Title: microRNA signature and expression of Dicer and Drosha can predict prognosis and delineate risk groups in neuroblastoma

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Keywords: miRNA, Dicer, Drosha, and neuroblastoma
Abbreviations: miRNA, microRNA; NB, neuroblastoma; PNN, Plausible Neural Networks

Running head: Expressions of microRNAs, Dicer and Drosha in neuroblastoma
Abstract

Neuroblastoma (NB) 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 NB, we have assessed the expression of 162 human miRNAs, Dicer and Drosha in 66 NB tumors by using real-time PCR methods. We found global downregulation of miRNA expression in advanced NB 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 NB tumors, which accounted for global downregulation of miRNAs in advanced disease and correlated with poor outcome. Notably, for patients with MYCN non-amplified tumors, low expression of Dicer can serve as a significant and independent predictor of poor outcome (HR=9.6, p=0.045, n=52). Using Plausible Neural Networks (PNN) to select a combination of 15 biomarkers which consist of 12 miRNAs signature, expression levels of Dicer and Drosha and age at diagnosis, we were able to segregate all patients into 4 distinct patterns which were highly predictive of clinical outcome. In vitro studies also showed that knockdown of either Dicer or Drosha promoted the growth of NB cell lines. Our results reveal that a combination of 15 biomarkers that can delineate risk groups of NB 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.
Introduction

microRNAs (miRNAs) are a class of small, evolutionarily conserved, endogenous noncoding RNAs that acts as negative regulator of gene expression by inhibiting translation or promoting RNA degradation. miRNAs are transcribed by RNA polymerase II to generate the primary miRNA transcript, 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 demonstrated that miRNAs are involved in the regulation of multiple physiological processes including apoptosis, proliferation and differentiation and implicated in the pathogenesis of various diseases. In cancer, miRNA have been shown to function as both tumor suppressors or oncogenes by targeting genes which are critical regulators in cancer development, and 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 (NB) 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
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 NB 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 NB patients is tailored to risk group assignment according to well-known prognostic factors in addition to MYCN-amplification, such as patient age at diagnosis, INSS stage (International Neuroblastoma Staging System), 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 NB patients. Thus, it will be important to search for NB-specific prognostic markers that can provide better prediction of tumor behavior and refine risk assessment.

Recent microarray studies of NB have shown that gene expression profile may be useful for molecular classification and clinical prognostication (17-19). Emerging evidence suggest 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 NB and assessed its usefulness in clinical prognostication, as well as its role in tumorigenesis of NB.
Materials and Methods

NB samples and cell lines

A total of 66 NB primary tumors with a tumor cell content of 80% or greater were obtained from three sources: Children's Oncology Group (COG), Pediatric Oncology Group (POG) and Cooperative Human Tissue Network (CHTN) 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. The clinicopathologic information was listed in Table 1 and Supplementary Table S1. The cohort was representative of the population with NB in general (21). The 5-yr event-free survival was 50.8% with a median follow-up time of 5.33 years; and the 5-yr overall survival rate was 56.9% with a median follow-up time of 6.08 years. The NB cell lines, Be2C, NMB7 and NB5, were 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 NB patients were analyzed by TaqMan miRNA Assays Human Panel-Early Access Kit (Applied Biosystems, Foster City, CA) 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 μl 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 control for miRNA quantification assays, U6 rRNA (24, 25), and all Ct values higher or equal to 36, which were considered as not expressed, were adjusted to 36.

Data analysis was performed by using GENESPRING software (version 7.2, Silicon Genetics, Redwood City, CA). 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 clinicopathological 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 were 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 algorithm of Prediction Analysis of Microarray (PAM) (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 RT-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.

**PNN analysis**

Plausible Neural Networks (PNN) (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 NB 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. Using these features, PNN was used to perform cluster analysis and predict the patients’ clinical outcome.

