Association of microRNA 21 With Biological Features and Prognosis of Neuroblastoma
Yaodong Zhou, MD, and Bo Sheng, MD

Background: The aim of this study was to assess the differences in microRNA 21 expression among neuroblastoma (NB), embryonic tissue, and normal adrenal tissue and to identify correlations between microRNA 21 expression, the biological features of the tumor, and prognosis.

Methods: A total of 70 patients with NB were selected from December 2005 and December 2007. Real-time polymerase chain reaction was used to assess microRNA 21 expression. All patients were followed-up for 5 years.

Results: Significant differences in microRNA 21 expression were found between the 3 groups, with the highest expression in the NB samples \((P < .001)\). The expression of microRNA 21 was highest in the high-risk group compared with the moderate- and low-risk groups \((P < .001)\). The microRNA 21 expression in the MYCN amplification group was higher than in the group without amplification \((P = .001)\). The 5-year overall survival rate of patients with NB was 71.4%.

Conclusions: The higher expression of microRNA 21 in NB samples compared with embryonic and normal tissue samples predicted a close correlation between microRNA 21 expression and the biological features of NB. In patients with NB, higher microRNA 21 expression correlated with lower rates of overall survival. Therefore, microRNA 21 expression may represent a novel risk factor for determining the prognosis of patients with NB.

Introduction
Neuroblastoma (NB) is the most frequently occurring solid tumor in children, with an incidence of 1.3 cases per 100,000 children aged 14 years and younger.\(^1\) In addition, it is the most common extracranial tumor in children. Clinically, NB is characterized by rapid growth, susceptibility to multidrug resistance, and metastasis. As is characteristic of embryonic tumors, neuroblasts are histologically indistinguishable from developing neuroblastic cells in the embryo. Despite many advances made during the last 30 years, NB has remained a challenge to clinical and basic research scientists.

To date, several approaches for determining the prognosis of NB have been described, including assessment of MYCN status or the Shimada classification. MYCN was originally found to be amplified in NB, and since then research has focused on the search for other genetic markers.\(^2\) The International Neuroblastoma Risk Group, which represents the major cooperative groups on pediatric cancer, met in 2005 to review data collected on 11,000 patients studied between 1974 and 2002.\(^3\) Consensus was reached to consider age (> or < 18 months), image-defined risk factors for surgery, International Neuroblastoma Staging System, and MYCN status as basic tools in the risk group schema, which included high-, moderate-, and low-risk groups.\(^3\) Elucidating the exact molecular signature of NB will allow for analysis of how specific markers, alone or in combination, can help to stratify disease in prospective studies. Currently, stratification is based on age, tumor stage, MYCN status, and Shimada pathology.

NB may be one of the first examples of a disease for which genetic tumor markers are used as a tool to define tumor behavior and to aid in clinical staging. microRNAs can act as oncogenes by posttranscriptionally repressing the expression of target tumor suppressor genes, or as tumor suppressors by repressing the expression of target oncogenes. microRNA 21 is up-regulated in many solid tumors, including lung, breast, prostate, and stomach carcinomas, as well as pancreatic endocrine tumors, hepatocellular carcinoma, and glioblastoma.\(^4,5\) Growing evidence suggests that microRNA 21 has an antiapoptotic function and promotes growth and chemosensitivity in tumor cells.\(^4,6,7\) However, these data were mainly derived from experiments on cell lines in vitro or in xenograft animal models. Thus, we previously used a microRNA array to screen for genes involved in NB and found that microRNA 21 was up-regulated,
and our previous data suggest a correlation between microRNA 21 expression and the biological features and prognosis of NB (Fig 1A–C).

The aim of this study was to assess the differences in microRNA 21 expression among NB, embryonic tissue, and normal adrenal tissue samples and to determine the correlation between microRNA 21 expression, the biological features of the tumor, and prognosis.

Materials and Methods

Patients and Specimens

A total of 70 patients with NB were selected from the Children's Hospital of Fudan University (Shanghai, China) between December 2005 and December 2007 (Table 1). Tumor samples were collected from each study participant during surgical resection, frozen in liquid nitrogen, and stored at –80 °C. Sections from each specimen were examined by a pathologist and histologically graded. Patients who had received neoadjuvant chemotherapy or radiation therapy prior to surgery were excluded from this study. Staging was retrospectively determined and based on surgery and pathology. A total of 120 samples were obtained; 60 samples of normal adrenal tissue were retrieved from patients with nephroblastoma and 60 samples of embryonic tissue were obtained from deceased donors.

