Supplementary Data

Supplementary Materials and Methods

Western Blot

Total protein extract was prepared by using RIPA lysis buffer plus protease inhibitors. 15 μg of each protein lysate was resolved on a 4-12% Bis-Tris NuPAGE gel (Invitrogen) and transferred to PVDF and probed with antibodies against Dicer, Drosha and β-actin (Abcam, Inc.) Proteins were visualized with ECF (Amersham Biosciences) and detected by Typhoon 9400 imager (GE Healthcare).

miRNA microarray

1 μg of MicroRNA-enriched small RNA samples were prepared from Dicer or Drosha-knockdown NB cell lines using the mirVAna™ miRNA Isolation Kit (Ambion) and labeled with DyLight™ DY-547(negative control, sh-Luc) or DyLight™ DY-647 (sh-Dicer or sh-Drosha) using T4 RNA ligase. The labeled samples were pooled and hybridized to miRNA OneArray (Phalanx Biotech, Hsing-Chu, Taiwan). The miRNA microarray contains 1,040 unique miRNA probes in triplicate, including 838 human miRNA (Sanger miRBase v11.0). Processed slides were scanned by using Axon GenePix 4000B scanner with laser set to 532nm and 635nm and a scan resolution of 10 μm. Signal intensities for each spot were calculated by subtracting local background from total

intensities and normalization were done by using U6 as internal control. Normalized data were hierarchically clustered by gene and are plotted as heat map.

Supplementary Results

PNN result validation

In further analysis, we assigned the NB patients into two data sets: a Training Set comprised of the first 38 patients diagnosed with NB, and a Testing Set containing the later-diagnosed 28 patients. We trained a new PNN to learn from the 15 biomarkers and clinical survival information (dead/alive status) of the NB patients in the Training Set. Then with the survival information removed from the Testing Set, we used the newly trained PNN to automatically classify each patient of the Testing Set as belonging to alive or dead groups. The accuracy of prediction for patient survival status in the Testing set was 25 correct out of 28(89%). We then performed another Kaplan-Meier survival analysis on these two patient groups (Supplementary Figure S4). The figure shows a clear distinction between the groups; thus, given a patient's 15 biomarkers, with no survival information known, one can distinguish NB patients who will survive from those who will die. These findings imply that a combination of these 15 biomarkers may serve as a powerful predictor of NB clinical outcome.

Supplementary Table S1

Summary of clinical characteristics of neuroblastoma patients

Variable	Cases No. (%) (n=66)
INSS stage	
Stage 1	7 (11)
Stage 2	18 (27)
Stage 3	12 (18)
Stage 4	22 (33)
Stage 4S	7 (11)
MYCN	
Amplification	13 (20)
Non Amplification	53 (80)
Risk	
Low	31 (47)
Intermediate	10 (15)
High	25 (38)
Survival	
Alive	49 (74)
Dead	17 (26)
Event	
no event	45 (68)
event	21 (32)
Age at diagnosis	
<1.5 year	43 (65)
≧1.5 year,<5 year	18 (27)
≧5 year	5 (8)
Shimada histology	
Favorable	29 (44)
Unfavorable	18 (27)
Missing	19 (29)
Sex	
Male	23 (35)
Female	36 (55)
missing	7 (10)
Sample source	
CCG (Children's Oncology Group)	35 (53)
POG (Pediatric Oncology Group)	21 (32)
CHTN (Cooperative Human Tissue Network)	10 (15)

Supplementary Table S2

miRNAs differentially expressed in comparison of clinically defined groups

Group	no. of miRNAs
Stage classification	
stage 1 (n=7) vs. 2 (n=18) vs.3 (n=12) vs. 4s (n=7) vs.4 (n=22)	33
stage 1~3 and 4s (n=44) vs. 4 (n=22)	78
stage 1 (n=7) vs. 2 (n=18) vs.3 (n=12) vs. 4s (n=7)	0[a]
stage 4 (n=22) vs. 4s (n=7)	33[b]
Risk classification	
Low (n=31) vs. Intermediate (n=10) vs. High (n=25)	53
Low and Intermediate (n=10) vs. High (n=25)	67
Low (n=31) vs. High (n=25)	59
Low (n=31) vs. Intermediate (n=25)	0[a]
MYCN classification	
all stage: NA (n=53) vs. Amp (n=13)	14
stage 4: NA (n=13) vs. Amp (n=9)	5[b]
Survival classification	
survival (n=49) vs. dead (n=17)	40
stage 4: survival (n=9) vs. dead (n=13)	1[a]
Event classification	
no event (n=45) vs. event (n=21)	21
stage 4: no event (n=8) vs. event (n=14)	1[a]
Age at diagnosis classification, yr	
<1.0 (n=32) vs. 1~5 (n=28) vs. <5 (n=5)	3[a]

