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Cancer Res Published OnlineFirst August 30, 2010.

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microRNA Signature and Expression of *Dicer* and *Drosha* Can Predict Prognosis and Delineate Risk Groups in Neuroblastoma

Ruey-Jen Lin^{1,2}, You-Chin Lin², Jeremy Chen³, Huan-Hsien Kuo², Yuan-Yan Chen³, Mitchell B. Diccianni⁴, Wendy B. London⁵, Chih-Hao Chang^{1,2}, and Alice L. Yu^{2,4}

Abstract

Neuroblastoma is a common childhood tumor and accounts for 15% of pediatric cancer deaths. To investigate the microRNA (miRNA) profile and role of *Dicer* and *Drosha* in neuroblastoma, we assessed the expression of 162 human miRNAs, *Dicer* and *Drosha* in 66 neuroblastoma tumors by using real-time PCR methods. We found global downregulation of miRNA expression in advanced neuroblastoma and identified 27 miRNAs that can clearly distinguish low- from high-risk patients. Furthermore, expression levels of *Dicer* or *Drosha* were low in high-risk neuroblastoma tumors, which accounted for global downregulation of miRNAs in advanced disease and correlated with poor outcome. Notably, for patients with non-*MYCN*-amplified tumors, low expression of *Dicer* can serve as a significant and independent predictor of poor outcome (hazard ratio, 9.6; $P = 0.045$; $n = 52$). Using plausible neural networks to select a combination of 15 biomarkers that consist of 12 miRNAs' signature, expression levels of *Dicer* and *Drosha*, and age at diagnosis, we were able to segregate all patients into four distinct patterns that were highly predictive of clinical outcome. *In vitro* studies also showed that knockdown of either *Dicer* or *Drosha* promoted the growth of neuroblastoma cell lines. Our results reveal that a combination of 15 biomarkers can delineate risk groups of neuroblastoma and serve as a powerful predictor of clinical outcome. Moreover, our findings of growth promotion by silencing *Dicer*/*Drosha* implied their potential use as therapeutic targets for neuroblastoma. *Cancer Res*; 70(20); 7841–50. ©2010 AACR.

Introduction

microRNAs (miRNAs) are a class of small, evolutionarily conserved, endogenous noncoding RNAs that act as negative regulators of gene expression by inhibiting translation or promoting RNA degradation. miRNAs are transcribed by RNA polymerase II to generate the primary miRNA transcripts, which are processed into the ~70-nt hairpin structured pre-miRNA by *Drosha*, a RNase III endonuclease in the nucleus. After being transported to cytoplasm by exportin 5, pre-miRNA is further processed by another RNase III endonuclease, *Dicer*, to generate the ~22-nt mature miRNA (1). Recent studies have shown that miRNAs are involved in the regulation of multiple physiologic processes, including

apoptosis, proliferation, and differentiation, and are implicated in the pathogenesis of various diseases. In cancer, miRNA have been shown to function as both tumor suppressors and oncogenes by targeting genes that are critical regulators in cancer development, and are also shown to be useful for cancer classification and prognostication (2). Furthermore, the components of miRNA biogenesis machinery, including *Drosha* and *Dicer*, have been implicated in tumorigenesis (3–10). These findings suggest that miRNAs may serve as both prognostic markers and therapeutic targets.

Neuroblastoma is a common childhood tumor derived from primitive sympathetic neuroblasts and accounts for 15% of pediatric cancer deaths. The hallmark of this tumor is its plethoric clinical behavior, ranging from malignant tumor progression to spontaneous regression or differentiation to benign ganglioneuroma (11). The biological variables, such as genomic amplification of the *MYCN* oncogene, allelic loss of chromosome 1p, 3p, 11q, and 14q, gain of 17q, and differential expression of neurotrophin receptors TrkA/TrkB have been associated with tumor behavior (12). However, the key prognostic indicator in neuroblastoma is *MYCN* amplification, which occurs in about 20% of cases and is associated with high tumor stage and poor outcome (13, 14). Currently, treatment of neuroblastoma patients is tailored to risk group assignment according to well-known prognostic factors in addition to *MYCN* amplification, such as patient age at diagnosis, International Neuroblastoma Staging System (INSS)

Authors' Affiliations: ¹Graduate Institute of Life Science, National Defense Medical Center, and ²Genomics Research Center, Academia Sinica, Taipei, Taiwan; ³PNN technologies Inc, Woodbridge, Virginia; ⁴Department of Pediatrics/Hematology-Oncology, University of California San Diego Medical Center, San Diego, California; and ⁵Children's Oncology Group Statistics and Data Center, University of Florida, Gainesville, Florida

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Corresponding Author: Alice L. Yu, Genomics Research Center, Academia Sinica, 128, Academia Road, Sec. 2, Taipei 115, Taiwan. Phone: 886-2-2789-9930; Fax: 886-2-2789-993; E-mail: aliceyu@ucsd.edu.

doi: 10.1158/0008-5472.CAN-10-0970

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stage, tumor histopathology, and DNA index (15, 16). Although risk group assignment is useful for therapeutic stratification, there is still a lack of reliable markers for predicting treatment failure in neuroblastoma patients. Thus, it will be important to search for neuroblastoma-specific prognostic markers that can provide better prediction of tumor behavior and refine risk assessment.

Recent microarray studies of neuroblastoma have shown that gene expression profile may be useful for molecular classification and clinical prognostication (17–19). Emerging evidence suggests that miRNA expression profiling may add another dimension to sort out subtypes of various cancers (20). In this study, we delineated miRNA expression profiling of neuroblastoma and assessed its usefulness in clinical prognostication, as well as its role in tumorigenesis of neuroblastoma.