This study was approved by the Institutional Review Board and Ethics Committee of the Children's Hospital of Fudan University. The parents or guardians of patients provided written informed consent.

Table 1.—Patient Demographics and Clinical Data

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of Patients</th>
</tr>
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<tbody>
<tr>
<td>Age, y</td>
<td></td>
</tr>
<tr>
<td>≤ 1</td>
<td>24</td>
</tr>
<tr>
<td>&gt; 1</td>
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<tr>
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<tr>
<td>Male</td>
<td>34</td>
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<tr>
<td>Female</td>
<td>36</td>
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<tr>
<td>International Neuroblastoma Staging System</td>
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</tr>
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<td>1/2</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
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<tr>
<td>Group</td>
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<tr>
<td>Moderate risk</td>
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</tr>
<tr>
<td>Low risk</td>
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</tr>
<tr>
<td>MYCN status</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>16</td>
</tr>
<tr>
<td>Negative</td>
<td>54</td>
</tr>
<tr>
<td>Shimada pathology classification</td>
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<tr>
<td>Unfavorable histology</td>
<td>28</td>
</tr>
<tr>
<td>Favorable histology</td>
<td>42</td>
</tr>
</tbody>
</table>

Tissue RNA Isolation

Total RNA was isolated from the tissues using Trizol reagent (Invitrogen, Grand Island, New York). Some of the total RNA specimens were further purified using a Qiagen midi column (Qiagen, Shanghai, China).

Real-Time Polymerase Chain Reaction

Primers for the analysis of microRNA 21 expression were designed as F: 5'-TAGCTTATCAGACTGATGTTGA-3' and R: 5'-TGCGTGTCGTGAGTC-3'. Mixtures of 1 μg of total RNA together with 50 nM reverse primer, 2 U of an RNAase inhibitor (Promega, Madison, Wisconsin), 5 U of M-MLV reverse transcriptase (TaKaRa Bio, Shiga, Japan), and 0.5 μM dNTP were used for each reverse transcription (RT) reaction. The expression of microRNA precursors was determined using a real-time quantitative polymerase chain reaction (PCR) assay as described, with the ex-

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Fig 1A–C.—(A) Gene screening results in tumor tissue, (B) embryonic adrenal tissue, and (C) normal adrenal tissue.
ception that 35 cycles of PCR were used. The re-
action parameters were as follows: incubation at
16 °C for 30 minutes, 42 °C for 42 minutes, and 85 °C
for 5 minutes. Fluorescence was then measured.

To generate the complementary DNA (cDNA)
template for the endogenous-control PCR reactions,
first-strand cDNA was synthesized using 1 μg of RNA
from the same samples for stem-loop RT and oligo
d(T) as the primer. The reaction parameters were as
follows: 95 °C for 5 minutes followed by 35 cycles of
95 °C for 10 seconds, 56 °C for 15 seconds, and 72 °C
for 20 seconds. Fluorescence was then measured.

Real-time PCR was performed on an Applied Bio-
systems 7500 detection system (Applied Biosystems,
Foster City, California) in a 15-μL reaction volume. All
reactions were performed in triplicate. For quantita-
tion of microRNA 21 expression, a 15-μL PCR reaction
mixture was used that included 1 μL of the microRNA
21 RT product, 1X SYBR-Green I Mastermix (Toyobo
USA, New York, New York), and 0.5 μM each of the
forward and reverse primers. For the endogenous
control (U6), 1 μL of cDNA synthesized using oligo
d(T) was used as a template. The housekeeping gene
primer was F: 5’ GCTTCGGCAGCACATATACTAAAAT
3’ and R: 5’ CGTTTCAGAATTTGCGTGTCAT 3’. The
reaction parameters were 95 °C for 5 minutes followed
by 35 cycles of 95 °C for 10 seconds, 56 °C for 15 sec-
onds, and 72 °C for 20 seconds. The cycle threshold
(Ct) was defined as the cycle number at which fluo-
rescence passed a predetermined threshold.