All using 1-way ANOVA analysis, $p<0.001$; except [a] : $p<0.05$, [b]: $p<0.01$

NA, not amplification

Supplementary Table S3

27 miRNAs differentially expressed in high- vs. low-risk NB

	miRNA	location	fold change*	*p*-value [a]	PAM score [b]	
					H-risk (n=10)	L-risk (n=31)
1	**hsa-miR-149**	2q37.3	-3.8	1.66E-05	-0.0918	0
2	**hsa-miR-129**	7q32.1 / 11p11.2	-8.5	3.44E-05	-0.0892	0.0194
3	**hsa-miR-27b**	9q22.32	-5.7	1.66E-05	-0.0835	0.0074
4	hsa-miR-23b	9q22.1	-4.4	1.66E-05	-0.0828	0
5	**hsa-miR-190**	15q22.2	-3.8	1.05E-04	-0.0822	0
6	hsa-miR-128a	2q21	-4.8	1.66E-05	-0.0746	0
7	hsa-miR-15a	13q14	-6.5	3.56E-05	-0.0661	0
8	hsa-miR-148a	7p15.2	-5.3	1.53E-04	-0.0656	0.0364
9	**hsa-miR-137**	1p21.3	-5.1	1.16E-04	-0.0616	0
10	**hsa-miR-30c**	1p34.2 / 6q13	-4.3	1.66E-05	-0.0514	0
11	hsa-miR-197	1p13	-4.1	2.13E-05	-0.0418	0
12	hsa-miR-195	17p13	-4.4	2.29E-04	-0.0297	0
13	hsa-miR-26b	2q35	-4.6	1.66E-05	-0.0273	0
14	hsa-miR-21	17q23.2	-3.7	5.52E-05	-0.0267	0
15	**hsa-miR-30b**	8q24.22	-3.4	1.66E-05	-0.0264	0
16	hsa-miR-135a	3p21.1 / 12q23.1	-4.0	2.40E-04	-0.0259	0
17	hsa-miR-126	9q34.3	-4.2	6.44E-05	-0.0215	0.014
18	hsa-miR-95	4p16	-3.7	8.63E-05	-0.0206	0
19	hsa-miR-142-5p	17q23	-3.8	8.26E-04	0	0.0201
20	hsa-miR-128b	3p22	-4.0	1.39E-04	-0.0171	0
21	hsa-miR-98	xp11.2	-4.7	8.69E-05	-0.0137	0
22	hsa-miR-142-3p	17q23	-4.4	8.26E-04	0	0.0131
23	hsa-miR-340	5q35.3	-2.5	3.44E-05	-0.0079	0
24	**hsa-miR-30e**	1p34.2	-4.6	1.94E-05	-0.0071	0
25	**hsa-miR-331**	12q22	-3.0	3.44E-05	-0.004	0
26	hsa-miR-140	16q22.1	-2.9	3.56E-05	-0.0032	0
27	**hsa-miR-324-5p**	17p13.1	-3.6	1.87E-04	-0.0022	0

* fold change in high-risk compared to low-risk group

[a] by ANOVA (Welch *t* test in the Genespring software package)

[b] Centroid scores for the two classes of the PAM

Bold, miRNAs also obtained in Chen and Stallings group

Supplementary Table S4 Cox regression analyses of the various clinical factors with *event-free survival* in NB patients (N=65)

Variables	HR（95% CI）	Favorable/Unfavorable	*p*-value
Univariate analysis			
Stage	4.38（1.76，10.88）	1, 2, 3, 4S/4	**0.0015**
Myc	7.34（3.07，17.57）	Non-amplification/Amplification	**<0.0001**
Gender	1.08（0.95，1.24）	Female/Male	0.2419
Risk	8.18（2.74，24.42）	Low, Middle/High	**0.0002**
Age at diagnosis_1.5	4.97（1.99，12.37）	<1.5 year/≧1.5 year	**0.0006**
Dicer	4.28（1.76，10.40）	High/Low	**0.0013**
Drosha	6.49（1.91，22.10）	High/Low	**0.0028**
Multivariate analysis			
Stage	1.83（0.67，4.99）	1, 2, 3, 4S/4	0.2361
Myc	2.78（1.01，7.66）	Non-amplification/Amplification	**0.0481**
Age at diagnosis_1.5	2.00（0.65，6.17）	<1.5 year/≧1.5 year	0.2273
Dicer	1.34（0.46，3.93）	High/Low	0.5898
Drosha	2.66（0.63，11.23）	High/Low	0.1840

Cox regression analyses of the various clinical factors with *overall survival* in NB patients (N=65)

