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Original Article

Identification of hsa-miR-21 as a target gene of tumorigenesis in neuroblastoma

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Abstract: MicroRNAs (miRNA) have a crucial role in tumorigenesis. We used genome-wide analysis of miRNA expression in neuroblastoma (NB) to identify novel targets for the further study of NB. Genome-wide analysis of miRNA expression in NB, fetal adrenal and normal adrenal tissues was conducted using a miRNAs microarray. The differential expressions of miRNAs were identified through fold-change filtering. Gene ontology and pathway analyses were performed using the standard enrichment computation method. Target miRNA correlated to embryonic development and tumorigenesis of NB was screened through bioinformatics. We found 30 miRNAs that were upregulated > 2-fold in the order NB > embryonic adrenal tissues > normal adrenal tissues. The predicted target genes of miRNAs involved in embryonic and tumorigenic related signal pathways were selected, including has-miR-21, -153, -155 and -24. The expression of has-miR-21 in NB tissues was significantly higher ($P < 0.05$) compared to fetal and normal adrenal tissues. Has-miR-21 is an important miRNAs related to embryonic development and the pathogenesis of NB.

Keywords: MicroRNA, neuroblastoma, has-miR-21, qRT-PCR, microarray

Introduction

MicroRNAs (miRNAs) are short (18-25 nt), single-stranded, endogenous non-coding RNAs that have a fundamental role in negative post-transcriptional regulation of gene expression. Regulation of gene expression by miRNAs is via either sequence-specific interactions with target mRNAs and subsequent mRNA degradation or miRNA-mediated translational repression [1]. They are encoded by independent transcriptional units in intergenic regions and transcribed by RNA polymerase II or III to form primary miRNAs, which are processed by ribonuclease III enzymes into a stem-loop miRNA: miRNA* duplex [2]. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC) with endonuclease argonaute proteins to guide the cleavage or translational repression of the target mRNA by complementary base pairing [3], resulting in downregulation of a wide variety of proteins.

Neuroblastoma (NB), the most common extracranial embryonic solid tumor, is derived from the peripheral sympathetic nervous system of the neural crest [4]. NB is an extremely heterogeneous tumor that can regress or mature spontaneously even without treatment. NB, which can have a very aggressive malignant phenotype with a poor response to current intensive multimodality therapies, accounts for 15% of pediatric cancer deaths [5].

Many miRNAs are involved in the biological functions of NB cells. Hsa-miR-181c inhibits NB cell growth and metastasis-related traits through suppression of Smad7; thus functioning as a tumor suppressor [6]. Hsa-miR-421 expression was higher in NB tissues compared to matched adjacent normal tissues. Forced over-expression of miR-421 substantially enhanced cell proliferation, cell-cycle progression, migration, and invasion of NB cells [7]. Hsa-miR-23a is key in the promotion of NB cell migration and invasion through its targeting of the CDH1 gene [8]. Hsa-miR-145 suppressed HIF-2a expression under normoxic conditions, thus inhibiting the aggressiveness and angiogenesis of NB [9]. Hsa-miR-338-3p suppressed NB proliferation, invasion and migration through targeting PREX2a [10]. Recently, Wu et al. [11]

reported hsa-miR-362-5p inhibits proliferation and migration of NB cells by targeting phosphatidylinositol 3-kinase-C2b. Given the multifaceted nature of the cellular effects, we hypothesized miRNAs are expressed differentially and are important during the onset and development of NB. In this study, we examined the differential expressions of miRNAs in NB, fetal adrenal tissues and normal adrenal tissues and explored the potential roles of these miRNAs in the tumorigenesis of NB.

Materials and methods

Patient samples

Group 1. Neuroblastoma tissues: biopsy of NB patients, period III, without chemotherapy, n = 3.

Group 2. Fetal adrenal tissues: normal fetus by fertility drug-induced voluntary labor, n = 3.

Group 3. Normal adrenal tissues: adult organ transplant donor, n = 3.

