High expression of *ncRAN*, a novel non-coding RNA mapped to chromosome 17q25.1, is associated with poor prognosis in neuroblastoma

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Abstract. Neuroblastoma shows complex patterns of genetic aberrations including *MYCN* amplification, deletion of chromosome 1p or 11q, and gain of chromosome 17q. The 17q gain is frequently observed in high-risk neuroblastomas, however, the candidate genes still remain elusive. In the present study, we integrated the data of comparative genomic hybridization of 236 tumors by BAC array and expression profiling of 136 tumors by using the in-house cDNA microarray carrying 5,340 genes derived from primary neuroblastomas. A novel candidate gene mapped to chromosome 17q25.1 with two splicing variants, *Nbla10727* and *Nbla12061,* was identified. The transcript size appeared to be 2.3 kb by Northern blot, however, the cDNA sequences had no obvious open reading frame. The protein product was undetectable by both *in vivo* and *in vitro* translation assays, suggesting that the transcript might not encode any protein product. Therefore, we named it as *ncRAN* (non-coding RNA expressed in aggressive neuroblastoma). In analysis of 70 patients with sporadic neuroblastoma, the high levels of *ncRAN* mRNA expression were significantly associated with poor outcome of the patients ($p<0.001$). The multivariate analysis showed that expression of *ncRAN* mRNA was an independent prognostic factor among age, stage, origin and *MYCN* expression. Ectopic expression of *ncRAN* induced transformation of NIH3T3 cells in soft agar, while knockdown of endogenous *ncRAN* with RNA interference significantly inhibited cell growth in SH-SY5Y cells. Collectively, our results suggest that *ncRAN* may be a novel non-coding RNA mapped to the region of 17q gain and act like an oncogene in aggressive neuroblastomas.

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Introduction

Neuroblastoma is one of the most common pediatric solid tumors in children and originates from sympathoadrenal lineage of the neural crest. Its clinical behavior is heterogeneous because the tumors often regress spontaneously when developed in patients under one year of age, while they grow rapidly and cause very poor clinical outcome when occurring in patients over one year of age (1). Recent cytogenetic analyses have revealed that given subsets of neuroblastoma with unfavorable prognosis often have *MYCN* amplification, gains of chromosome 1q, 2p, and 17q as well as allelic losses of 1p, 3p, and 11q (1). However, the precise molecular mechanisms underlying pathogenesis and progression of neuroblastoma still remain unclear.

Accumulating evidence shows that gain of chromosome 17 or 17q is the most frequent genetic abnormality in neuroblastoma (1-4). We have previously conducted microarray-based comparative genomic hybridization (array-CGH) with a DNA chip carrying 2,464 BAC clones to examine genomic aberrations in 236 primary neuroblastomas (5). Our array-CGH analysis demonstrated three major groups of genomic aberrations in sporadic neuroblastomas (n=112) that can well define the prognoses of neuroblastomas: a genetic group of silent chromosomal aberration (GGS, 5-year cumulative survival rate: 68%), a genetic group of partial chromosomal gains and/or losses (GGP, 43%), and a genetic group of whole chromosomal gains and/or losses (GGW, 80%). The classification of three genetic groups corresponded well with the pattern of chromosome 17 abnormalities, namely, no gain of either chromosome 17 or 17q, gain of chromosome

17q, and gain of whole chromosome 17, respectively (5). Thus, 17q gain has been implicated in close correlation with aggressiveness of neuroblastoma (5-7). The region has been narrowed down to 17q21-qter, in which several important candidate genes such as *Survivin* and *PPM1D* were suggested to be involved in acquiring aggressiveness of neuroblastoma (4,7,8).

In the present study, by combining with our previous array-CGH data, we searched for the candidate 17q gain gene(s) by applying the results of our gene-expression profiling obtained from the analysis of 136 neuroblastoma samples using an in-house cDNA microarray carrying 5,340 genes isolated from primary neuroblastomas (9,10). This approach has led us to identify a novel non-coding RNA as the candidate mapped to the region of chromosome 17q gain. Its high expression is significantly associated with aggressiveness of primary neuroblastomas.