Variables	HR（95% CI）	Favorable/Unfavorable	*p*-value
Univariate analysis			
Stage	7.71（2.50，23.78）	1, 2, 3, 4S/4	**0.0004**
Myc	12.82（4.67，35.23）	Non-amplification/Amplification	**<0.0001**
Gender	1.12（0.97，1.29）	Female/Male	0.1183
Risk	34.14（4.51，258.65）	Low, Middle/High	**0.0006**
Age at diagnosis_1.5	5.67（1.99，16.16）	<1.5 year/≧1.5 year	**0.0012**
Dicer	3.37（1.28，8.90）	High/Low	**0.0142**
Drosha	4.66（1.34，16.26）	High/Low	**0.0158**
Multivariate analysis			
Stage	3.82（1.10，13.30）	1, 2, 3, 4S/4	**0.035**
Myc	6.04（1.61，22.63）	Non-amplification/Amplification	**0.0077**
Age at diagnosis_1.5	1.72（0.47，6.38）	<1.5 year/≧1.5 year	0.4155
Dicer	1.05（0.32，3.45）	High/Low	0.9365
Drosha_	1.01（0.20，5.23）	High/Low	0.9881

HR: Hazard Ratio; 95% CI, 95% confidence interval

Supplementary Table S5.

(A)

	miR-29a	miR-30c	miR-30e	miR-95	miR-128a	miR-128b	miR-135a	miR-135b	miR-137	miR-138	miR-148a	miR-195	Dxage	Dicer	Drosha
Pattern A	1.85	1.56	1.13	1.24	1.48	2.3	1.28	2.53	1.19	2.02	3.13	1.46	662.08	-4.05	-5.13
Pattern B	1.9	0.36	0.44	0.44	0.33	0.46	0.66	9.38	0.36	1.29	0.44	0.62	1045.22	-5.46	-6.23
Pattern C	9.07	4.08	6.38	5.9	6.04	5.88	7.58	18.1	5.64	11.07	4.6	7.22	664.38	-3.52	-5.24
Pattern D	2.77	3.65	2.19	2.79	3	4.83	5.82	12.59	2.7	2.51	4.82	3.33	216.43	-2.48	-3.82

(B)

PNN pattern	Dead		Event		MYCN-amp		Risk					
							H		M		L	
	No.	% of pattern	No.	% of pattern	No.	% of pattern	No.	%	No.	%	No.	%
A (n=19)	14	74%	15	79%	9	47%	18	95%	0	0%	1	5%
B (n=25)	3	12%	5	20%	4	16%	6	24%	5	20%	14	56%
C (n=13)	0	0%	1	8%	0	0%	0	0%	2	15%	11	85%
D (n= 9)	0	0%	0	0%	0	0%	1	11%	3	33%	5	56%

The 15 biomarkers can delineate clinical risk groups and refine risk assessment in NB.

(A), Based on PNN analysis, 15 biomarkers including 12 miRNAs, Dicer, Drosha and age at diagnosis (Dxage), were self-organized into four cluster patterns. (B) Lower table shows the distribution of patient characteristics for each of the four PNN patterns.