**shRNA design and transfection**

All the shRNA clones were obtained from National RNAi Core Facility (Genomic Research Center, Academia Sinica,). shRNA against target sequence
for human Dicer (shDicer) was 5’-GCTGGCTGTAAAGTACGACTA-3’ (clone ID TRCN0000051262), for Drosha (shDrosha) was 5’-GCCAGATGAGACTGAAGACAT-3’ (TRCN0000022253) and for firefly luciferase (as the negative control, shNC) was 5’-TCCTAAGGTTAAGTCGCCCTCG-3’ (TRCN0000072243). The shRNAs plasmids were transfected into NB cell lines with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Knockdown efficiency of Dicer and Drosha was confirmed by q-RT PCR and western blot analysis at 48h~72h post-transfection.

Cell proliferation and soft-agarose colony forming assay

Cells were seeded at a density of 5×10^3 in a 96-well plate 24h before transfection with different shRNAs or negative control shLeu plasmids using Lipofectamine 2000 (Invitrogen). 48h post-transfection, proliferation rate were determined daily by incubated in 10% AlamarBlue for 4h and followed by measurement of fluorescence with excitation at 544 nm and emission at 590 nm by spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA).

For soft-agarose colony forming assay, 60 mm dishes were coated with 0.6% agarose in RPMI-1640 supplemented with 10% FBS. This layer was then overlaid with a suspension of 1000 cells, which were transfected with shRNA for 48h, in 0.36% agarose/RPMI/FBS and allowed to grow for two weeks at 37°C. The number of cell colonies larger than 1 mm in diameter were counted after staining with 5% MTT for 15 min. Experiments were performed in triplicate using three NB cell lines (Be2C, NMB7 and NB5).
**Statistical analysis**

The best cut-off 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 Kaplan-Meier method and compared by log-rank test using GraphPad Prism software (version 4, GraphPad Software, San Diego, CA). Cox regression model analysis 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 NB tumor

The expression levels of 162 miRNAs in 66 primary NB 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 Figure S1. Notably, 23 of the 162 miRNAs were not detectable in any NB sample. In stage 4 tumors, most of the remaining miRNAs were also down-regulated, especially in those with MYCN amplification. Overall 33 miRNAs were differentially expressed among different stages (p<0.01, Supplementary Table S2), and miRNA profiles can 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 NB patients from low-risk group

To identify the smallest set of miRNAs predictive of NB 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 has 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 intermediated-risk had good outcome. In addition, cluster analysis based on these 27 selected miRNAs generated a tree that can 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 comprised almost exclusively of cases without features of aggressive NB and all survived (n=34). None of these patients harbored MYCN-amplified tumors and 28 of the 34 cases were diagnosed at <1.5yr. 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 COG system. Among 12 stage 3 patients, 7 patients classified as intermediate-risk and one patient with high-risk fell into this cluster, whereas the other 4 cases classified as high risk did not. Furthermore, 6 out of the 7 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 3 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, 7 of the 9 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 NB.

**Downregulation of Dicer and Drosha in advanced NB**

From the above cluster tree view and statistical analysis, the most prominent feature of miRNA expression profile was a widespread down-regulation in high-risk samples, especially stage 4 tumors. Since 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 down-regulation in stage 4 NB could be due to reduced expression of Dicer and/or Drosha. Using Real-time RT-PCR, Dicer and Drosha expression was determined in 65 of 66 NB samples (one sample had insufficient RNA). As expected, expression levels of Dicer and Drosha were significantly lower in stage 4 (p<0.05) than 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 NB 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 NB, although significant difference was only noted for Drosha comparing stage 4 vs. non-stage 4 samples (Supplementary Fig. S3).