The Ethics Committee of the Children's Hospital of Fudan University, Shanghai, China approved this study. Informed written consent was given by patients or their guardians. All tissue samples were confirmed by pathology, frozen in liquid nitrogen and stored at -80°C.

Microarray analysis of miRNA

Groups 1-3 were subjected to miRNA microarray analysis. Total RNA was extracted using TRIzol® reagent (Takara Bio Inc., Kyoto, Japan) and labeled with Hy3™/Hy5™ fluorescent dye using the miRCURY™ array power labeling kit (Exiqon A/S, Vedbaek, Denmark), following the manufacturer's protocol. Labeled RNA was mixed with and hybridized to the miRCURY™ Array version 9.2 (Exiqon). Arrays were scanned with a Genepix® 4000B Microarray Scanner (Molecular Devices) and the images were analyzed using Genepix® Pro 6.0 software with the robust multichip analysis normalization method. Changes of gene expression by 2-fold up- or downregulation were considered significantly different after normalization.

Bioinformatics analysis of putative miRNA targets

The analysis of miRNA predicted targets used the following algorithms: TargetScan (www.targetscan.org), PicTar (pictar.bio.nyu.edu) and miRanda (microrna.sanger.ac.uk). The predicted target genes were input into the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/), which used gene ontology (GO) to identify the molecular function represented in the gene profile. Further, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.ad.jp/kegg/) and BioCarta (http://www.biocarta.com) to analyze the potential functions of these target genes in the pathways. We set the level of statistical significance at $P \leq 0.05$.

Reverse transcription polymerase chain reaction (RT-PCR) confirms the expression of hsa-miR-21

To detect the expression levels of mature hsa-miR-21, total RNA was extracted from groups 1-3 using TRIzol® reagent (Takara Bio Inc.) and then reverse transcribed using gene-specific reverse transcription primers from the TaqMan microRNA assays (Applied Biosystems, Foster City, CA, USA) and the TaqMan microRNA Reverse Transcription kit (Takara Bio Inc.) according to the manufacturer's instructions. RT-PCR used 0.5 μl of diluted cDNA, TaqMan 2×RT-PCR MasterMix and the TaqMan probe in a total volume of 10 μl. RT-PCR was run on the Rotor-Gene 3000™ system and rotor gene 6.1.90 detection software (Corbett Life Science, Sydney, Australia) under the following conditions: 3 min at 95°C followed by 40 cycles at 95°C for 15 s, 58°C for 30 s, 72°C for 30 s. All reactions were run in triplicate and the relative quantities of mature miRNAs were calculated using the comparative 2-ΔΔCt method. All data were normalized to the expression of U6. The primers used for RT-PCR were as follows: U6 forward primer, 5-ATTGGAACGATACAGAGAAGATT-3; U6 reverse primer, 5-GGAACGCTTCACGAATTTG-3; miR-21 forward primer, 5-ACGTTGTGTAGCTTATCAGACTG-3; miR-21 reverse primer, 5-AATGGTTGTTCTCCACACTCTC-3.

Statistical analysis

All data were analyzed using the software package SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA) and are presented as the mean and the standard error of the mean (S.E.M.). Multiple groups of values were compared by one-way analysis of variance (ANOVA) followed by a post hoc test. Differentially ex-

Table 1. Expression of 30 miRNAs upregulated by > 2-fold in NB tissues compared to fetal adrenal tissues, in fetal adrenal tissues compared to normal adrenal tissues, and in NB tissues compared to normal adrenal tissues