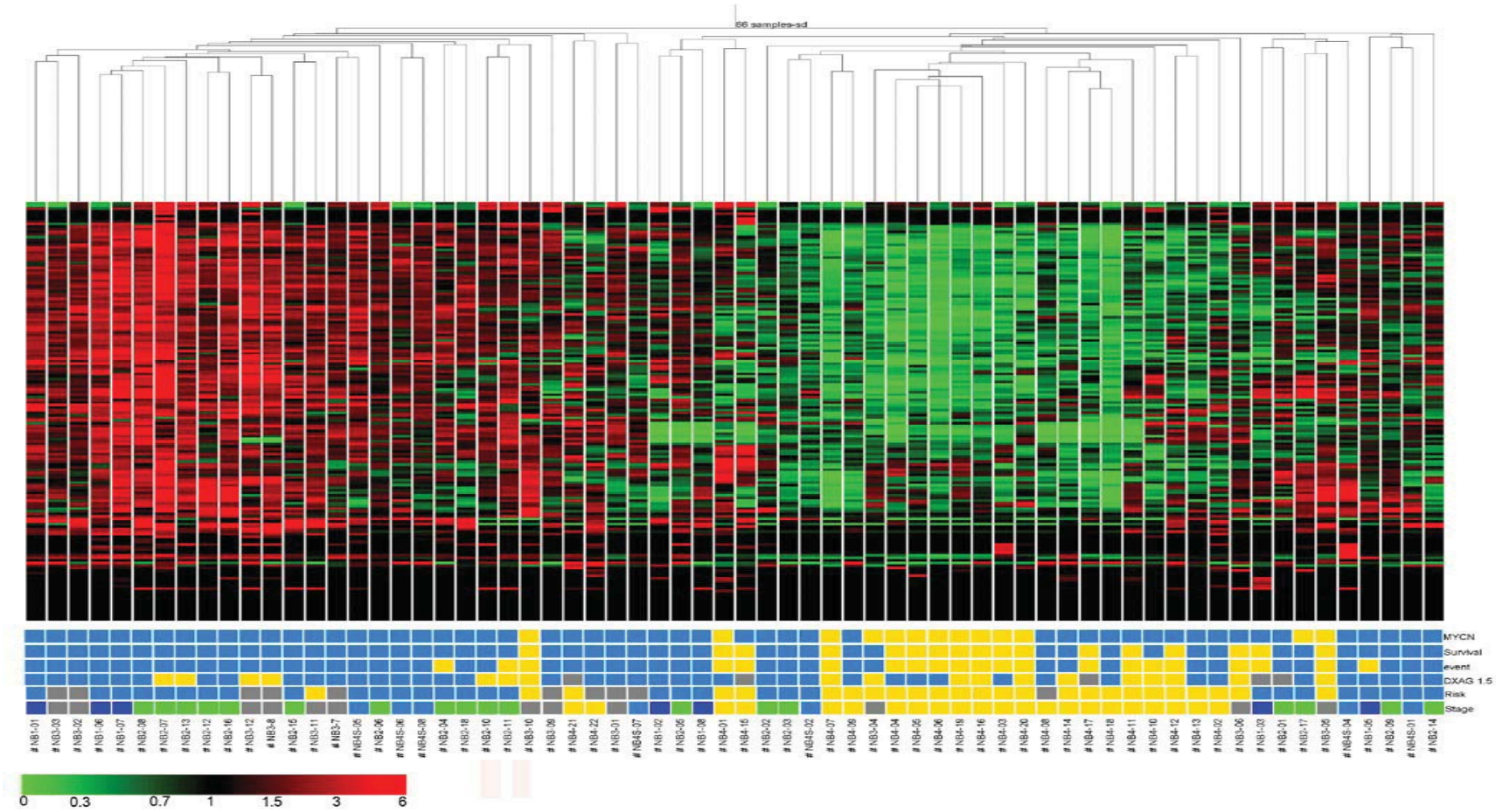
Supplementary Table S6 Cox regression analysis of the variables associated with event-free survival in existing NB microarray data sets (E-TABM-38 and E-MTAB-16)

all NB patients (n=251)

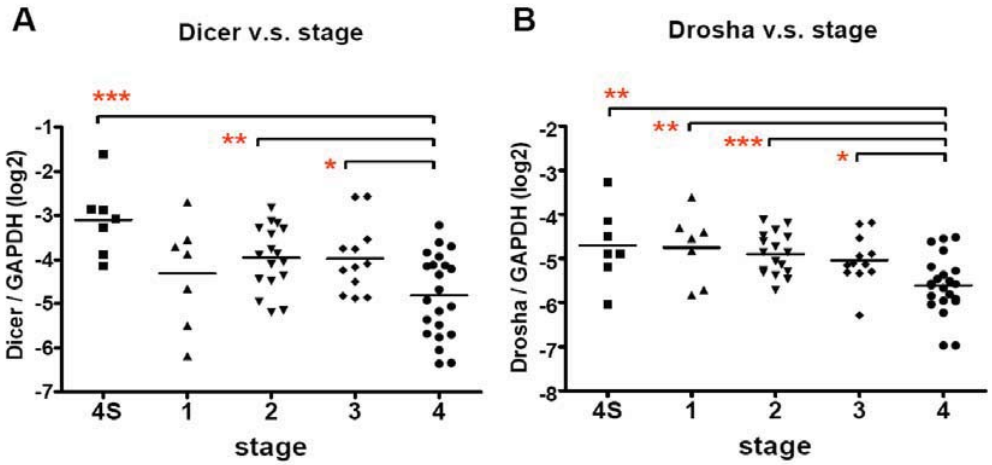
Variables	HR（95% CI）	Favorable/Unfavorable	*p*-value
Univariate analysis			
In E-TABM-38			
Stage	2.99（1.73，5.17）	1, 2, 3, 4S/4	**<0.001**
MYCN	2.32（1.31，4.08）	Non/Amplified	**0.004**
Gender	0.68（0.43，1.09）	Female/Male	0.113
Age at diagnosis_1.5	1.61（0.90，2.87）	<1.5 year/ ≧1.5 year	0.110
Dicer	1.24（0.76，2.01）	High/Low	0.386
In E-MTAB-16			
Dicer	1.29（0.81，2.40）	High/Low	0.287

NB patients without MYCN amplification (n=218)