Materials and methods

Patients. Tumor specimens were collected from the patients with neuroblastoma who had undergone biopsy or surgery at various institutions in Japan. Two hundred and thirty-six and 136 tumor samples were used for array-CGH and expression profiling, respectively (5,10). Among them, sporadic cases were 112 and 70, respectively. The clinical stage of tumor was classified according to the INSS criteria (11). Expression data for the latter 70 sporadic neuroblastomas, which were composed of 15 stage 1, 8 stage 2, 17 stage 3, 25 stage 4, and 5 stage 4s tumors, were used for the Kaplan-Meier analysis. The status of *MYCN* amplification in each tumor had been determined as described previously (8). Patients were treated according to previously described protocols (12,13). The procedure of this study was approved by the Institutional Review Board of the Chiba Cancer Center (CCC19-9).

Microarray-based comparative genomic hybridization (array-CGH) and gene expression profiling. Array-based CGH experiments for 236 neuroblastomas by using a chip carrying 2,464 BAC clones which covers the whole human genome at ~1.2-Mb resolution were performed as described previously (5). For the gene expression profiling of 136 neuroblastomas, we employed an in-house cDNA microarray, carrying 5,340 cDNAs obtained from the oligo-capping cDNA libraries generated from anonymous neuroblastoma tissues (10,14-16). The array-CGH and gene expression profile data are available at NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE 5784 and GSE 5779, respectively.

Cells, culture and transfection. NIH3T3, COS7 and human neuroblastoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 medium containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) and antibiotics. Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C. COS7 and NIH3T3 cell lines were transiently transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Construction of expression plasmid. The full-length cDNAs of *Nbla10727* and *Nbla12061* were cloned from the established full length-enriched cDNA libraries which we made from the primary neuroblastomas as described (14-16). The full-length cDNAs were then inserted into pcDNA3 or pcDNA3-FLAG plasmids.

In vitro transcription and translation assay. *In vitro* translation was carried out in the presence of [^{35}S]-methionine using TNT T7 Quick coupled transcription/translation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The products were resolved by SDS-PAGE and detected by autoradiography.

In vivo [^{35}S]-labeling experiment. COS7 cells were transfected with the FLAG-tagged *ncRAN* expression vectors or the HA-tagged MEL1 expression plasmid. After 24 h, cells were rinsed with 1X PBS 3 times and recultured in fresh growth medium without methionine and antibiotics. Two hours later, [^{35}S]-methionine (GE Healthcare, Tokyo, Japan) was added to the medium to a final concentration of 0.1 mCi/ml, and cells were further incubated. Cells were harvested and whole cell lysates were subjected to immunoprecipitation using a monoclonal anti-Flag antibody or a polyclonal anti-HA antibody. Immunoprecipitates were resolved by SDS-PAGE and detected by autoradiograph.

RNA isolation and semi-quantitative reverse transcription-PCR (RT-PCR). Total RNA was isolated from frozen tumor tissues by an AGPC method (8). Total RNA (5 μg) was employed to synthesize the first-strand cDNA by means of random primers and SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. We prepared appropriate dilutions of each single stranded cDNA for subsequent PCR by monitoring an amount of glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* as a quantitative control. The PCR amplification was carried out for 28 cycles (preheat at 95˚C for 2 min, denature at 95˚C for 15 sec, annealing at 55˚C 15 sec, and extension at 72˚C 20 sec) for *ncRAN* (*Nbla10727* and *Nbla12061*). The primers used were: *ncRAN* (*Nbla10727*) 5'-CAGTCAGCCTCAGTTTC CAA-3' (forward); 5'-AGGCAGGGCTGTGCTGAT-3' (reverse), *ncRAN* (*Nbla12061*) 5'-ATGTTAGCTCCCA GCGATGC-3' (forward); 5'-CTAACTGCCAAAAGGTTT TCC-3' (reverse).

Northern blot analysis. Total RNA (20 μg) was subjected to electrophoresis and Northern blotting. The cDNA insert (*Nbla10727*) was labeled with [α-^{32}P]-dCTP (GE Healthcare) by the BcaBEST™ labeling kit (Takara, Tokyo, Japan) and used for the hybridization probe.

Soft agar assay. NIH3T3 cells were transfected with FLAG-*Nbla10727*, FLAG-*Nbla12061* or empty vector, and resuspended in 0.33% agar (wt/vol) in DMEM with 10% FBS at a density of 500 cells/plate. Cell suspensions were poured on the top of the base layer (0.5% agar (wt/vol) in fresh medium, and grew in a 5% CO_2 incubator for 14 days. Colonies >100 μm were counted under an Olympus microscope.