For expression analysis, the experiment was de-
signed to use the matched normal adrenal tissue and
embryonic adrenal tissue as the control, so the rela-
tive quantification of microRNA 21 in tumor tissue
was calculated using the equation:

\[
\text{Amount of target} = 2^{-\Delta\Delta Ct} (17), \Delta\Delta Ct = (Ct_{\text{microRNA 21}} - Ct_{\text{U6}})_{\text{tumor}} - (Ct_{\text{microRNA 21}} - Ct_{\text{U6}})_{\text{matched nontumor}}
\]

For the matched, normal adrenal tissue control
samples, ΔΔCt was 0 and 2−ΔΔCt was 1. We set the nor-
mal adrenal value as 1 and calculated the value of em-
byronic adrenal and NB tissue. Melting curves were
generated, and 8% polyacrylamide gel electrophoresis
was performed for each real-time PCR to verify the
amplification of the desired product alone.

**MYCN Detection**

Amplified MYCN was used as a major prognostic fac-
tor of localized NB. Fluorescence in situ hybridiza-
tion was applied to identify MYCN, as previously de-
scribed.8

**Pathological Categorization**

Pathological classification of all tumor tissues was
performed according to the modified Shimada clas-
sification. After confirming the histological diagnosis,
2 morphological features were considered for prog-
nostic categorization: degree of differentiation of the
neuroblasts and proportion of mitotic and karyor-
rhectic cells (MKCs) to determine the mitotic karyor-
rhectic index (MKI). The proportion of tumor cells
showing mitosis and karyorrhexis was used to clas-
sify whether the NB tissue had a low (< 2% MKCs),
intermediate (2%-4% MKCs), or high (> 4% MKCs)
MKI. The average number of MKCs was assessed in
approximately 10 hpf, depending on the cell density
of the NB tissue (total number of tumor cells in the
chosen hpf ≤ 5,000).

All pathological sections were stained with he-
matoxylin and eosin and observed under an optical
microscope. Samples were classified as unfavorable
or favorable histology based on a classification sys-
tem according to the amount of Schwannian stroma,
degree of differentiation, MKI, and age at diagnosis.9

**Clinical Follow-Up**

All patients were followed for up to 5 years. We col-
lected and reviewed clinical data of patients from our
center during a 5-year period to evaluate the correla-
tion between microRNA 21 expression and NB prog-
nosis. We combined the data obtained from regular
visits to the hospital every 3 months and follow-up
phone calls.

**Data Analysis**

Statistical analyses were performed using SPSS 15.0
(SPSS, Chicago, Illinois). The results are expressed as
mean ± standard error, and less than .05 was con-
sidered statistically significant. The Wilcoxon test was
used to assess the difference in the microRNA levels
among NB, embryonic adrenal, and normal adrenal
tissues. The Mann-Whitney test or the Tukey-Kramer
test was selected to assess the associations between
mature microRNA levels and clinicopathological pa-
rameters.

**Results**

**Groups Analyzed in the microRNA Array**

Three groups were assessed in this study, including
a NB group (4 samples), an embryonic adrenal tis-
sue group (4 samples), and an adrenal tissue group
(4 samples; see Fig 1).

**Expression of microRNA 21 in Tumor and
Matched Nontumor Samples**

All tissues were assessed by real-time PCR. As shown
in the representative amplification curves in Fig 2,
the Ct value of microRNA 21 in tumor tissue was
lower than that of microRNA 21 in nontumor tis-
sue, suggesting that the expression level of mi-
croRNA 21 in tumor samples was higher than that
Of the 70 study patients, MYCN amplification was observed in 16 cases (Fig. 3). In addition, 28 cases had favorable histology and 42 cases had unfavorable histology (Fig. 4A–B). The expression of microRNA 21 in NB, normal adrenal, and embryonic adrenal tissues significantly differed, with NB samples having the highest expression followed by normal embryonic tissue and then adrenal tissue ($P < .001$; Table 2, Fig. 5).