Materials and Methods

Neuroblastoma samples and cell lines

A total of 66 neuroblastoma primary tumors with a tumor cell content of $\geq 80\%$ were obtained from three sources: the Children's Oncology Group, the Pediatric Oncology Group, and the Cooperative Human Tissue Network collected from 1986 to 1995. The samples were fully encoded and examined under a protocol approved by the Institutional Review Board of Human

Subjects Research Ethics Committee (University of California San Diego, La Jolla, CA). The clinicopathologic information is listed in Table 1 and Supplementary Table S1. The cohort was representative of the population with neuroblastoma in general (21). The 5-year event-free survival was 50.8% with a median follow-up time of 5.33 years, and the 5-year overall survival rate was 56.9% with a median follow-up time of 6.08 years. The neuroblastoma cell lines Be2C, NMB7, and NB5 were maintained as described previously (22, 23). Total RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions.

Real-time PCR quantification of miRNAs and computational analysis

Expressions of 162 mature miRNAs in 66 human neuroblastoma patients were analyzed by TaqMan miRNA Assays Human Panel-Early Access Kit (Applied Biosystems) according to the manufacturer's protocol. Briefly, gene-specific reverse transcription was performed for each miRNA from 2.5 ng of total RNA in 15 μ L reaction volume by using TaqMan miRNA Reverse Transcription Kit, followed by q-PCR amplification using sequence-specific primers from TaqMan miRNA assays Human Panel on the 7300 Sequence Detection System (Applied Biosystems). Total RNA input was normalized based on threshold cycle (Ct) values of common internal

Table 1. Relationships between expression levels of *Dicer* and *Drosha* and various clinicopathologic characteristics

Characteristics	Cases	Dicer			Drosha		
		High	Low	<i>P</i> *	High	Low	<i>P</i> *
Sex							
Male	35	23	12	1.0	17	18	1.0
Female	23	15	8		12	11	
Age at diagnosis							
<1.5 years	43	36	7	<0.001	22	21	0.301
≥ 1.5 years	22	8	14		8	14	
INSS stage							
1	7	4	3	0.038 [†]	5	2	0.006 [†]
2	17	14	3		10	7	
3	12	9	3		6	6	
4	22	10	12		4	18	
4S	7	7	0		5	2	
Risk							
Low and intermediate	40	32	8	0.013	26	14	<0.001
High	25	12	13		4	21	
Histology							
Favorable	29	24	5	0.004	16	13	0.079
Unfavorable	18	7	11		5	13	
MYCN							
Nonamplified	52	37	15	0.321	28	24	0.015
Amplified	13	7	6		2	11	

*Two-sided Fisher's exact test.

[†]Comparing stage 1, 2, and 4S with stage 3 and 4.

control for miRNA quantification assays, U6 rRNA (24, 25), and all Ct values ≥ 36 , which were considered as not expressed, were adjusted to 36.

Data analysis was performed by using GENESPRING software (version 7.2, Silicon Genetics). First, data transformation was set to real-time PCR and then normalization was performed by using U6, allowing comparison among samples. To highlight miRNAs that characterize each clinicopathologic factors, a per-gene on median normalization was first performed with subsequent statistical comparisons performed by ANOVA with the Benjamin and Hochberg correction for false positive reduction. Hierarchical clustering for both miRNAs and conditions was generated by using standard correlation as a measure of similarity.

Further analyses to identify differentially expressed miRNAs among risk and classified samples were performed by using the prediction analysis of microarray (PAM) algorithm (26). We identified the miRNAs that result in best-risk tumor classification across all samples by using the method of the nearest shrunken centroids, as implemented in PAM. The prediction error was calculated by means of 10-fold cross-validation and then the miRNAs were selected yielding the minimum misclassification error.

Real-time reverse transcriptase-PCR of *Dicer* and *Drosha*

Total RNA was reverse transcribed to cDNA using SuperScript First-Strand Synthesis System with random hexamer primers (Invitrogen). Real-time quantitative PCR was performed using cDNA transcribed from 10 ng total RNA, 1X SYBR Green Master Mix (Applied Biosystems), and either *Dicer*, *Drosha*, or *GAPDH* primers as previously described (4) on an Applied Biosystems PRISM 7300-HT. All reactions were run in triplicate with expression levels normalized against *GAPDH*.

Plausible neural network analysis

Plausible neural networks (PNN; refs. 27, 28) is an intelligent self-organizing neural networks system. PNN performs unsupervised learning, associative memory, clustering, classification, function approximation, and belief judgment in single-network architecture. One specific advantage of PNN is the capability to deal with all kinds of measurement simultaneously. PNN was trained using all of the neuroblastoma patient information and biomarkers including *Dicer*, *Drosha*, and miRNAs to form multivariate patterns. PNN performs feature selection by calculating the joint mutual information relationship between the biomarkers and the patterns. With these features, PNN was used to perform cluster analysis and predict the patients' clinical outcome.

Short hairpin RNA design and transfection

All the short hairpin RNA (shRNA) clones were obtained from National RNAi Core Facility (Genomics Research Center, Academia Sinica). shRNA against target sequence was 5'-GCTGGCTGTAAAGTACGACTA-3' (clone ID TRCN0000051262) for human *Dicer* (shDicer), 5'-GCCAGATGAGACTGAAGACAT-3' (TRCN0000022253) for *Drosha*

(shDrosha), and 5'-TCCTAAGGTTAAGTCGCCCTCG-3' (TRCN0000072243) for firefly luciferase as the negative control (shLuc). The shRNAs plasmids were transfected into neuroblastoma cell lines with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Knock-down efficiency of *Dicer* and *Drosha* was confirmed by quantitative reverse transcriptase-PCR and Western blot analysis at 48 to 72 hours posttransfection.