Table I. The comparison of *ncRAN/Nbla10727/Nbla12061* expression level among three major groups of genomic aberrations in neuroblastomas.

Genetic group	n	*ncRAN* expression Mean ± SD (log2 ratio)	p-value	
ncRAN-long/Nbla10727				
GGS (silent)	n=10	-1.12±0.39	p=0.004	
GGP (partial 17q+)	n=26	-0.60±0.48		p=0.952
GGW (whole 17+)	n=35	-1.11±0.48	p<0.001	
ncRAN-short/Nbla12061				
GGS (silent)	n=10	-1.60±0.33	p=0.070	
GGP (partial 17q+)	n=26	-1.23±0.59		p=0.163
GGW (whole 17+)	n=35	-1.81±0.43	p<0.001	

n, number of samples; GGS, Genetic group silent (normal 17); GGP, Genetic group partial gains/losses (17q gain); GGW, Genetic group whole gains/losses (17 gain); *ncRAN* expression levels are shown as normalized log2 ratio of microarray data. p-values were calculated based on statistical t-test.

RNA interference. Oligonucleotides for knocking down the *ncRAN* with *Sac*I and *Xho*I extension were inserted into pMuni vector. The oligonucleotides used were: 5'-CCC CATCCTCTAGTAGCCACGGTTTCAAGAGAACCGT GGCTACTAGAGGATTTTTTGGAAAC-3' and 5'-TCG AGTTTCCAAAAAATCCTCTAGTAGCCACGGTTCTCT TGAAACCGTGGCTACTAGAGGATGGGGAGCT-3'. The plasmids containing the oligonucleotide sequence were transfected into SH-SY5Y cells by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Statistical analysis. The Student's t-tests were used to explore possible associations between *ncRAN* expression and other factors, such as age. Kaplan-Meier curves were calculated and survival distributions were compared using the log-rank test. Univariate and multivariate analyses were made according to the Cox hazard models. q-value was also calculated because *ncRAN* expression was measured with 5340 genes in the microarray (17). Statistical significance was set at $p<0.05$.

Results

Identification of a novel Nbla10727/12061 gene mapped to chromosome 17q25.1 upregulated in advanced neuroblastomas with gain of chromosome 17q. To explore the candidate genes for therapeutic target against aggressive neuroblastomas, the genomic and molecular characteristics specific to high-risk tumors were surveyed. We previously conducted array-CGH analysis with a microarray carrying 2,464 BAC clones to examine genomic aberrations in 236 primary neuroblastomas and found that the gain of chromosome 17q was most strongly correlated with the patient's prognosis (5). The genetic group of 'silent chromosomal aberrations' (GGS) could be defined as the tumor group without apparent abnormalities in chromosome 17, and the genetic group of 'whole chromosomal gains and/or losses' (GGW) as that with gain of whole chromosome 17 (5-year cumulative survival rate in 112 sporadic neuroblastomas: 68 and 80%, respectively, according to ref. 5). On the other hand, the genetic group of 'partial chromosomal gains and/or losses' (GGP) with gain of chromosome 17q showed poor prognosis (43%).

According to the different grade of aggressiveness among the genetic groups, we hypothesized that the GGP tumors may have higher levels of expression of the activated 17q candidate gene(s) that is (are) involved in defining the grade of malignancy of neuroblastoma than the GGS or GGW tumors. We then used our data set of gene expression profile in 136 neuroblastomas to subtract the genes mapped to the commonly gained region of chromosome 17q and differentially expressed in the GGP tumors at high levels and the GGS or GGW tumors at low levels. Consequently, we found two cDNA clones *Nbla10727* and *Nbla12061* (Fig. 1A) on our in-house microarray carrying 5,340 cDNAs obtained from oligo-capping cDNA libraries generated from different subsets of primary neuroblastomas (10,14-16), both of which were splicing variants of the same gene mapped to chromosome 17q25.1 (Table I and Fig. 1B, expression in GGP more than that in GGS or GGW). Database searching showed that both 2,087-bp and 2,186-bp insert sequences (Genbank/DDBJ accession numbers: AB447886 and AB447887) did not exhibit significant similarity to any previously known genes. As the size of mRNA was ~2.3 kb by Northern blot (Fig. 1C), the clones *Nbla10727* and *Nbla12061* appeared to be almost full-length cDNAs. Therefore, *Nbla10727*/12061 appeared to be the gene activated for its expression in neuroblastomas with partial gain of chromosome 17q, but not activated in those with diploid or triploid pattern of whole chromosome 17.