Systematic name	NB tissues/fetal adrenal tissues	Fetal adrenal tissues/ normal adrenal tissues	NB tissues/normal adrenal tissues
hsa-miR-100	2.84979168	2.394143859	6.822811251
hsa-miR-106a	2.07176792	3.40813232	7.06085921
hsa-miR-135b	2.376697816	7.443996508	17.69213024
hsa-miR-140-5p	2.354743573	2.142091969	5.044077296
hsa-miR-142-3p	4.668981881	5.330269104	24.88692987
hsa-miR-146a	2.596168883	14.29574089	37.11415766
hsa-miR-146b-5p	2.528712349	7.42561627	18.77724756
hsa-miR-153	2.901638029	11.27321282	32.71078302
hsa-miR-17	2.329895151	2.522529255	5.759322163
hsa-miR-181d	2.070417701	11.13317291	23.05031826
hsa-miR-199a-5p	2.600231105	2.222150157	5.778103959
hsa-miR-21	7.867051197	2.140276637	16.83766588
hsa-miR-221	2.764282383	2.389430978	6.605061956
hsa-miR-24-1/24-2	2.693764975	2.230135579	6.007461113
hsa-miR-302c	2.183283787	3.997701828	8.728117585
hsa-miR-30e	2.187156165	2.940838127	6.432072238
hsa-miR-331-5p	2.256557047	3.521266704	7.945939195
hsa-miR-338-5p	2.073467136	2.033992023	4.217415614
hsa-miR-373	2.073211787	7.646179129	15.85214869
hsa-miR-504	2.276215468	2.073765208	4.720336443
hsa-miR-587	2.566833641	3.18590797	8.177695755
hsa-miR-604	2.414045924	3.250400035	7.846614956
hsa-miR-652	2.28448194	7.352095316	16.79572897
hsa-miR-876-3p	2.194228758	3.963738344	8.697348663
hsa-miR-93	3.707648592	8.173800087	30.30557839
miRPlus_17821	2.36418888	2.328163517	5.504218297
miRPlus_17828	2.048633962	2.210252554	4.527998448
miRPlus_17897	2.488712617	5.636606409	14.02789349
miRPlus_17951	2.194642763	2.042248699	4.482006327
miRPlus_28534	3.208542051	2.107600657	6.762325336

pressed genes were then identified through fold change as well as *P* value calculated with t-test. The threshold set for up and down-regulated genes was a fold change ≥ 2.0. We set the level of statistical significance at $P \leq 0.05$.