Groups with a more advanced tumor stage and greater risk had high expression of microRNA 21 (see Table 2 and Fig. 5). Of the 70 study patients, MYCN amplification was detected in 16 tumors. In addition, microRNA 21 expression in amplification cases was higher than in nonamplification cases ($P = .001$). In the 70 study patients, expression of microRNA 21 in samples with unfavorable histology was higher than in those with favorable histology ($P = .008$).

**Patient Follow-Up**

The median follow-up for all patients was 5 years and was
conducted from December 2005 to December 2012. The 5-year overall survival (OS) rate of patients with NB was 71.4%. Twenty participants died during the study, among whom 12 had stage 4 (OS, 55.6%), 5 had stage 3 (OS, 75%), and 3 had either stage 1 or 2 NB (OS, 87.0%). The 5-year OS rates were 48.0% in the high-risk group, 78.3% in the moderate-risk group, and 90.9% in the low-risk group. Among the 20 study patients who died, MYCN amplification was present in 9 cases (45%), and 12 cases (60%) had unfavorable histology. The 5-year OS rate in the MYCN amplification group was lower than that in the group without amplification (43.5% vs 82.9%, respectively; \( P = .001 \)). The 5-year OS rate for patients with unfavorable histology was 57.1% compared with 81.0% for patients with favorable histology (\( P = .02 \)). In addition, microRNA 21 expression was higher in participants who died compared with those who were alive at the end of the study (70.5 ± 4.16 vs 25.3 ± 1.24, respectively; \( P = .03 \)). In addition, we found that higher Ct values for microRNA 21 correlated with a shorter rate of OS (Fig 6).

Table 2. — microRNA 21 Expression in Various Tissues, Tumor Stages, and Risk Groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>2-ΔΔCt Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>70</td>
<td>40.34 ± 3.16</td>
</tr>
<tr>
<td>Embryonic adrenal tissue</td>
<td>60</td>
<td>3.13 ± 0.52</td>
</tr>
<tr>
<td>Normal adrenal tissue</td>
<td>60</td>
<td>1.09 ± 0.10</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2</td>
<td>23</td>
<td>12.66 ± 1.78</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>24.89 ± 2.77</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>60.35 ± 5.23</td>
</tr>
<tr>
<td>Group</td>
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<td></td>
</tr>
<tr>
<td>Low risk</td>
<td>22</td>
<td>10.66 ± 1.12</td>
</tr>
<tr>
<td>Moderate risk</td>
<td>23</td>
<td>30.53 ± 6.45</td>
</tr>
<tr>
<td>High risk</td>
<td>25</td>
<td>61.95 ± 11.16</td>
</tr>
</tbody>
</table>

\( P = .001 \).

Ct = cycle threshold.

Fig 5A–C. — (A) Mean values of microRNA 21 expression in NB tissue vs normal and embryonic tissue. \( P = .001 \) for NB tissue vs embryonic adrenal tissue and NB tissue vs normal adrenal tissue; \( P = .002 \) for embryonic adrenal tissue vs normal adrenal tissue. (B) Mean values of microRNA 21 expression in NB samples of the indicated tumor stages. \( P = .001 \) for stage 4 vs stage 3; \( P = .000 \) for stage 4 vs stages 1/2; \( P = .003 \) for stage 3 vs stages 1/2. (C) Mean values of microRNA 21 expression in samples classified according to the indicated risk groups. \( P = .02 \) for the high-risk group vs moderate-risk group; \( P < .001 \) for the high-risk group vs low-risk group; and \( P = .006 \) for the moderate-risk group vs low-risk group.

Ct = cycle threshold, NB = neuroblastoma.
Discussion

To the best of our knowledge, this is the first study to assess microRNA expression patterns in patients with NB using microRNA microarrays. microRNA 21 seems to play an important oncogenic role in malignant tumors, and it has generated more research interest than any other microRNA given its involvement in various cancers, cardiac hypertrophy, and neointimal formation.10-12 Widespread overexpression of MIR21 in cancer has led to the hypothesis that it is oncogenic.5 Similar to many other malignant tumors, the development, progression, invasion, and metastasis of NB are caused by multiple genetic alterations, but the mechanism has not been fully elucidated until now. It has been hypothesized that 1 microRNA may simultaneously down-regulate multiple target genes; therefore, microRNAs may act as efficient regulators of tumor-related genes.6 To reduce error caused by gene-expression differences between individuals, we used matched nontumor tissue as a control and used 2^-ΔΔCt to represent the level of microRNA 21 expression in various cancers, cardiac hypertrophy, and neointimal formation.10-12 To reduce error caused by gene-expression differences between individuals, we used matched nontumor tissue as a control and used 2^-ΔΔCt to represent the level of microRNA 21 expression in tumor samples relative to matched nontumor samples. Unfavorable and favorable histology classification is a common method for evaluating the prognosis of NB.