The *Nbla10727*/12061 gene was expressed in multiple human tissues with preferential expression in heart, kidney, lung, spleen, mammary gland, prostate and liver, but with low expression in neuronal tissues such as brain and cerebellum, fetal brain and adrenal gland (Fig. 1D).

High expression of Nbla10727/12061 is associated with poor prognosis of neuroblastoma. The analysis by semi-quantitative RT-PCR in a panel of cDNAs obtained from 8 favorable (stage 1, <1-year-old, single copy of *MYCN* and high expression

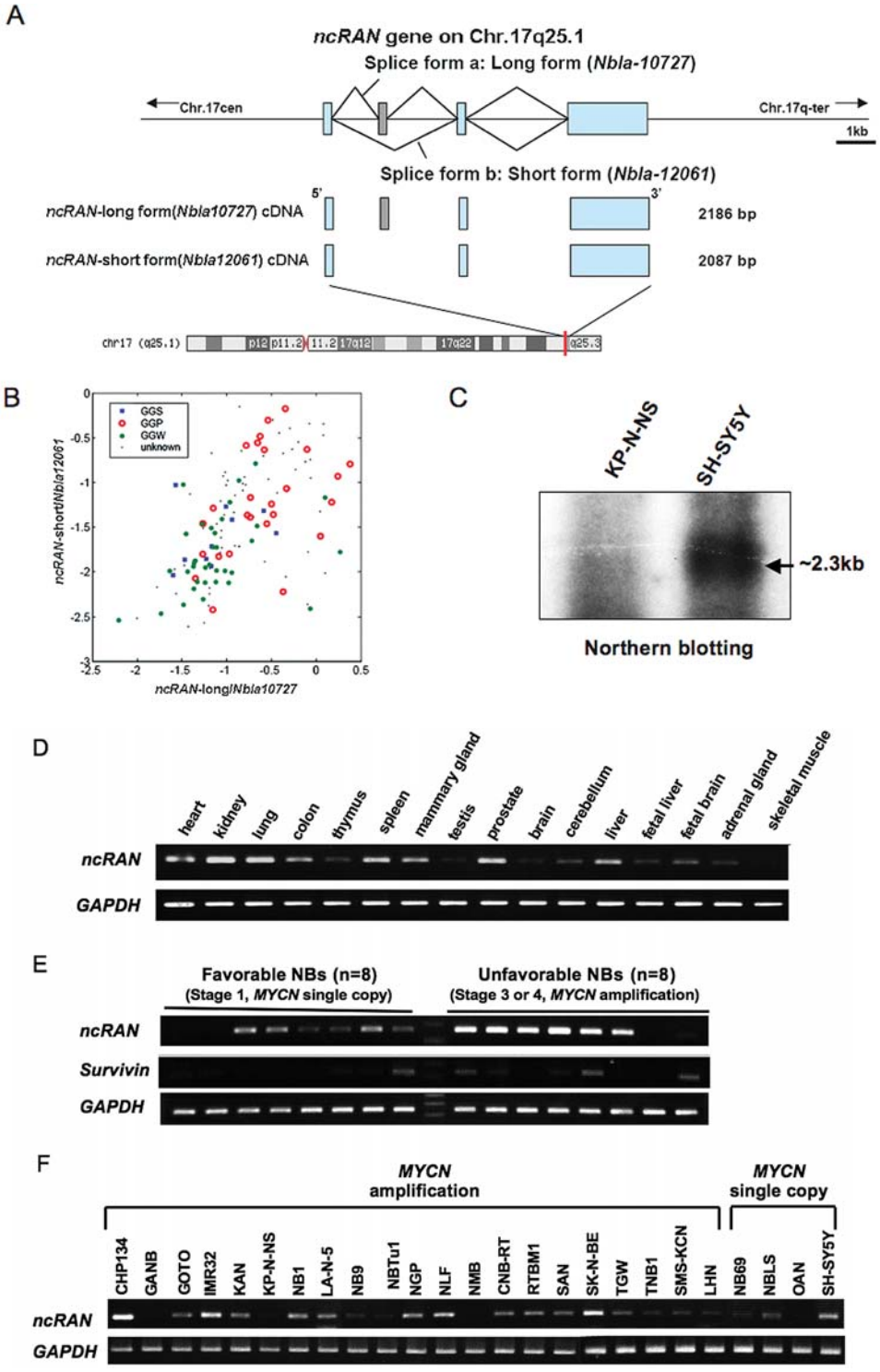

Figure 1. *ncRAN* is mapped to the 17q gain region. A, Genomic structure of *ncRAN* region on chromosome 17q25.1. Splicing variants, whose sequences were contained in cDNAs as *ncRAN-long/Nbla10727* and *ncRAN-short/Nbla12061*, are schematically shown. These are transcribed from a single gene, *ncRAN* (see text). B, High expression of *ncRAN* is associated with high malignant subset of neuroblastoma. Scatter plot of the expression levels of the *ncRAN-long/Nbla10727* and *ncRAN-short/Nbla12061* in 71 primary neuroblastomas with both accompanying expression and aCGH data. Blue, red, green, and black spots denote GGS, GGP, GGW and unknown genomic group samples, respectively. As shown in Table I, the expression levels of the *ncRAN* were significantly higher in GGP tumors (+17q gain) than in GGS (no 17 gain) or GGW (+ whole 17 gain) tumors ($p=0.004$ and $p<0.001$ for *ncRAN-long/Nbla10727*, and $p=0.070$ and $p<0.001$ for *ncRAN-short/Nbla12061*, respectively), whereas