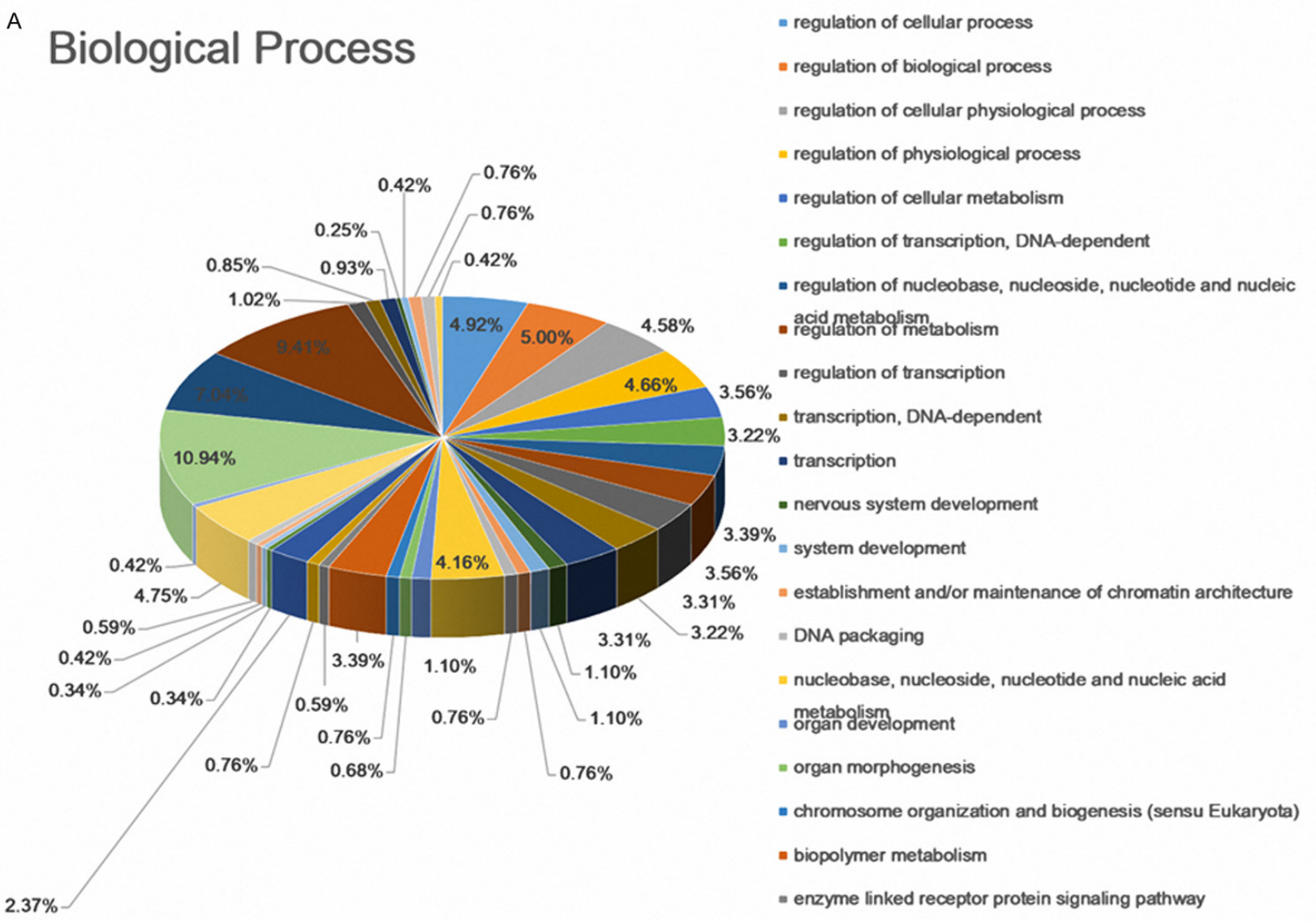
Results

Microarray analysis of miRNA

Using the microarray, we first assessed the different miRNA expression profiles (fold change > 2.0, $P < 0.01$) in NB versus the fetal and normal adrenal tissues. We found 123 miRNAs were upregulated and 30 were downregulated in NB compared to fetal adrenal tissues; 354 were upregulated and 15 were downregulated in fetal compared to normal adrenal tissues; 437 were upregulated and 22 were downregulated in NB compared to normal adrenal tissues, The 30 miRNAs were upregulated > 2-fold in the order NB > embryonic adrenal tissues > normal adrenal tissues (**Table 1**).