The average number of MKCs was assessed in approximately 10 hpf depending on the cell density of NB. Expression of microRNA 21 in unfavorable histology samples was higher than that in favorable histology and the 5-year OS in samples with unfavorable histology were lower than that in those with favorable histology, so it is possible that microRNA 21 expression may signify poor prognosis.

In our study, the 5-year OS rate in the MYCN amplification group was lower than that in the group without amplification. MYCN amplification indicates poor prognosis, and the expression of microRNA 21 in amplification cases was higher than that in non-amplification cases, indicating that microRNA 21 expression may be related to NB prognosis. In NB, MIR17/92 expression has been shown to correlate with MYCN amplification and adverse outcomes, a finding also confirmed by Schulte et al.13,14 Chen and Stallings8 further suggested that MYCN may mediate a tumorigenic effect, in part through directly or indirectly regulating the expression of microRNAs involved in neural cell differentiation, apoptosis, or both. Thus, these findings warrant further study of microRNAs as potential therapeutic targets.

In our study, microRNA 21 was up-regulated in patients with MYCN amplification, unfavorable histology, and stages 3/4 NB, as well as those in high- and moderate-risk groups, which predicted a close correlation between microRNA 21 expression and the biological features of NB. However, further research is needed to understand the mechanistic link between MYCN and microRNA 21.

Significant differences were found in the expression of microRNA 21 in NB, normal adrenal, and embryonic adrenal tissues, with NB samples having the highest microRNA 21 expression. These results indicate that microRNA 21 may play a role in the function and development of NB, but this hypothesis requires additional data for verification.

We found that the 5-year OS of all patients with NB was 71.4%. In addition, expression of microRNA 21 was higher in the patients who died than in those who remained alive at the end of the study. Several risk factors can affect the prognosis of NB, such as MYCN status, unfavorable histology, and favorable histology. However, these factors have a binary classification index that cannot definitively determine risk. Therefore, microRNA 21 expression analysis could potentially solve this problem. In our study, the relative expression value in the high-risk group was approximately 60, whereas it was 30 and 10 in the moderate- and low-risk groups, respectively. Thus, the expression values of microRNA 21 may be useful in determining risk in patients with NB. An expression level close to 60 may indicate poor prognosis, whereas a value less than 10 may not indicate poor prognosis. Thus, the predictive value of microRNA 21 expression may be advantageous in the clinical setting, but further studies are needed for clarification.

High expression of microRNA 21 in NB suggests that microRNA 21 may be an effective therapeutic target, and numerous studies have confirmed that inhibiting microRNA 21 expression blocks tumor growth in various tumor types.6,8 Other reports have shown that inhibiting the expression of microRNA 21 can increase the sensitivity of tumors to chemotherapy.10,15 Therefore, future studies should focus on targeting microRNA 21 as a cancer treatment modality.16 Nevertheless, the utility of microRNA 21 expression as a prognostic factor or molecular target in the treatment of NB will require further clarification with more samples and patients from multicenter studies.

Conclusions

The higher expression of microRNA 21 in neuroblastoma samples compared with embryonic and normal tissue samples predicted a close correlation between microRNA 21 expression and the biological features of neuroblastoma. In patients with neuroblastoma, higher rates of microRNA 21 expression correlated with lower rates of overall survival. Therefore, microRNA 21 expression may represent a novel risk factor for determining the prognosis of patients with neuroblastoma.

Acknowledgment: We thank Reginald C. Tsang, MD, for his help in editing this manuscript.
References


2. Reiter JL, Brodeur GM. MYCN is the only highly expressed gene from the core amplified domain in human neuroblastomas. Genes Chromosomes Cancer. 1998;23(2):134-140.


