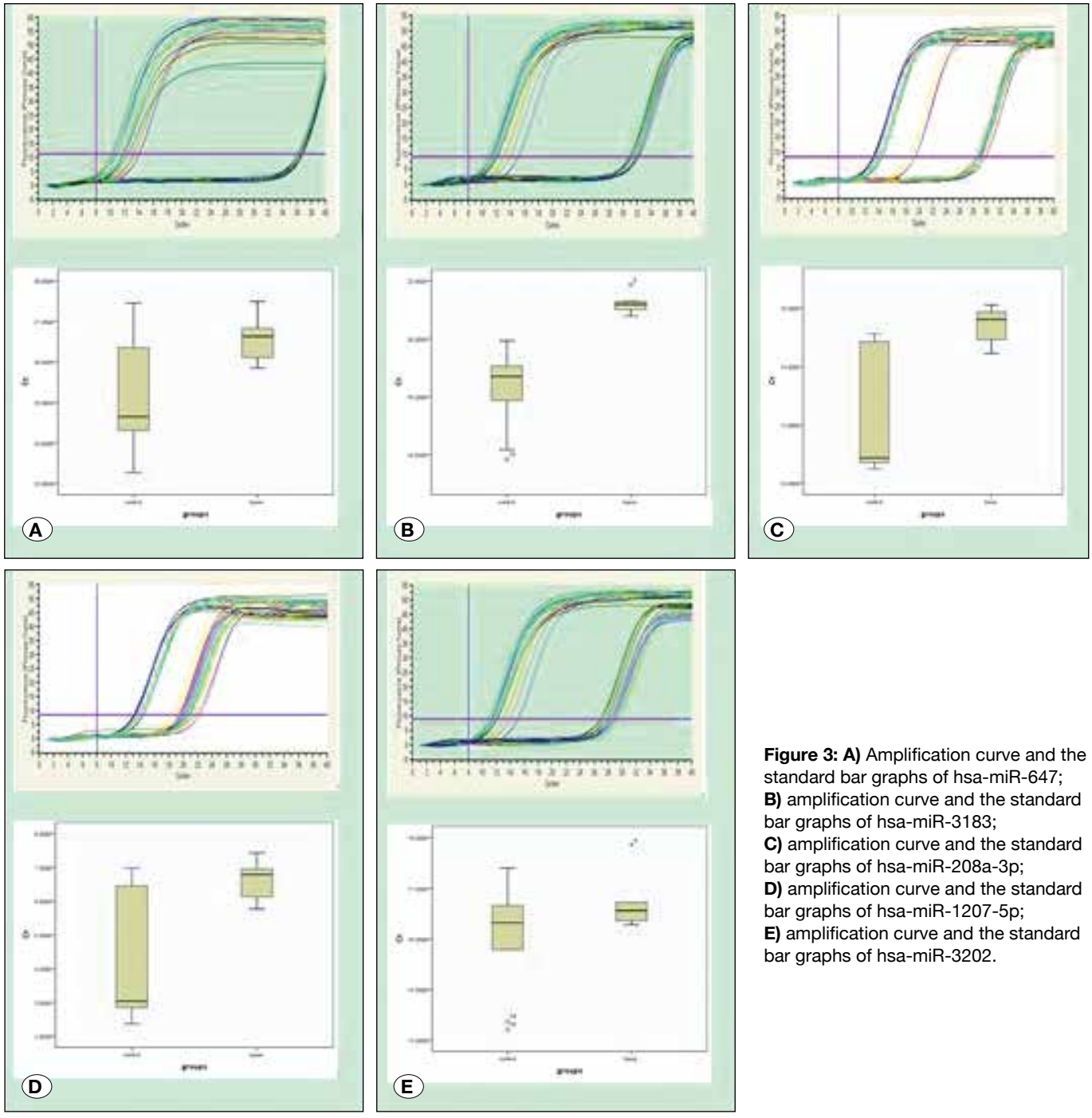

Figure 3: A) Amplification curve and the standard bar graphs of hsa-miR-647; **B)** amplification curve and the standard bar graphs of hsa-miR-3183; **C)** amplification curve and the standard bar graphs of hsa-miR-208a-3p; **D)** amplification curve and the standard bar graphs of hsa-miR-1207-5p; **E)** amplification curve and the standard bar graphs of hsa-miR-3202.