Cell proliferation and soft-agarose colony forming assay

Cells were seeded at a density of 5×10^3 in a 96-well plate 24 hours before transfection with different shRNAs or negative control shLuc plasmids using Lipofectamine 2000 (Invitrogen). Forty-eight hours posttransfection, proliferation rates were determined daily by incubation in 10% alamarBlue for 4 hours and followed by measurement of fluorescence with excitation at 544 nm and emission at 590 nm by spectrophotometer (Spectramax 190, Molecular Devices).

For soft-agarose colony-forming assay, 60-mm dishes were coated with 0.6% agarose in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). This layer was then overlaid with a suspension of 1,000 cells, which were transfected with shRNA for 48 hours, in 0.36% agarose/RPMI/FBS and allowed to grow for two weeks at 37°C. The cell colonies >1 mm in diameter were counted after staining with 5% MTT for 15 minutes. Experiments were performed in triplicate using three neuroblastoma cell lines (Be2C, NMB7, and NB5).

Statistical analysis

The best cutoff value for separating two groups in terms of gene expression levels (*Dicer*, *Drosha*) were determined by Student's *t*-test; these values were found to be -4.5 and -5.13 for *Dicer* and *Drosha*, respectively, which are close to their mean values (-4.2 and -5.13, respectively). The association between various clinical characteristics and expression levels of *Dicer* and *Drosha* were examined by Fisher's exact test. Event-free survival (EFS) and overall survival (OS) were estimated with the Kaplan-Meier method and were compared by log-rank test using GraphPad Prism software (version 4, GraphPad Software). Cox regression model was used for analysis of factors potentially related to EFS or OS. All statistical analyses were performed with SAS software (version 9.0, SAS Institute Inc). A *P* value <0.05 was considered as significant.

Results

Global downregulation of miRNA expression profile in advanced neuroblastoma tumors

The expression levels of 162 miRNAs in 66 primary neuroblastoma samples were quantified by real-time PCR method using TaqMan MiRNA Assay kit. Global miRNA expression profiles were generated using unsupervised agglomerative hierarchical clustering and are shown in Supplementary Fig. S1. Notably, 23 of the 162 miRNAs were not detectable in any neuroblastoma sample. In stage 4 tumors, most of the remaining miRNAs were also downregulated, especially in

those with *MYCN* amplification. Overall 33 miRNAs were differentially expressed among different stages ($P < 0.01$, Supplementary Table S2), and miRNA profiles could discriminate tumors with low- or intermediate-risk features from most of stage 4 *MYCN*-amplified tumors, which were clustered together (Supplementary Fig. S1).

miRNA signature can discriminate high-risk neuroblastoma patients from low-risk group

To identify the smallest set of miRNAs predictive of neuroblastoma risk group, we used the PAM algorithms to find 27 miRNAs that could discriminate between high- and low-risk groups. These miRNAs also have ANOVA P value of <0.001 in risk examination (Supplementary Table S3). Based on these 27 selected miRNAs, PAM correctly classified 23 of the 25 (92%) high-risk samples and 26 of the 31 (84%) low-risk samples. Using this signature, 9 of 10 intermediate-risk samples were classified to low risk and one stage 4 sample was grouped to high risk, and all 10 patients with intermediate risk had good outcome. In addition, cluster analysis based on these 27 selected miRNAs generated a tree that could discriminate risk groups and predict clinical outcome (Fig. 1). The tree consisted of a left branch (S1) and a right branch which could be subdivided into S2 and S3. The S1

branch was composed almost exclusively of cases without features of aggressive neuroblastoma and all survived ($n = 34$). None of these patients harbored *MYCN*-amplified tumors and 28 of the 34 cases were diagnosed at <1.5 years. The patients in this cluster belonged to stage 1, 2, 3, and 4S; all of them except for those stage 3 were classified as low risk by the current Children's Oncology Group system. Among 12 stage 3 patients, 7 were classified as intermediate risk and 1 patient with high risk fell into this cluster, whereas the other 4 cases classified as high risk did not. Furthermore, six of the seven stage 4S fell into S1 and one into S3 group, but all survived.

On the other hand, the right branch (S2 + S3) consisted of cases that were nearly all associated with more advanced disease and poor outcome. The smaller subbranch S2 ($n = 5$) included three cases with *MYCN* amplification at stage 2 or 3 and one case at stage 1 who died. The other subbranch S3 ($n = 27$) consisted of all stage 4 patients ($n = 22$). Remarkably, seven of the nine samples with stage 4 and *MYCN* amplification status were exclusively clustered together under this subbranch.

Our findings suggest that miRNA expression profile is significantly correlated with clinical and biological features of neuroblastoma.