Bioinformatics analysis of putative miRNA targets

By inputting the differential expressions of the miRNAs into the miRBase database (http://microrna.sanger.ac.uk/) and Pubmed (http: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system development
establishment and/or maintenance of chromatin architecture
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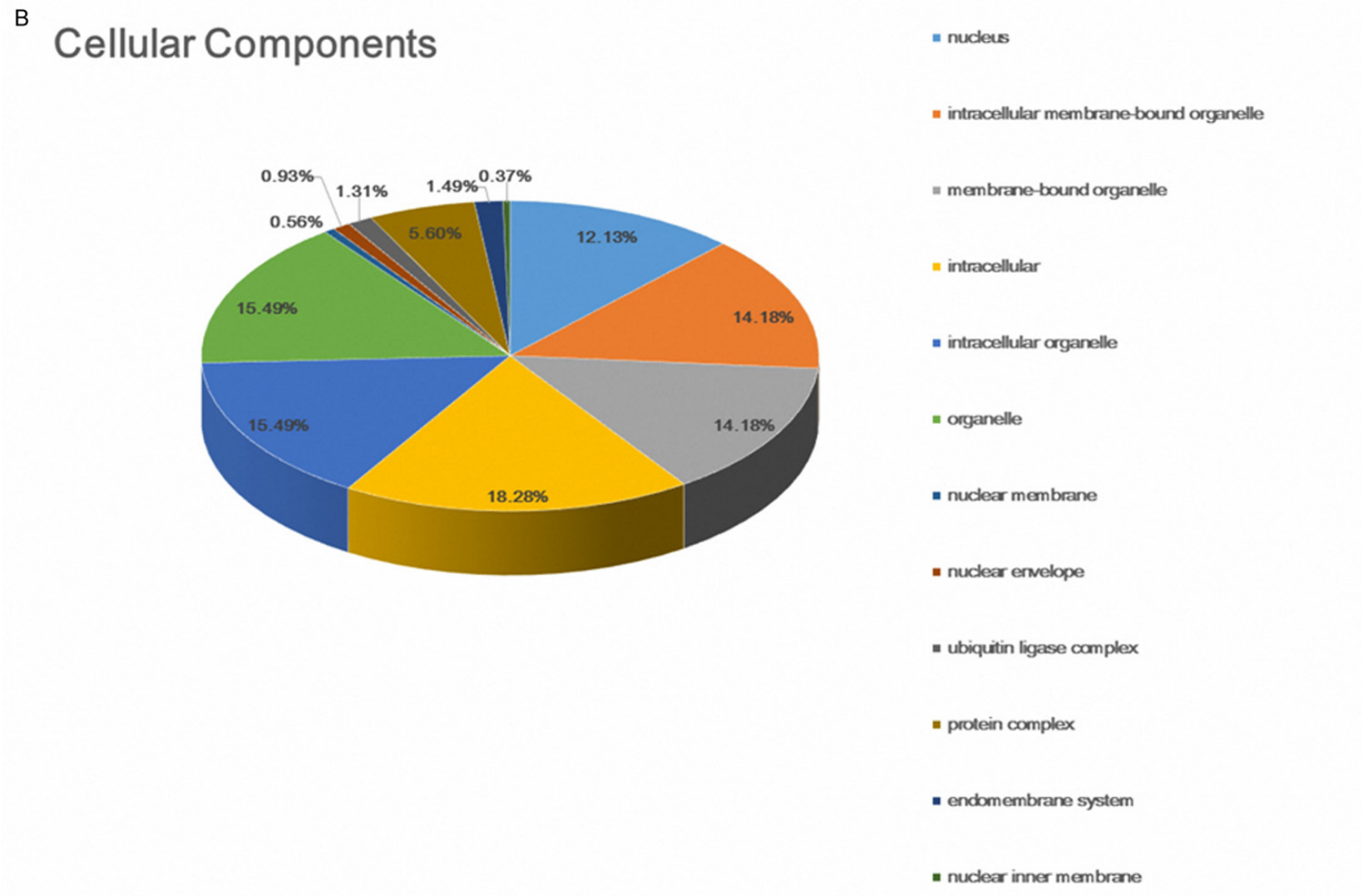
B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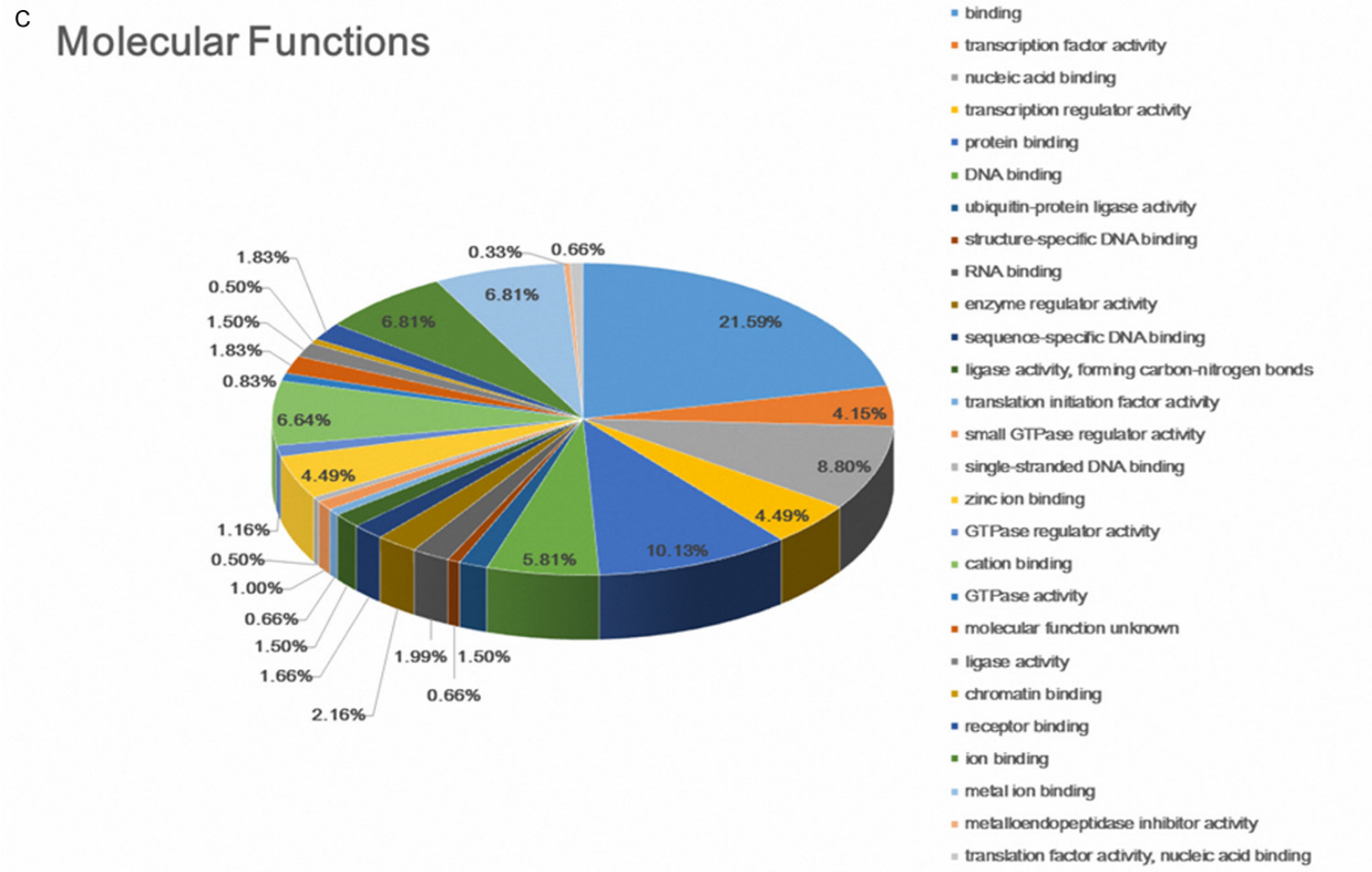