their expression levels in GGS and GGW tumors were comparable ($p=0.952$ for *ncRAN-long/Nbla10727*, and $p=0.163$ for *ncRAN-short/Nbla12061*, see also Table I), suggesting that the acquired allele(s) at 17q might be silenced at least for the *ncRAN* expression in GGW tumors, and that high expression of *ncRAN* is associated with high malignant subset of neuroblastoma. C, Northern blot analysis of *ncRAN*. Total RNA (20 μg) prepared from neuroblastoma cell lines, SH-SY5Y and KP-N-NS were used. A 2.3-kb band was visible in only SH-SY5Y cells. The cDNA insert *(Nbla10727)* was labeled with [α-^{32}P]-dCTP and used for the hybridization probe. D, Semiquantitative RT-PCR of *ncRAN* in multiple human tissues and neuroblastoma cell lines. Total RNA of 25 adult tissues and two fetal tissues were purchased from Clontech Co. Ltd. The expression of *GAPDH* is also shown as a control. E, Semi-quantitative RT-PCR of *ncRAN* in favorable and unfavorable subsets of primary neuroblastomas. The mRNA expression patterns for *ncRAN* and *Survivn*, a known oncogene identified at 17q, were detected by semi-quantitative RT-PCR procedure in eight favorable (lanes: 1-8, stage 1, with a single copy of *MYCN*) and eight unfavorable (lanes: 9-16, stage 3 or 4, with *MYCN* amplification) neuroblastomas. F, Semiquantitative RT-PCR of *ncRAN* in neuroblastoma cell lines. Twenty-one neuroblastoma cell lines with *MYCN* amplification and 4 cell lines with a single copy of *MYCN* were used for this study as templates.