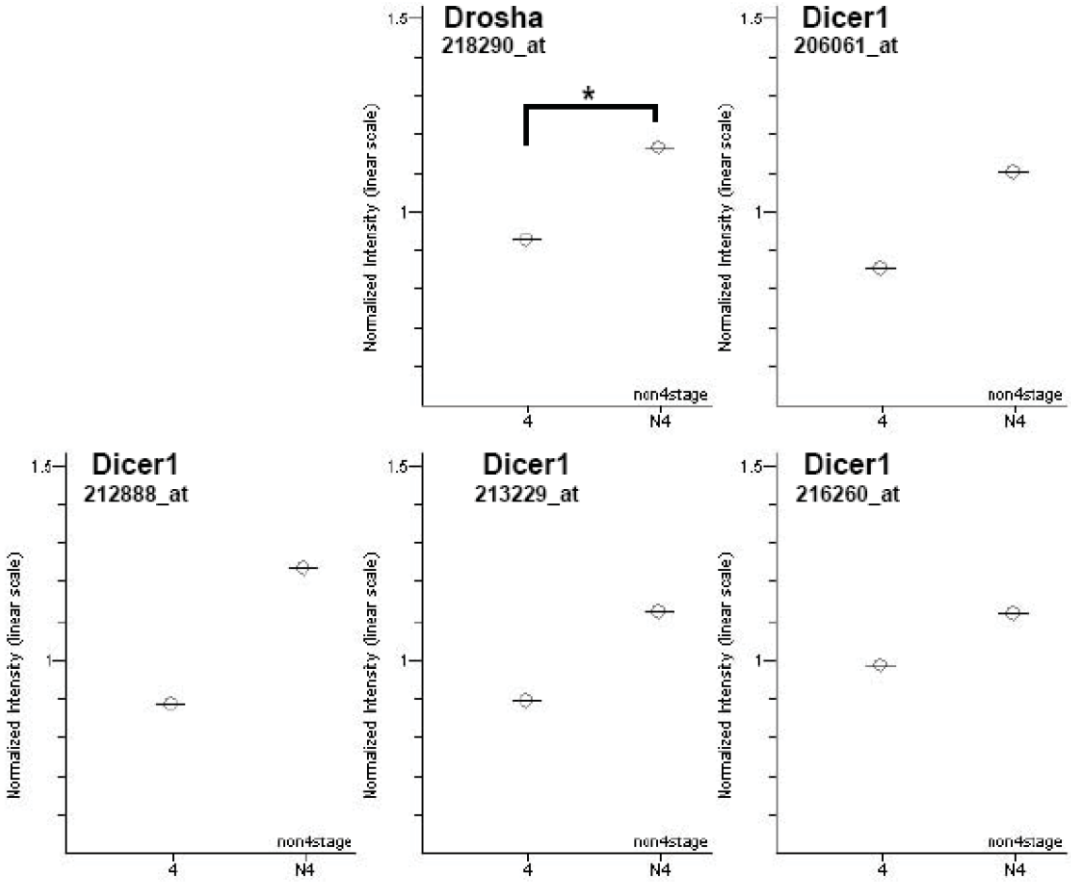
Variables	HR（95% CI）	Favorable/Unfavorable	*p*-value
Univariate analysis			
In E-TABM-38			
Stage	3.01（1.59，5.69）	1, 2, 3, 4S/4	**0.001**
Gender	0.60（0.34，1.05）	Female/Male	0.072
Age at diagnosis_1.5	2.19（1.15，4.14）	<1.5 year/ ≧1.5 year	**0.016**
Dicer	1.55（0.85，2.82）	High/Low	0.149



Supplementary Figure S1. Hierarchical clustering of 162 miRNAs expression profile in 66 neuroblastoma samples by using Standard Correlation as measure of similarity. Samples are shown in columns and 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=1.5~5 year and high-risk group 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 group are shown in grey. NB samples were coded as #NBX-01, NBX-02, etc., where X refers to INSS stage.