Figure 1. GO enrichment analysis of miRNA-targets. GO analysis of miRNA target genes according to (A) biological process, (B) cell component and (C) molecular function.

Table 2. The predicted target of has-miR-21 with KEGG pathways analysis

Category	Term	RT	Genes	Count	%	*P*-Value	Benjamini
KEGG_PATHWAY	JAK-STAT SIGNALING PATHWAY	RT		6	0.5	1.20E-02	9.10E-01
KEGG_PATHWAY	CYTOKINE-CYTOKINE RECEPTOR INTERACTION	RT		7	0.6	2.80E-02	9.30E-01
KEGG_PATHWAY	MAPK SIGNALING PATHWAY	RT		7	0.6	3.90E-02	9.20E-01
KEGG_PATHWAY	TGF-BETA SIGNALING PATHWAY	RT		4	0.3	4.30E-02	8.80E-01
KEGG_PATHWAY	APOPTOSIS	RT		4	0.3	5.30E-02	8.30E-01

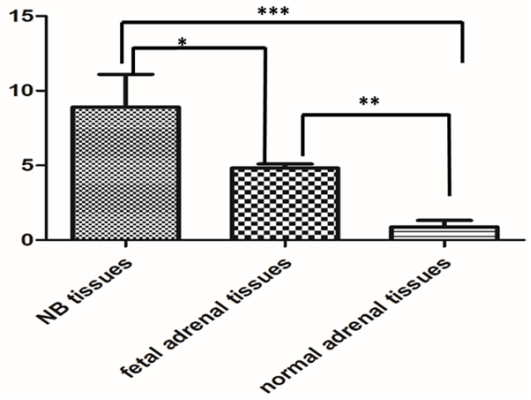

Figure 2. RT-PCR showed a higher level of expression of hsa-miR-21 in the order NB > fetal adrenal tissues > normal adrenal tissues. NB tissues versus fetal adrenal tissues *P = 0.025; fetal adrenal tissue versus normal adrenal tissues **P = 0.029; and NB tissues versus normal adrenal tissues ***P < 0.001.