For the purpose of clinical correlative analyses, we used the student t-test to select a cut-off value that could best discriminate high and low expression groups for Dicer and Drosha. As shown in Table 1, Dicer and Drosha expression was 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 NB patients with low expression
levels of Dicer had a significantly shorter event-free survival (32.4% vs. 79.9%,  
\( p=0.0005 \)) and overall survival (45.5 vs. 82.2%,  \( p=0.0093 \)) than those with high 
levels of Dicer, respectively (Fig. 2A). Similarly, low Drosha expression was 
significantly associated with shorter event-free survival (44.7% vs. 88.5%,  
\( p=0.0006 \)) and overall survival (55.4% vs. 88.5%,  \( p=0.0079 \)) than high Drosha 
expression (Fig. 2B). Furthermore, univariate Cox regression analysis of various 
parameters with overall survival showed that in addition to the well-known 
prognostic factors for NB 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 to be independently predictors. This is 
also true for the correlation between event-free survival 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 Meir Survival estimates (Supplementary Fig. S7) and 
Cox regression analysis (Supplementary Table S6), with HR=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 NB patients without MYCN amplification

In the above analyses of the entire 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 NB patients without MYCN-amplification revealed that the low expression levels of Dicer or Drosha were significantly correlated with shorter event-free survival and overall survival (Fig. 2C and 2D). Notably, further analysis by univariate and multivariate Cox regression model identified Dicer expression as a significant and independent predictor for overall survival (HR=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 NB 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 Plausible Neural Networks (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 NB samples except outcome information. During training, PNN uses an unsupervised learning algorithm that makes use of each biomarker to provide a 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 the 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; which consist of 12 miRs signature, expression of Dicer and Drosha, and age at diagnosis. We then used the 15 biomarkers to train the network again to form new cleaner patterns, and let PNN assign the NB patients into four different natural clusters (Supplementary Table 256 on April 13, 2017. © 2010 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2010 American Association for Cancer Research.
Figure 3 shows the Kaplan-Meier survival curve of overall survival for NB patients according to their cluster (group) assignment. It reveals that group A contains mostly high-risk patients (18/19) with a fatal outcome (74%); in group B, 76% (19/25) of the patients are 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 of one patient. It shows that multivariate patterns found by PNN analysis can distinguish between short-term and long-term NB patient survival; better than using Dicer and Drosha expression levels alone to determine NB patient survival. These results were further confirmed by assigning NB patients into training and test set to correlate with patient’s survival (see details in Supplemental results, and Supplementary Fig. S4).

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

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

To elucidate the role of Dicer and Drosha on NB tumorigenesis, we repressed the expression of Dicer and Drosha in three NB cell lines (Be2C, NMB7 and NB5) with shRNA. The extent of knockdown at mRNA and protein levels varied from
33–81% 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 Fig. S5 and Fig. 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 NB.
Discussion

In this study, we determined expression profile of miRNAs, Dicer and Drosha in primary NB samples and identified 27 miRNAs that can clearly distinguish low-from high-risk patients, using the PAM algorithm. Furthermore, using PNN system to select a combination of 15 biomarkers which consist of 12 miRNAs, expression levels of Dicer and Drosha and age at diagnosis, we were able to separate all patients into 4 distinct patterns which correlated well with clinical-pathologic characteristics of patients, and more importantly, with patient’s survival. If proven in future prospective study, this means that a combination of 12 miRNAs with Dicer Drosha and age at diagnosis may serve as NB-specific biomarkers for risk group assignment and selection of optimal therapy, as well as a powerful predictor of clinical outcome.