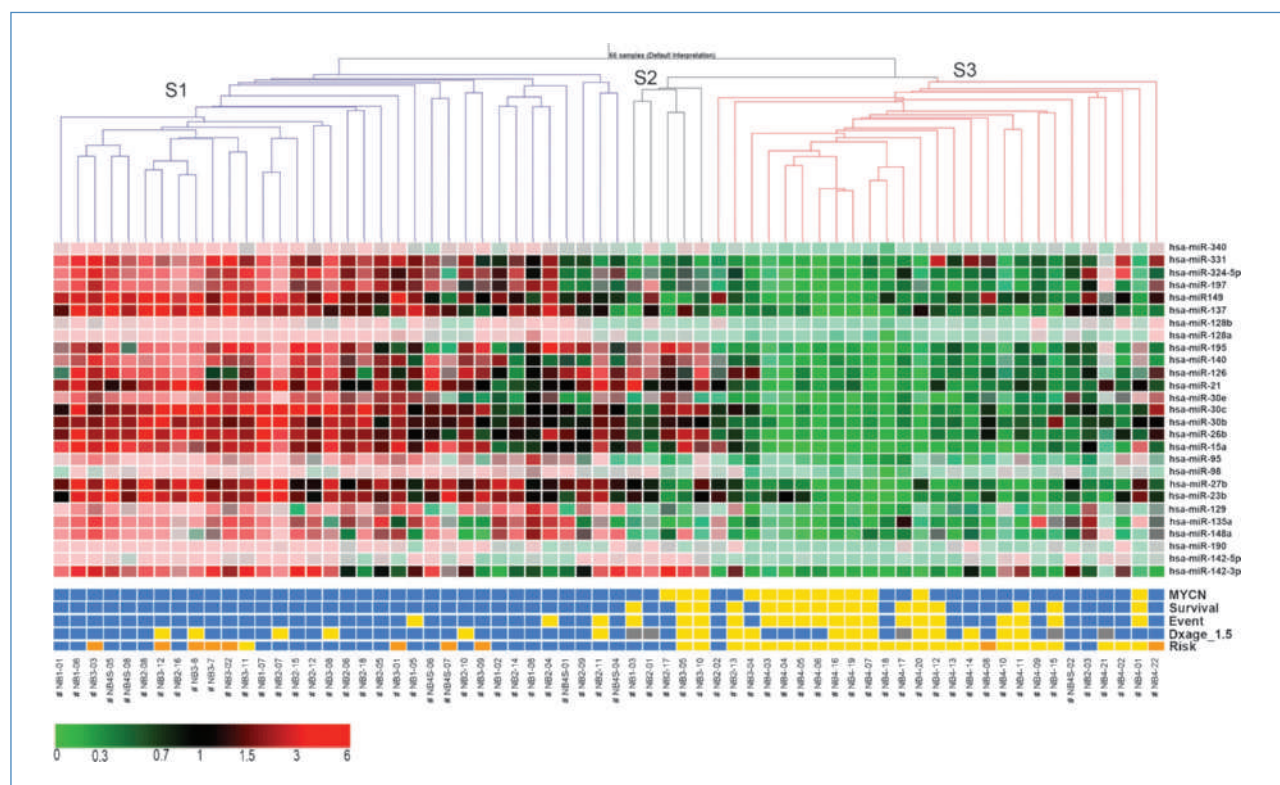


Figure 1. Hierarchical clustering of 66 primary neuroblastoma samples for 27 miRNAs selected, showing three main samples groups, S1 (blue), S2 (black), and S3 (red), using standard correlation as measure of similarity. Columns, samples; rows, miRNAs. Color scale, expression level relative to mean expression of a miRNA across all samples (red, high expression; green, low expression). Clinical characteristics: yellow, *MYCN* amplification, dead, event, age at diagnosis (Dxage) = 1.5–5 years, and high-risk; blue, *MYCN* not amplified, alive, no event, age at diagnosis <1.5 years, and low risk; gray, age at diagnosis >5 years; orange, intermediate risk. Neuroblastoma samples were coded as #NBx-01, NBx-02, etc., where x refers to INSS stage.