Table II: Significantly Differentially Expressed miRNAs

miRNA s	multiple
upregulated	
hsa-miR-4518	7.49977
hsa-miR-3651	6.07914
hsa-miR-196a-3p	4.14677
hsa-miR-5689	3.19520
hsa-miR-5096	3.78320
hsa-miR-647	2.14198
hsa-miR-3183	2.13916
…	
downregulated	
hsa-miR-138-5p	0.07818
hsa-miR-5010-5p	0.16658
hsa-miR-3665	0.19920
hsa-miR-208a-3p	0.21806
hsa-miR-1283	0.23606
hsa-miR-1207-5p	0.31042
hsa-miR-3202	0.38940
…	

samples and control samples (t=4.384, p<0.05), an average of $2^{-\Delta\Delta Ct} = 2^{-3.266} = 0.10$, was downregulated in cancer tissue, and was consistent with the result of chip. The ΔCt of hsa-miR-1207-5p was significantly different between tumor samples and control samples (t=3.671, p<0.05), an average of $2^{-\Delta\Delta Ct} = 2^{-2.496} = 0.18$, was downregulated in cancer tissue, and was consistent with the result of chip. The ΔCt of hsa-miR-3202 was not significantly different between tumor samples and control samples (t=1.650, p>0.05), an average of $2^{-\Delta\Delta Ct} = 2^{-0.647} = 0.64$, had no obvious difference in expression between tumor tissue and tissue adjacent to a tumor, but was not consistent with the result of chip.

DISCUSSION

Studies have shown that miRNAs can serve as oncogenes or tumor suppressor genes in regulation of cellular proliferation and apoptosis (16), and play an important role in cancer. Using a combination of chip technology and real time quantitative PCR, we found for the first time that hsa-miR-208a-3p and hsa-miR-1207-5p expression was significantly downregulated in medulloblastoma. Liu et al. (13) reported that hsa-miR-208a induced epithelial cells converted to mesenchymal cells, promoted the pancreatic cancer cell metastasis and invasion, the overexpression was concerned with the activation of AKT/GSK-3β/snail signaling pathway. Li et al. (12) have reported that hsa-miR-208a promoted the proliferation of human esophageal squamous cell carcinoma by inhibiting the expression of SOX6. Chen et al. (1) have reported that hsa-miR-1207-5p inhibited the growth of gastric cancer cells by acting on telomerase reverse transcriptase. Our finding that the expressions of hsa-miR-208a-3p and hsa-miR-1207-5p were

Table III: Pathway Analysis Results

Gene set name	Number of genes	Significance probability	Genes
KEGG_WNT_SIGNALING_PATHWAY	14	6.99E-07	*NLK,CHP,PPP3R1,GSK3B,WNT1,CCND1,FZD5,FZD3,PPP2CA,CAMK2G,PPP2R5E,CSNK2A2,DKK2,VANGL2*
KEGG_MAPK_SIGNALING_PATHWAY	15	7.62E-05	*NLK,CHP,PPP3R1,TGFBR1,STK4,FGF12,MEF2C,RAPGEF2,MAP3K2,CACNA2D1,SRF,DUSP3,MAPK8IP3,DUSP16,TAOK2*
KEGG_PATHWAYS_IN_CANCER	16	1.37E-04	*GSK3B,WNT1,CCND1,FZD5,FZD3,TGFBR1,STK4,FGF12,PTCH1,SUFU,RXRA,RARA,IGF1,CCDC6,ARNT,ETS1*
KEGG_ENDOCYTOSIS	11	7.15E-04	*SRC,SH3KBP1,SH3GLB2,SMURF1,RAB5B,ARF6,ARFGAP3,AGAP1,VPS37A,EHD2,SMAP2*
BIOCARTA_WNT_PATHWAY	5	7.15E-04	*NLK,GSK3B,WNT1,CCND1,PPP2CA*
KEGG_BASAL_CELL_CARCINOMA	6	2.20E-03	*GSK3B,WNT1,FZD5,FZD3,PTCH1,SUFU*
KEGG_MELANOGENESIS	7	7.71E-03	*GSK3B,WNT1,FZD5,FZD3,CAMK2G,GNAQ,CREB3L2*
BIOCARTA_GABA_PATHWAY	3	7.71E-03	*SRC,GABRA1,GABRA3*
BIOCARTA_P35ALZHEIMERS_PATHWAY	3	8.59E-03	*GSK3B,PPP2CA,CDK5R1*
BIOCARTA_GSK3_PATHWAY	4	8.59E-03	*GSK3B,WNT1,CCND1,PPP2CA*

downregulated in medulloblastoma suggests a role for these miRNAs in the tumor, but whether they are associated with tumor cell proliferation or invasion remains to be determined.