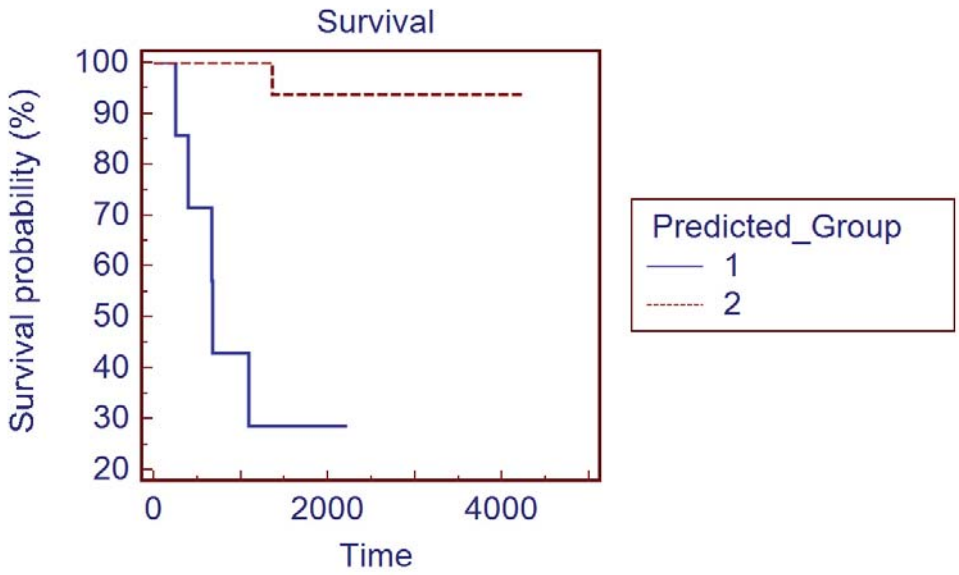


Supplementary Figure S2. Expression of Dicer and Drosha are significantly lower in stage 4 NB than other stages. Expression of Dicer (A) and Drosha (B) are determined by qRT-PCR in samples from each stage of NB patients. (*p* values were obtained using T-test: *, p<0.05; **, p<0.01; ***, p<0.001)

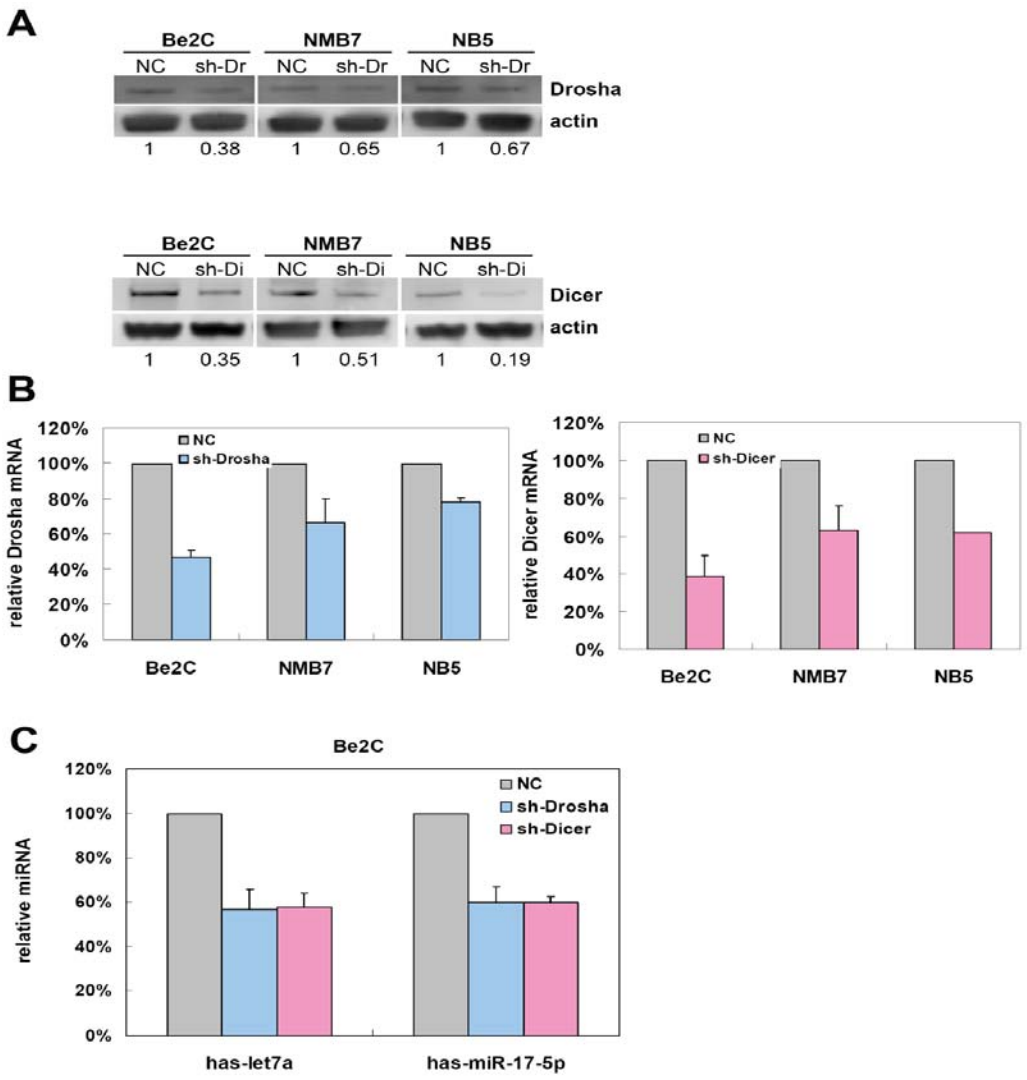


Gene	Probe set	stage 4 (N=20)	non-stage (N=10)
Drosha	218269_at	0.925 (0.473 to 1.266)	1.166 (0.9 to 1.564)
Dicer	206061_s_at	0.855 (0.316 to 2.71)	1.103 (0.494 to 2.093)
Dicer	212888_at	0.888 (0.355 to 2.134)	1.237 (0.844 to 1.954)
Dicer	213229_at	0.896 (0.43 to 1.926)	1.123 (0.823 to 1.687)
Dicer	216260_at	0.985 (0.565 to 3.201)	1.121 (0.796 to 1.742)