Our finding of down-regulation of miRNAs in NB is consistent with several recent reports showing that the expression of most miRNAs are 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 down-regulation of miRNAs in high-risk tumors, especially those with stage 4 and MYNC amplification. Similar findings were reported in a study of 157 miRNAs in 35 primary NB tumors by Chen and Stalling (24). Ten of the 20 miRNAs
associated with MYCN in their study are among 27 miRNAs we identified to correlate with tumor risk (Supplementary Table S3). Thus, down-regulation of miRNAs was associated with more advanced NB, 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 NB caused by over-expression 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 demonstrated that lower expression of Dicer and Drosha was responsible for global down-regulation of miRNAs, and expression levels of Dicer and Drosha were significantly lower in stage 4 than 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 event free survival and overall survival of NB patients. Thus, expression of Dicer and Drosha appeared to be another significant prognostic factor for NB. Moreover, in cases without MYCN-amplification, low expression levels of Dicer and Drosha correlated with worse patient survival and multivariate analysis.
demonstrated that, low expression of Dicer could serve as a significant and independent prognostic factor for MYCN non-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 NB 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 NB but our data showed further that low expression of either Dicer or Drosha seemed to have prognostic impact. On the other hand, up-regulation of Dicer has also been reported in prostate adenocarcinoma with correlation to more advanced clinical stage and lymph node status (7). Similarly, up-regulation of Drosha was reported to be associated with increased cell proliferation and poor prognosis in esophageal cancer (6). In cervical cancer, copy-number driven over-expression of Drosha and changes in miRNAs profile seems 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 exist 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 over-expression in cervical squamous cell carcinoma (9) have been reported. As to NB, loss of heterogeneity (LOH) for 14q 23-32 (which may encompass Dicer locus) occurs in 22% of primary NB tumors and is more frequently associated with presence of 11q LOH and inversely correlated with MYCN-amplification (39). However, a conflicting report showed that deletions of 14q32 were only seen in the low-and intermediated-risk group (40). It is not clear whether the deletion encompassed Dicer gene in 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 NB await further investigation.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

We are grateful to Children's Oncology Group (COG), Pediatric Oncology Group (POG) and Cooperative Human Tissue Network (CHTN) for providing neuroblastoma samples. We also thank the National RNAi Core of Academia Sinica for providing RNAi for Dicer and Drosha.

Grant Support

This work was supported in part by Academia Sinica and grant FD-R-002319 from the Orphan Products Development Office of the U. S. Food and Drug Administration.
References


**Figure Legends**

**Figure 1.** Hierarchical clustering of 66 primary NB samples for 27 miRNAs selected, showing three main samples groups, S1 (blue), S2 (black), and S3 (red) using Standard correlation as measure of similarity. Samples are shown in columns, miRNAs in rows. Color scale represents expression level relative to mean expression of a miRNA across all samples (red: high expression, green: low expression). Clinical characteristics: MYCN amplification, dead, event, age at diagnosis (Dxage) =1.5~5 year and high-risk are shown in yellow; MYCN not amplified, alive, no event, age at diagnosis< 1.5 year and low-risk are shown in blue; age at diagnosis >5 year, intermediated-risk are shown in grey. NB 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 NB patients as a whole and those without MYCN amplification. Kaplan-Meier estimated of event-free survival and overall survival for NB patients were shown according to the expression levels of Dicer (A & C) or Drosha (B & D) for sixty-five NB patients (A & B) and for fifty-two NB patients without MYCN amplification (C & D). p-values were obtained using the log-rank test.
**Figure 3.** The 15 biomarkers can delineate clinical risk groups and refine risk assessment in NB.

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 overall survival for NB patients according to their pattern assignment by PNN analysis.

**Figure 4.** Knockdown of Dicer or Drosha enhances cell proliferation of NB cell lines.

NB 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 (A) alamarBlue assay or (B) colony formation assay in soft agar and expressed as percentage of growth as compared to negative control, shLuc. Values are mean ± s.e.m. from at least three independent experiments.
Table 1. Relationships between expression levels of Dicer and Drosha and various clinicopathologic characteristics

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<td>13</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Histology</td>
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<td></td>
</tr>
<tr>
<td>Favorable</td>
<td>29</td>
<td>24</td>
<td>5</td>
<td>0.004</td>
<td>16</td>
</tr>
<tr>
<td>Unfavorable</td>
<td>18</td>
<td>7</td>
<td>11</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>MYCN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-amplified</td>
<td>52</td>
<td>37</td>
<td>15</td>
<td>0.321</td>
<td>28</td>
</tr>
<tr>
<td>Amplified</td>
<td>13</td>
<td>7</td>
<td>6</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$ Two-sided Fisher’s exact test
$^b$ compare stage 1, 2 and 4S with stage 3 and 4
Table 2. Cox regression analyses of the various factors associated with event-free survival in NB patients without MYCN (N=52)