Given that hsa-miR-208a-3p and hsa-miR-1207-5p have largely unknown biological functions, we used bioinformatics methods to identify potential target genes and biological pathways. We used the Targetscan, microRNA.ORG and miRDBA databases to predict target genes. Hsa-miR-208a-3p had eight target genes shared across the three databases, and hsa-miR-1207-5p had 81 target genes. Because of the complex regulation network of miRNAs, a miRNA could control multiple target genes, or one gene could be regulated by multiple miRNAs at the same time. Therefore, we focused on enriched biological pathways for the target genes to gain a broader understanding of miRNA function in medulloblastoma. Target genes were mainly enriched in the Wnt signaling pathway, MAPK signaling pathway and other cancer pathways, suggesting that the candidate target genes of hsa-miR-208a-3p and hsa-miR-1207-5p were closely involved in cancer's biological processes. Hence hsa-miR-208a-3p and hsa-miR-1207-5p were likely to control these genes and participate in the occurrence of medulloblastoma.

Particularly notable was the target gene enrichment in the Wnt signaling pathway, which plays an important role in a variety of biological processes, including cell proliferation and differentiation (14). Abnormal expression or activation of the Wnt pathway could lead to a variety of diseases and even cancer (11). β-catenin is the key member in the classic Wnt pathway (7). When Wnt protein binds to Fz transmembrane receptors, protein degradation is disrupted, and accumulated β-catenin moves to the nucleus and forms a complex with Tcf/Lef transcription factors to activate the expression of downstream target genes. Current research suggests that Wnt signal transduction pathways are closely associated with carcinogenesis of medulloblastoma (9).

This study showed that hsa-miR-208a-3p and hsa-miR -1207-5p expression in medulloblastoma was significantly downregulated, and target gene prediction showed that Nemo-like kinase (NLK), which participates in Wnt signaling, was a potential target gene of hsa-miR-208a-3p. NLK is an evolutionarily conserved serine/threonine kinase that suppressed the transcriptional activity of β-catenin/T-cell factor complex through phosphorylation of T-cell factor (10). The Wnt/β-catenin signaling is thought to play a critical role in human carcinogenesis, so it is possible that NLK can act as a tumor suppressor by regulating the Wnt/β-catenin pathway. Cui et al.(3) found that NLK induced apoptosis in glioma cells via the activation of caspases, and suggested that NLK might be a useful independent prognostic indicator for glioma. Gene therapeutic approaches aimed at upregulating NLK expression could be developed for treatment of glioma. Wang et al.(17) found that miR-92b could regulate Wnt/β-catenin signaling pathway by NLK, promoting cell proliferation and invasion of glioma.

The MAPK signaling pathway is another important pathway for extracellular signals to trigger reactions in the nuclei. In 2001, MacDonald et al.(15) reported that in metastatic medulloblastoma, the Ras/MAPK (Ras proteins mediated MAPK activation) signaling pathway was upregulated; this was closely related to upregulation of the upstream gene platelet derived growth factor receptor alpha (PDGFRA). Previous research had shown that the activation of signaling pathways was related to gene mutation (18). But in 2006 Gilbertson et al.(6) suggested that the upregulation of the pathway in medulloblastoma might be caused by other mechanisms, through the sequencing of hot gene mutations in the pathway. This study showed that hsa-miR-208a-3p and hsa-miR-1207-5p expression in medulloblastoma was significantly downregulated, and target gene prediction showed that the NLK was potential target gene of hsa-miR-208a-3p. RAPGEF2, CACNA2D1, DUSP3, MAPK8IP3 were identified as potential target genes of hsa-miR-1207-5p, all of which are involved in the MAPK signaling pathway. Zhang et al.(19) found that in *in-vitro* experiments, NEDD4-1 could regulate the migration and invasion of glioma cells through CNrasGEF ubiquitination.

CONCLUSION

Using bioinformatics methods to predict potential target genes for hsa-miR-208a-3p and hsa-miR-1207-5p narrowed the scope of our investigation to verify the real target genes. We found that downregulation of hsa-miR-208a-3p and hsa-miR-1207-5p might be involved in the occurrence of medulloblastoma through modulation of the Wnt and MAPK signaling pathways. Further research is needed to confirm this hypothesis and identify the key players in modulating medulloblastoma.

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