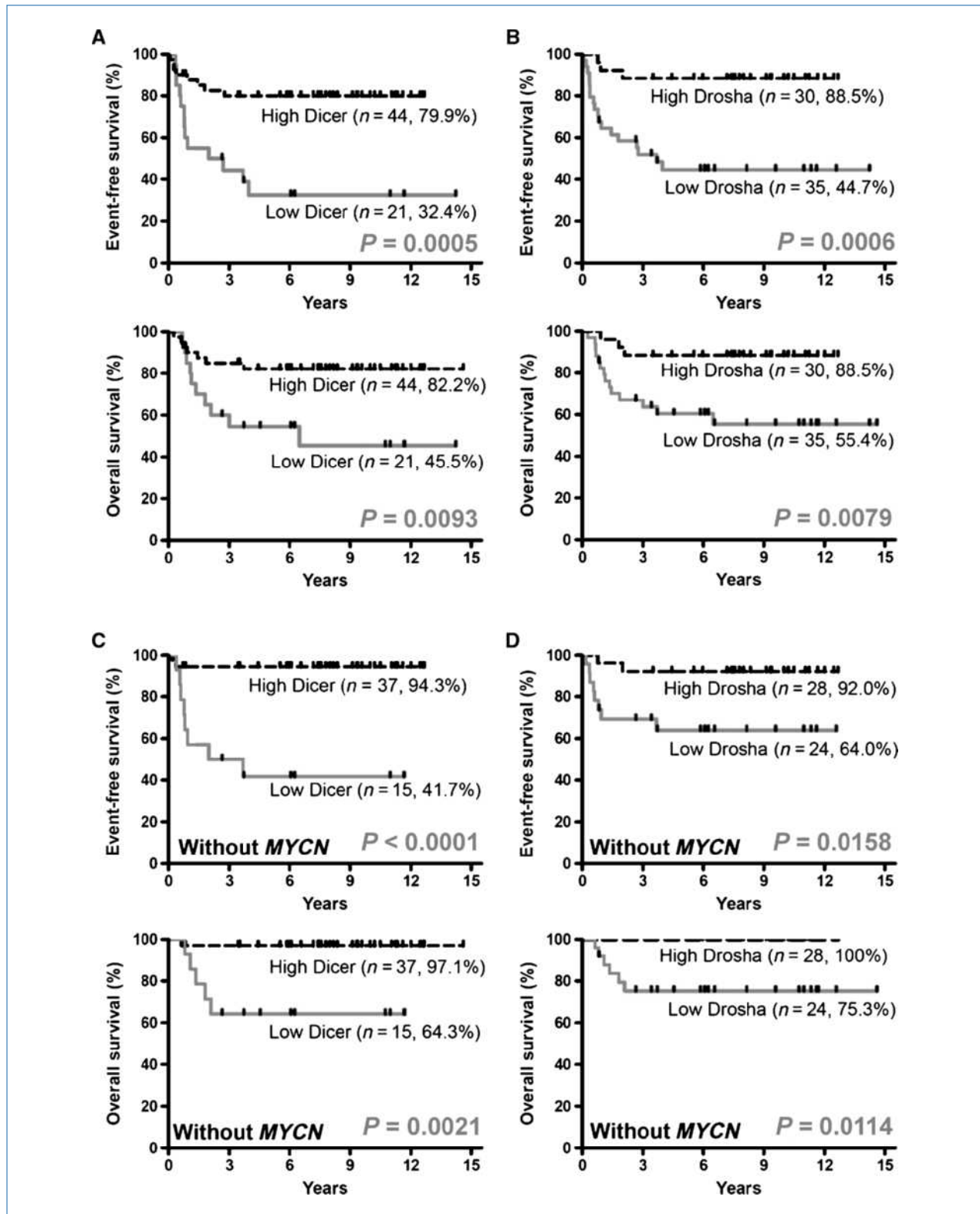


Figure 2. Expression levels of *Dicer* and *Drosha* were significantly correlated with survival of neuroblastoma patients as a whole and those without *MYCN* amplification. Kaplan-Meier estimates of EFS and OS for neuroblastoma patients are shown according to the expression levels of *Dicer* (A and C) and *Drosha* (B and D) for 65 neuroblastoma patients (A and B) and for 52 neuroblastoma patients without *MYCN* amplification (C and D). P values were obtained using the log-rank test.

Table 2. Cox regression analyses of the various factors associated with event-free survival and overall survival in neuroblastoma patients without MYCN ($n = 52$)

Event-free survival			
Variables	HR (95% CI)	Favorable/Unfavorable	P
Univariate analysis			
Stage	2.66 (0.77-9.17)	1, 2, 3, 4S/4	0.123
Gender	3.66 (0.78-7.26)	Female/Male	0.102
Risk	4.45 (1.25-15.77)	Low, Middle/High	0.021
Age at diagnosis_1.5	14.03 (2.97-66.36)	<1.5 years/≥1.5 years	0.001
Dicer	12.20 (2.57-57.82)	High/Low	0.002
Drosha	5.48 (1.16-25.84)	High/Low	0.032
Multivariate analysis			
Age at diagnosis_1.5	9.72 (1.37-69.10)	<1.5 years/≥1.5 years	0.023
Dicer	1.88 (0.22-16.23)	High/Low	0.568
Drosha	4.12 (0.69-24.68)	High/Low	0.121
Overall survival			
Variables	HR (95% CI)	Favorable/Unfavorable	P
Univariate analysis			
Stage	5.86 (1.07-32.06)	1, 2, 3, 4S/4	0.041
Gender	4.06 (0.47-34.81)	Female/Male	0.648
Risk	16.32 (1.90-139.92)	Low, Middle/High	0.011
Age at diagnosis*	594.6 (0.01-∞)	<1.5 years/≥1.5 years	0.242
Dicer	13.39 (1.56-114.77)	High/Low	0.018
Drosha	2.51 (0.46-13.74)	High/Low	0.288
Multivariate analysis			
Stage	3.22 (0.56-18.55)	1, 2, 3, 4S/4	0.190
Dicer	9.60 (1.05-87.44)	High/Low	0.045

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

*Because there was no death among the patients with diagnosis less than 1.5 years or 1 year.

Downregulation of Dicer and Drosha in advanced neuroblastoma

From the above cluster tree view and statistical analysis, the most prominent feature of miRNA expression profile was a widespread downregulation in high-risk samples, especially stage 4 tumors. Because Dicer and Drosha are essential for miRNA biogenesis and alterations of their expression levels have been reported in some human tumors (4, 6, 7, 29), we explored the possibility that global miRNA downregulation in stage 4 neuroblastoma could be due to reduced expression of Dicer and/or Drosha. With the use of real-time reverse transcriptase-PCR, *Dicer* and *Drosha* expression were determined in 65 of 66 neuroblastoma samples (one sample had insufficient RNA). As expected, expression levels of *Dicer* and *Drosha* were significantly lower in stage 4 ($P < 0.05$) than in other stages (except *Dicer* expression in stage 1), with the most striking difference in *Dicer* expression between stage 4 and 4S ($P < 0.001$), whereas no statistically significant difference among neuroblastoma tumors of other stages was noted (Supplementary Fig. S2). By probing the GSE13136 microarray dataset, we also observed lower expression of *Drosha* and *Dicer* in stage 4 neuroblastoma, although signif-

icant difference was only noted for *Drosha* comparing stage 4 versus non-stage 4 samples (Supplementary Fig. S3).

For the purpose of clinical correlative analyses, we used Student's *t*-test to select a cutoff value that could best discriminate high- and low-expression groups for Dicer and Drosha. As shown in Table 1, *Dicer* and *Drosha* expression were in general well correlated with various characteristics except for gender. Low *Drosha* expression was frequently seen in tumors of stage 4 (18 of 22, 82%), tumors of high risk (21 of 25, 84%), and MYCN-amplified tumors (11 of 13, 85%). Low *Dicer* expression was also significantly correlated with unfavorable age at diagnosis, stage, risk, and Shimada histology ($P < 0.001$, 0.038, 0.013, and 0.004, respectively; Table 1). Thus, low expression of *Dicer* and/or *Drosha* was significantly associated with high-risk phenotype.