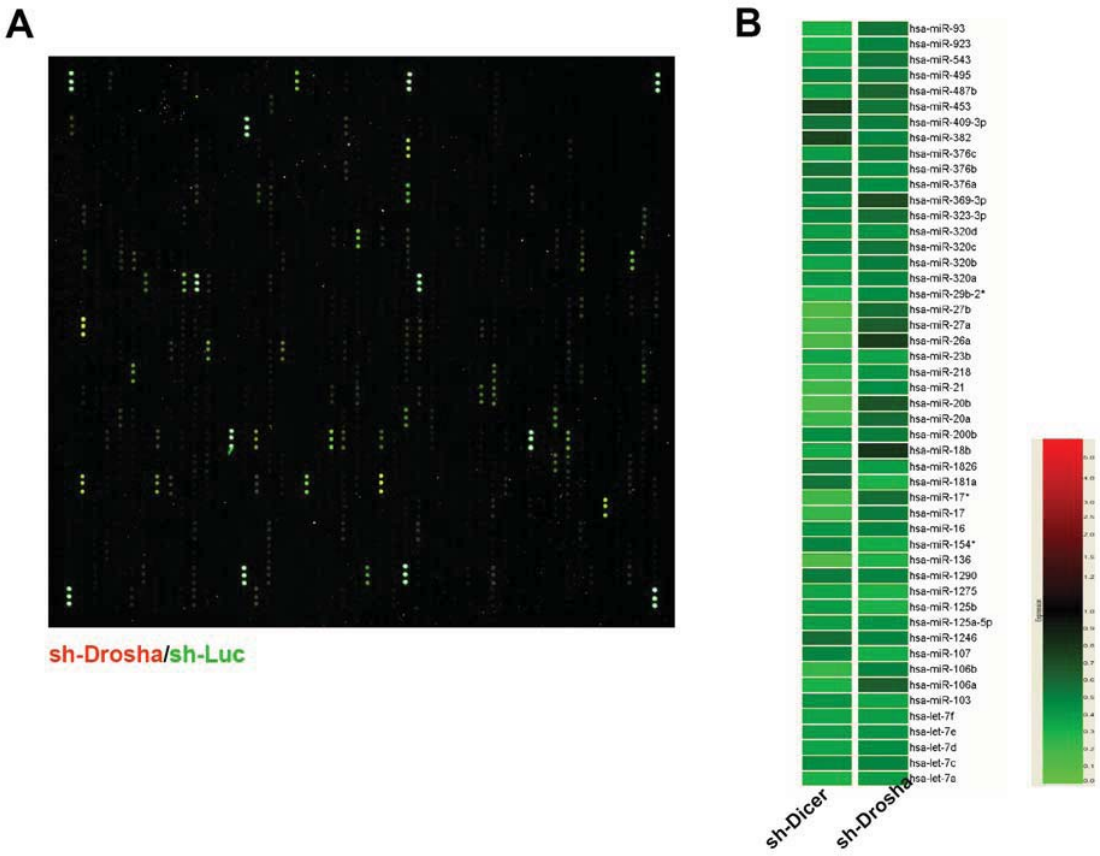
Supplementary Figure S3. Low expressions of Drosha and Dicer in stage 4 neuroblastoma based on the published GSE13136 microarray dataset. (*Oncogene* 2007 Nov 22;26(53):7432-44). There were four probe sets for Dicer and one set for Drosha. All 5 probe sets exhibited lower expression in stage 4 samples (N =20) than in non-stage 4 samples (N = 10); however, the difference reached statistical significance only in Drosha probe set. Raw data of GSE13136 were processed and analyzed in GeneSpring GX 7.3 software (Agilent Technologies, Palo Alto, CA, USA) by the default normalization setting with an additional perchip normalization to GAPDH control gene. *, $p<0.05$ was established by the default 1-way ANOVA setting.