//www.ncbi.nlm.nih.gov/pubmed/), we found that a quarter of miRNAs were reported to be involved in tumorigenesis, embryonic development and neuronal differentiation. Among them, 30 miRNAs were upregulated > 2-fold in the order NB > fetal adrenal tissues > normal adrenal tissues. The common predicted targets were found by analyzing the miRNA predicted targets, using getScan, Pictar and miRanda. The predicted target genes were input into the database using GO and pathway analyses. Through GO analysis, we found the under-regulated and upregulated transcripts of miRNAs were associated with cellular processes (ontology: biological process) (**Figure 1A**), cell (ontology: cellular component) (**Figure 1B**) and binding (ontology: molecular function) (**Figure 1C**). Combined with the analysis of the KEGG pathway, we selected hsa-miR-21, -153, -155 and -24, because their targets were associated with the tumorigenesis and embryonic development pathways. The predicted target gene hsa-miR-21 was related to the following pathways: TGF-β [12], JAK-STAT [13], MAPK [14], CYTOKINE-CYTOKINE RECEPTOR and APOPTOSIS [15] (**Table 2**). We reviewed the literature and found hsa-miR-21 is one of the most highly expressed miRNAs in adult tumors. As such, we chose hsa-miR-21 as worthy of further investigation.

RT-PCR confirmed the expression of hsa-miR-21

The expression of hsa-miR-21, as confirmed by RT-PCR (**Figure 2**), was much higher in NB tissues compared to fetal adrenal tissues and normal adrenal tissues.

Discussion

The molecular mechanisms responsible for NB development have been studied extensively; the pathogenesis of this disease is vague, however, and most of the altered gene expressions and regulations involved remain to be elucidated. The miRNAs are non-coding small RNAs important in tissue and cell functionality, including embryo development, human disease and carcinogenesis.

To our knowledge, there is no report of differentially expressed miRNAs in NB, fetal adrenal tissues or normal adrenal tissues. Our data are the first to show 30 expressed miRNAs upregulated by fold changes of > 2 in the order NB > fetal adrenal tissues > normal adrenal tissues, indicating these miRNAs contribute to NB development. In this study, to understand the functions of the miRNAs better, we used pathway analysis to associate these differentially expressed miRNAs with their target genes. GO and pathway analyses predicted downregulated and upregulated transcripts of miRNAs were associated with cellular processes (ontology: biological process), cell (ontology: cellular com-