Lower expression of Dicer or Drosha is associated with shorter survival

Next, we examined the prognostic value of *Dicer* and *Drosha* expression. The Kaplan-Meier survival analyses showed that neuroblastoma patients with low expression levels of *Dicer* had a significantly shorter EFS (32.4% versus

79.9%; $P = 0.0005$) and OS (45.5 versus 82.2%; $P = 0.0093$) than those with high levels of *Dicer*, respectively (Fig. 2A). Similarly, low *Drosha* expression was significantly associated with shorter EFS (44.7% versus 88.5%, $P = 0.0006$) and OS (55.4% versus 88.5%; $P = 0.0079$) than high *Drosha* expression (Fig. 2B). Furthermore, univariate Cox regression analysis of various parameters with OS showed that in addition to the well-known prognostic factors for neuroblastoma such as clinical stage, risk, and *MYCN* status, low expression of *Dicer* ($P = 0.0142$) or *Drosha* ($P = 0.0158$) was another significant predictive factor for poor outcome (Supplementary Table S4). However, when further analyzed by multivariate Cox regression, only *MYCN* amplification ($P = 0.0077$) and stage ($P = 0.035$) remained independent predictors. This is also true for the correlation between EFS and expression of *Dicer* and *Drosha* (Supplementary Table S4). Further supporting evidence was obtained by analysis of two large and independent cohorts of microarray database. A similar trend of association was noted between lower *Dicer* and decreased EFS, as determined by Kaplan-Meier survival estimates (Supplementary Fig. S7) and Cox regression analysis (Supplementary Table S6), with hazard ratios of 1.24 and 1.29 for E-TABM-38 ($n = 251$) and E-MTAB-16 ($n = 262$), respectively.

Expression of *Dicer* is an independent predictor for survival in neuroblastoma patients without *MYCN* amplification

In the above analyses of all 66 patients, *MYCN* is such a strong prognostic factor that it may have masked the effect of other factors in subgroup of patients. Analysis of neuroblastoma patients without *MYCN* amplification revealed that the low expression levels of *Dicer* or *Drosha* were significantly correlated with shorter EFS and OS (Fig. 2C and D). Notably, further analysis by univariate and multivariate Cox regression model identified *Dicer* expression as a significant and independent predictor for overall survival (hazard ratio, 9.6; $P = 0.045$; Table 2) in this subgroup.

These findings suggest that low expression of *Dicer* and *Drosha* may account for global reduction of miRNAs in advanced neuroblastoma and may significantly impact on patient outcome. Notably, expression levels of *Dicer* can serve as an important and independent predictor in patients without *MYCN* amplification.

Identification of a unique signature of 12 miRNAs, *Dicer*, *Drosha*, and age at diagnosis that can delineate clinical risk group by PNN analysis

Next, we investigated whether a combination of all biomarkers including *Dicer*, *Drosha*, and miRNAs can provide a better prediction for clinical outcome. To facilitate multivariate biomarker analysis, we utilize the theory of PNN (27), which is a self-organizing neural network model with plausible reasoning capability.

Using PNN Solution software, we trained a PNN on all the biomarkers and clinical information of the neuroblastoma samples except outcome information. During training, PNN uses an unsupervised learning algorithm that makes use of each biomarker to provide partial-weighted

evidence to form multivariate patterns. The network then uses these patterns to assign the patient samples into different natural clusters.

Then we used PNN Solution to calculate mutual information content to measure the joint relationship between the biomarkers and the patterns. Because of their strong mutual information relationship, the top-ranked biomarkers provide the strongest information to distinguish the patterns. Thus, we kept only the top 15 biomarkers for further PNN analysis; these consisted of 12 miRNAs' signature, expression of *Dicer* and *Drosha*, and age at diagnosis. We then used the 15 biomarkers to train the network again to form new clearer patterns, and let PNN assign the neuroblastoma patients into four different natural clusters (Supplementary Table S5). Figure 3 shows the Kaplan-Meier survival curve of OS for neuroblastoma patients according to their cluster (group) assignment. It reveals that group A contains mostly high-risk patients (18 of 19) with a fatal outcome (74%); in group B, 76% (19 of 25) of the patients are of low/intermediate risk, and 12% are high-risk patients that have fatality. Groups C and D consist of low/intermediate-risk patients that are alive, except for one patient. It shows that multivariate patterns found by PNN analysis can distinguish between short-term and long-term neuroblastoma patient survival, better than using *Dicer* and *Drosha* expression levels alone to determine neuroblastoma patient survival. These results were further confirmed by assigning neuroblastoma patients into training and test set to correlate with patient survival (see details in Supplementary Results, and Supplementary Fig. S4).

These findings imply that a combination of these 15 biomarkers may serve as a powerful predictor of neuroblastoma clinical outcome.

Knockdown of *Dicer* or *Drosha* can promote neuroblastoma cell proliferation and transformation

To elucidate the role of *Dicer* and *Drosha* on neuroblastoma tumorigenesis, we repressed the expression of *Dicer* and

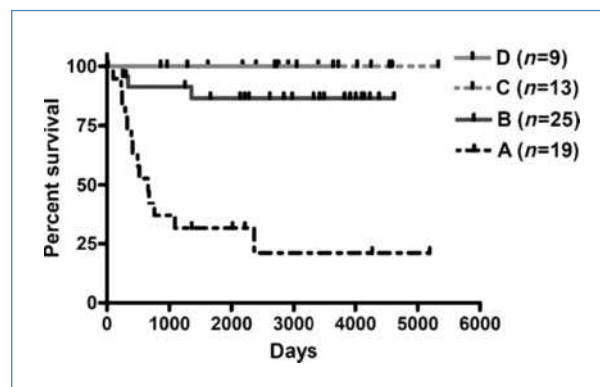


Figure 3. The 15 biomarkers can delineate clinical risk groups and refine risk assessment in neuroblastoma. Based on PNN analysis, 15 biomarkers, including 12 miRNAs, *Dicer*, *Drosha*, and age at diagnosis (Dxage), were self-organized into four cluster patterns. Kaplan-Meier estimates of OS for neuroblastoma patients according to their pattern assignment by PNN analysis.