<table>
<thead>
<tr>
<th>Variables</th>
<th>HR (95% CI)</th>
<th>Favorable/Unfavorable</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>2.66 (0.77, 9.17)</td>
<td>1, 2, 3, 4S/4</td>
<td>0.123</td>
</tr>
<tr>
<td>Gender</td>
<td>3.66 (0.78, 7.26)</td>
<td>Female/Male</td>
<td>0.102</td>
</tr>
<tr>
<td>Risk</td>
<td>4.45 (1.25, 15.77)</td>
<td>Low, Middle/High</td>
<td>0.021</td>
</tr>
<tr>
<td>Age at diagnosis_1.5</td>
<td>14.03 (2.97, 66.36)</td>
<td>&lt;1.5 year/≥1.5 year</td>
<td>0.001</td>
</tr>
<tr>
<td>Dicer</td>
<td>12.20 (2.57, 57.82)</td>
<td>High/Low</td>
<td>0.002</td>
</tr>
<tr>
<td>Drosha</td>
<td>5.48 (1.16, 25.84)</td>
<td>High/Low</td>
<td>0.032</td>
</tr>
<tr>
<td><strong>Multivariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis_1.5</td>
<td>9.72 (1.37, 69.10)</td>
<td>&lt;1.5 year/≥1.5 year</td>
<td>0.023</td>
</tr>
<tr>
<td>Dicer</td>
<td>1.88 (0.22, 16.23)</td>
<td>High/Low</td>
<td>0.568</td>
</tr>
<tr>
<td>Drosha</td>
<td>4.12 (0.69, 24.68)</td>
<td>High/Low</td>
<td>0.121</td>
</tr>
</tbody>
</table>

Cox regression analyses of the various factors associated with overall survival in NB patients without MYCN (N=52)

<table>
<thead>
<tr>
<th>Variables</th>
<th>HR (95% CI)</th>
<th>Favorable/Unfavorable</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>5.86 (1.07, 32.06)</td>
<td>1, 2, 3, 4S/4</td>
<td>0.041</td>
</tr>
<tr>
<td>Gender</td>
<td>4.06 (0.47, 34.81)</td>
<td>Female/Male</td>
<td>0.648</td>
</tr>
<tr>
<td>Risk</td>
<td>16.32 (1.90, 139.92)</td>
<td>Low, Middle/High</td>
<td>0.011</td>
</tr>
<tr>
<td>Age at diagnosis#</td>
<td>594.6 (0.01, ∞)</td>
<td>&lt;1.5 year/≥1.5 year</td>
<td>0.242</td>
</tr>
<tr>
<td>Dicer</td>
<td>13.39 (1.56, 114.77)</td>
<td>High/Low</td>
<td>0.018</td>
</tr>
<tr>
<td>Drosha</td>
<td>2.51 (0.46, 13.74)</td>
<td>High/Low</td>
<td>0.288</td>
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<tr>
<td><strong>Multivariate analysis</strong></td>
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<td></td>
</tr>
<tr>
<td>Stage</td>
<td>3.22 (0.56, 18.55)</td>
<td>1, 2, 3, 4S/4</td>
<td>0.190</td>
</tr>
<tr>
<td>Dicer</td>
<td>9.60 (1.05, 87.44)</td>
<td>High/Low</td>
<td>0.045</td>
</tr>
</tbody>
</table>

HR, hazard ratio; 95% CI, 95% confidence interval

#: because there was no death among the patients with diagnosis less than 1.5 yr or 1yr.
Cancer Research


microRNA signature and expression of Dicer and Drosha can predict prognosis and delineate risk groups in neuroblastoma


Cancer Res  Published OnlineFirst August 30, 2010.

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