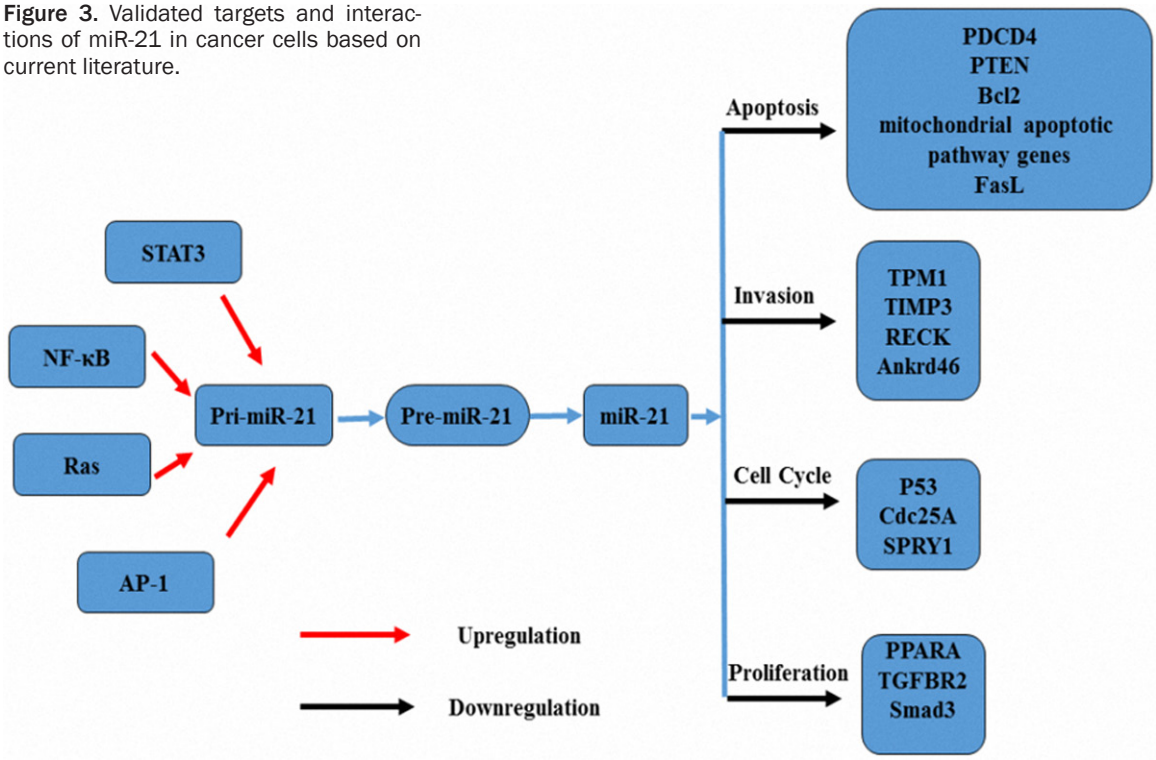

Figure 3. Validated targets and interactions of miR-21 in cancer cells based on current literature.

ponent) and binding (ontology: molecular function). We selected the miRNAs that had predicted target genes associated with tumorigenic and embryonic development, including hsa-miR-21, -153, -155 and -24. Finally, we confirmed hsa-miR-21 expression was higher in the order NB > fetal adrenal tissues > normal adrenal tissues. These results suggest hsa-miR-21 has an important role in NB tumorigenesis and embryo development.

Hsa-miR-21 is a miRNA with putative, anti-apoptotic and tumor promoting activities, and is highly expressed in a variety of solid tumors [16, 17]. Si et al. [18] found has-miR-21 was highly over-expressed in breast tumors compared to match normal breast tissues, suggesting has-miR-21 functions as an oncogene and might serve as a novel therapeutic target. Asangani et al. [19] reported that has-miR-21 downregulates the tumor suppressor PDCD4 post-transcriptionally and stimulates invasion, intravasation and metastasis in colorectal cancer. Liu et al. [20] showed has-miR-21 targeted the tumor suppressor RhoB and regulated proliferation, invasion and apoptosis in colorectal cancer cells. Increased expression of miR-21 in these tumors is associated with cell proliferation, migration, invasion and metastasis, suggesting miR-21 is a key regulatory molecule in cancer initiation and progression. We provided a schematic representation of validated targets and interactions of miR-21 in cancer cells based on current literature (**Figure 3**) [15, 21, 22].

Hsa-mir-21 is encoded on chromosome 17q, which is involved frequently in unbalanced translocations in NB cell lines [16]. Afanasyeva et al. [23] reported hsa-miR-21 is among the most frequently detected miRNAs in primary NB tumors. Buechner et al. [24] found hsa-miR-21 was expressed in all NB cell lines investigated and hsa-miR-21 expression was correlated with MYCN mRNA expression, which was associated with patients' prognosis. This is in accord with the results presented here.

We showed miRNAs likely have a role in NB development and progression. NB is the most common malignant neoplasm of children, yet its etiology, pathophysiology and molecular mechanisms are largely unknown. Further study is needed to understand this disease in detail, in order to aid effective control of it in the future. This study of the potential link between hsa-miR-21 and NB presents a novel area for further investigation into the target

genes of hsa-miR-21, which could lead to the development of therapeutic strategies for the disease.

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Disclosure of conflict of interest

None.

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