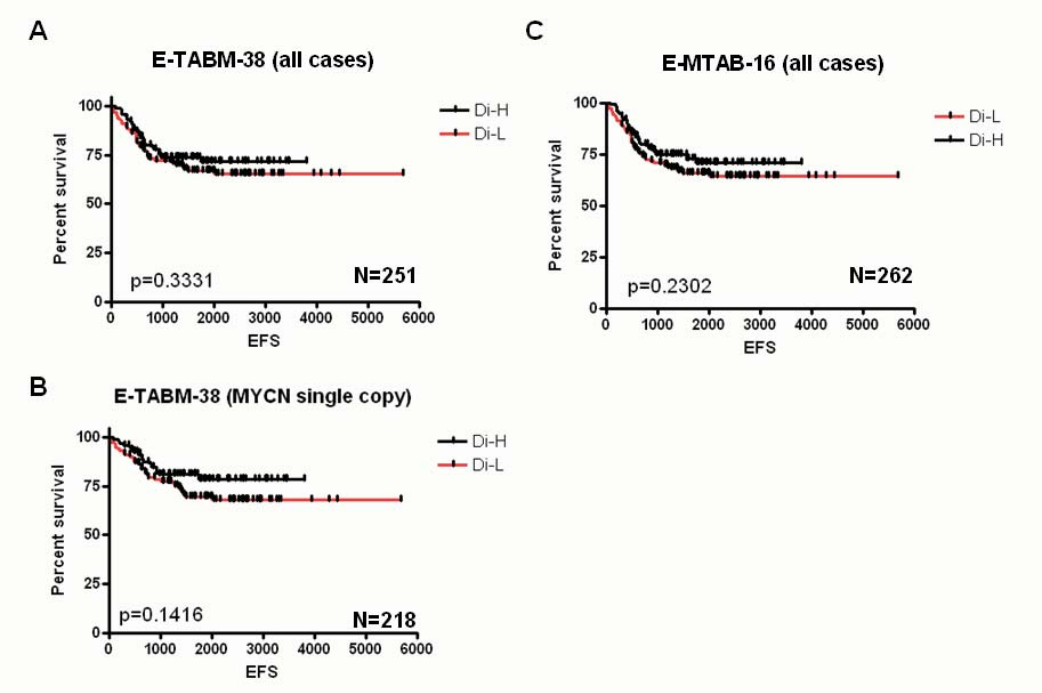
Supplementary Figure S4. Kaplan-Meier survival curve of predicted outcome in Testing set samples. This analysis was done by training PNN on the Training Set (38 samples), using the 12 miRs signature, expression of Dicer and Drosha, and age at diagnosis, and then using the trained network to predict the survival status of the 28 Testing Set samples. Using the predicted outcomes, we separated the patients into (dead) group 1 (N=7), and (alive) group 2(N=21) for Kaplan-Meier survival analysis.



Supplementary Figure S5. Efficiency of shRNA-mediated Drosha or Dicer knockdown in NB cell lines. NB cell lines, Be2C, NMB7, and NB5, were transfected with shRNA against Drosha (sh-Dr), Dicer (sh-Di), or luciferase (NC, negative control). (a) Immunoblot analysis of Dicer and Drosha was performed at 72h after transfection. The expression of actin was analyzed as an internal control. Expression levels relative to NC were shown under each lane. (b) q-RT-PCR analyses of Dicer and Drosha at 48h after transfection. Expression levels of Dicer and Drosha were shown as percent relative to luciferase negative control after normalization to GAPDH. Values are mean ± s.e.m. (n=3). (c) Downregulation of mature has-let7a and has-mir-17-5p by Dicer and Drosha in Be2C cells 2 day after transfection. Expression levels relative to negative control were shown after normalization to U6. Data shown in (a) and (c) were representatives from three independent experiments.



Supplementary Figure S6. miRNA microarray analysis of miRNA expression in Dicer or Drosha knockdown NB cells. Comparison of microRNA expression of microRNA extracted from sh-Luc (DY-547, green) and sh-Dicer or sh-Drosha (DY-647, red) of Be2C cells. In brief, 1ug of enriched microRNA per channel was labeled with DY-547 or DY-647 using RNA ligase and hybridized to antisense strand microRNA chips. (a) Representative miRNA array image from sh-Drosha (DY-647)/sh-Luc (DY-547) samples. (b) The normalized data were hierarchically clustered by gene and plotted as a heat map. Red denotes high expression and green denotes low expression relative to the negative control, sh-Luc; only the signal intensity of miRNAs large than 500 in both channel are shown here.



Supplementary Figure S7. Kaplan-Meier survival estimates for event-free survival (EFS) of NB were shown according to the expression levels of Dicer in (A) all NB patients or (B) NB patients without MYCN-amplification in E-TABM-38 cohort; (C) all NB patients in E-MTAB-16 cohort. All normalized array data were obtained from ArrayExpress (http://www.ebi.ac.uk/arrayexpress). Probe set AL122105 of customized array chip-A-MEXP-255 was used to measure expression of Dicer in both data sets; the analysis was performed by separating the cases from each cohort into High and Low group using the mean expression level of Dicer.