Drosha in three neuroblastoma cell lines (Be2C, NMB7, and NB5) with shRNA. The extent of knockdown at mRNA and protein levels varied from 33% to 81% inhibition (Supplementary Fig. S5). As expected, knockdown of either Dicer or Drosha led to a reduction in expression of miRNAs as reflected by qPCR detection of miR-17-5p and let-7a, and miRNA array (Supplementary Figs. S5 and S6). As compared with the negative control (shLuc), Drosha knockdown not only enhanced cell proliferation (Fig. 4A), but also induced greater number and size of colonies when cultured in soft agarose (Fig. 4B). Similar results were obtained with knockdown of Dicer (Fig. 4). These results were also confirmed by other independent shRNA clones against each gene (data not shown). These *in vitro* findings coupled with the above-mentioned clinical correlative studies suggest that Dicer and Drosha deregulate expression of miRNAs and promote tumor progression in neuroblastoma.

Discussion

In this study, we determined the expression profile of miRNAs, *Dicer*, and *Drosha* in primary neuroblastoma samples and identified 27 miRNAs that can clearly distinguish low- from high-risk patients, using the PAM algorithm. Furthermore, using the PNN system to select a combination of 15 biomarkers that consist of 12 miRNAs, expression levels of *Dicer* and *Drosha*, and age at diagnosis, we were able to separate all patients into four distinct patterns that correlated well with clinical-pathologic characteristics of patients, and more importantly, with patient survival. If proven in future prospective study, this means that a combination of 12 miRNAs with *Dicer* and *Drosha* and age at diagnosis may serve as neuroblastoma-specific biomarkers for risk-group assignment and selection of optimal therapy, as well as a powerful predictor of clinical outcome.

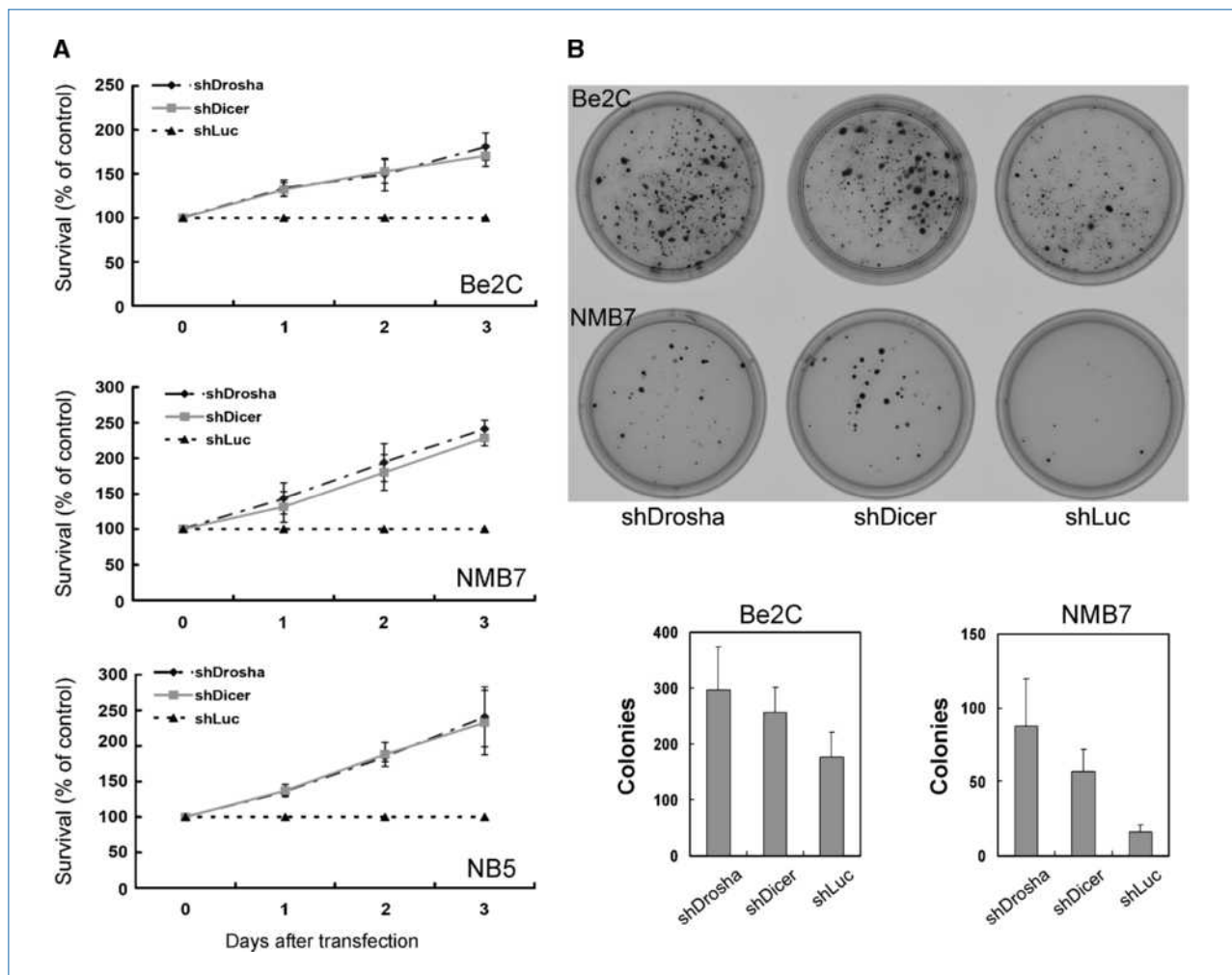


Figure 4. Knockdown of Dicer or Drosha enhances cell proliferation of neuroblastoma cell lines. Neuroblastoma cell lines Be2C, NMB7, and NB5 were transfected with shRNA against *Drosha* (shDrosha), *Dicer* (shDicer), or luciferase (shLuc). Cell proliferation was determined at indicated day after transfection using alamarBlue assay (A) or colony formation assay (B) in soft agar and expressed as percentage of growth as compared with negative control, shLuc. Values are mean \pm SE from at least three independent experiments.

Our finding of downregulation of miRNAs in neuroblastoma is consistent with several recent reports showing that the expression of most miRNAs is markedly reduced in a variety of human tumors, including breast cancer (30), thyroid anaplastic carcinoma (31), and prostate cancer (32). Notably, we found striking global downregulation of miRNAs in high-risk tumors, especially those with stage 4 and *MYCN* amplification. Similar findings were reported in a study of 157 miRNAs in 35 primary neuroblastoma tumors by Chen and Stalling (24). Ten of the 20 miRNAs associated with *MYCN* in their study are among the 27 miRNAs we identified to correlate with tumor risk (Supplementary Table S3). Thus, downregulation of miRNAs was associated with more advanced neuroblastoma, suggesting that overall repression of miRNAs may play a role in tumor progression.

Recent evidence suggested that expression of certain miRNAs was regulated by *MYCN* (33, 34). Furthermore, a widespread dysregulation of miRNA expression in neuroblastoma caused by overexpression of *MYCN* and large-scale chromosomal imbalances was noted, and a 15-miRNA signature was found to be predictive of clinical outcome (33). Although there are some overlapping in the selected miRNAs with those noted in our study, the contribution of Dicer and Drosha was not addressed in these studies. We showed that lower expression of *Dicer* and *Drosha* was responsible for global downregulation of miRNAs, and expression levels of *Dicer* and *Drosha* were significantly lower in stage 4 than in other stages, with a striking dichotomy in *Dicer* expression between stage 4 and 4S. Importantly, low expression of *Dicer* and *Drosha* significantly correlated with shorter EFS and OS of neuroblastoma patients. Thus, expression of *Dicer* and *Drosha* seemed to be another significant prognostic factor for neuroblastoma. Moreover, in cases without *MYCN* amplification, low expression levels of *Dicer* and *Drosha* correlated with worse patient survival, and multivariate analysis showed that low expression of *Dicer* could serve as a significant and independent prognostic factor for non-*MYCN*-amplified patients. Until now, no single marker or gene-expression profile can reliably predict outcome in tumor without *MYCN* amplification, which represents 80% of neuroblastoma (35, 36). If further validated in a larger and prospective cohort study, lower expression of *Dicer* may aid the prognostication of this large group of neuroblastoma patients.

Emerging evidence has linked the miRNA processing machinery to cancer. It has been reported that reduced expression of Dicer is associated with poor prognosis in non-small cell lung carcinomas (4) and ovarian cancer (10). This is consistent with our finding in neuroblastoma, but our data showed further that low expression of either *Dicer* or *Drosha* seemed to have prognostic impact. On the other hand, upregulation of Dicer has also been reported in prostate adenocarcinoma with correlation to more advanced clinical stage and lymph

node status (7). Similarly, upregulation of Drosha was reported to be associated with increased cell proliferation and poor prognosis in esophageal cancer (6). In cervical cancer, copy number-driven overexpression of *Drosha* and changes in miRNA profile seem to be important for cervical cancer progression (9). In addition, *Dicer1* mutation (missense mutation, L1583R) with truncation of a functional domain of RNase III has recently been identified in familial pleuropulmonary blastoma (37). These studies revealed that dysregulation of Dicer and Drosha exists in many types of human cancer, which may affect tumor progression and prognosis by altering miRNA expression profile.

Until now, the mechanism that regulates expression of Dicer and Drosha remains unclear. Zhang et al. have reported that copy number of *Dicer* and *Ago2* at genomic locus are abnormal in breast cancer, ovarian cancer, and melanoma (38). In addition, deletion of *Dicer* locus at chr14q32.13 in lung cancer (29) and genomic amplification of *Drosha* locus at chr5p13.3 leading to Drosha overexpression in cervical squamous cell carcinoma (9) have been reported. As to neuroblastoma, loss of heterogeneity for 14q 23-32 (which may encompass *Dicer* locus) occurs in 22% of primary neuroblastoma tumors and is more frequently associated with presence of 11q loss of heterogeneity and inversely correlated with *MYCN* amplification (39). However, a conflicting report showed that deletions of 14q32 were only seen in the low-and intermediate-risk group (40). It is not clear whether the deletion encompassed the *Dicer* gene in the above-mentioned reports and whether the locus of *Dicer* at 14q32.13 is aberrant in our samples set. The possibility of genetic abnormalities at *Dicer* and *Drosha* loci and the existence of other mechanisms such as epigenetic regulation of Dicer and Drosha in neuroblastoma await further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Children's Oncology Group, the Pediatric Oncology Group, and the Cooperative Human Tissue Network for providing neuroblastoma samples, and the National RNAi Core of Academia Sinica for providing RNAi for Dicer and Drosha.

Grant Support

Academia Sinica and grant FD-R-002319 from the Orphan Products Development Office of the U. S. Food and Drug Administration.

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Received 03/22/2010; revised 07/31/2010; accepted 08/19/2010; published OnlineFirst 08/30/2010.

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