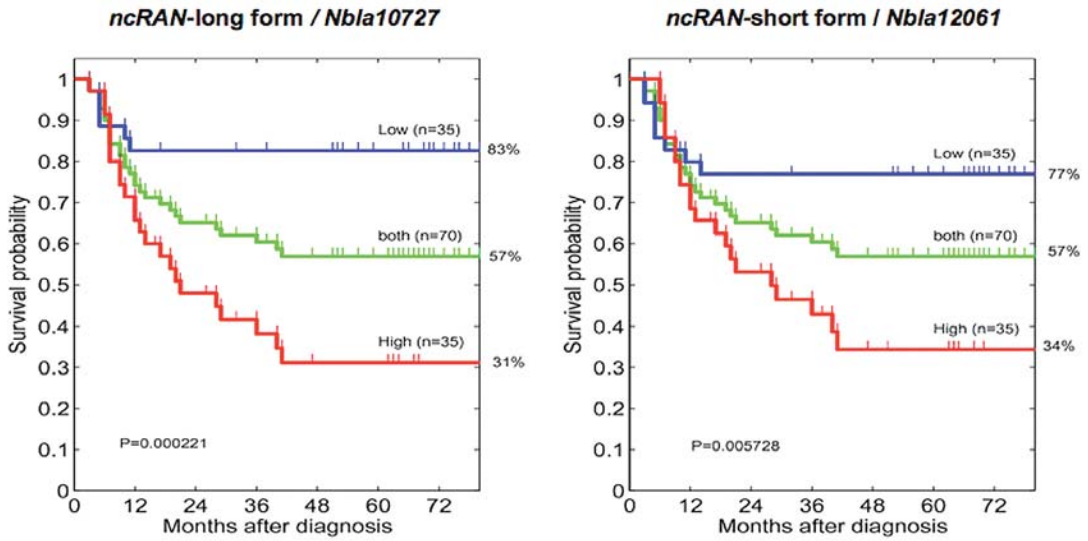

Figure 2. The high expression of *ncRAN/Nbla10727/12061* mRNA is a prognostic indicator of unfavorable neuroblastomas. The Kaplan-Meier survival curves were drawn from the results of the cDNA microarray data of 70 sporadic neuroblastomas (log-rank test, p=0.000221 and p=0.005728, respectively).

of *TrkA*) and 8 unfavorable (stage 3 or 4, >1-year-old, amplified *MYCN* and low expression of *TrkA*) primary neuroblastomas confirmed that this novel gene was expressed at significantly high levels in the latter compared to the former (Fig. 1E), such as *Survivin* which we have previously reported as one of the candidate genes mapped at the region of 17q gain (9). Among neuroblastoma cell lines, high or moderate levels of expression of *Nbla10727/12061* was observed in cell lines with *MYCN* amplification most of which had 17q gain, whereas it was relatively low in those with a single copy of *MYCN* and without the 17q gain (Fig. 1F).

As shown in Fig. 2, our microarray data of 70 sporadic neuroblastomas showed that the high levels of *Nbla10727/12061* expression were significantly associated with poor prognosis (log-rank test, p=0.000221 and p=0.005728, respectively). The multivariate analysis using Cox proportional hazard model demonstrated that expression of *Nbla10727/12061* was an independent prognostic factor among age at diagnosis, disease stage, tumor origin and *MYCN* expression (Table II). Thus, the expression level of *Nbla10727/12061* is a novel prognostic factor of neuroblastoma that is closely associated with gain of chromosome 17q.

Nbla10727/12061 is involved in inducing enhancement of cell growth in neuroblastoma cells and transformation of NIH3T3 cells. To investigate function of *Nbla10727/12061*, we transfected SH-SY5Y neuroblastoma cells with the siRNA, since SH-SY5Y cells have 17q gain in their genome as well as higher mRNA expression of *Nbla10727/12061*. As shown in Fig. 3A, suppression of endogenous levels of *Nbla10727/12061* transcripts significantly inhibited cell growth in SH-SY5Y neuroblastoma cells as compared with the control cells. On the other hand, the soft agar colony formation assay showed that the enforced expression of *Nbla10727/12061* significantly enhanced the anchorage-independent growth of NIH3T3 mouse fibroblast cells (Fig. 3B). These results suggested that *Nbla10727/12061* was a novel candidate gene of the region of 17q gain with an oncogenic function.

ncRAN-Nbla10727/12061 is a large non-coding RNA. Several lines of evidence from the gene structure analysis as well as the comparative genomic analysis described below further suggested that *Nbla10727/12061* is a non protein-coding but functional RNA. We therefore tentatively named this gene as *ncRAN* (non-coding RNA expressed in aggressive neuroblastoma).

First, the full-length cDNA sequences of *ncRAN*, which are suggested to be relevant to both *Nbla10727* and *Nbla12061* cDNAs by Northern blot analysis (Fig. 1C), did not contain any long-enough open reading frames (>200 bp). Bioinformatic analysis indicated that there were no ESTs longer than those two cDNAs at the genomic locus, and that the CpG island was located at the 5' region of the cDNA sequences.

Second, no protein product was translated both *in vivo* and *in vitro* from the *ncRAN* transcripts (Fig. 4). Though only the possible open reading frames (>150 bp) within the *ncRAN* cDNA were from n.t. 190 to 354 (55 amino acids) and from 293 to 469 (59 amino acids) in *Nbla10727*, none of the putative translation start sites contains the Kozak consensus sequence. In addition, these predicted protein products of 55 and 59 amino acids did not exhibit significant similarity to any other known protein or protein domain. Furthermore, *in vivo* transcription and translation of the full-length *ncRAN* did not lead to the synthesis of any peptide or protein (Fig. 4B), though endogenously and ectopically expressed *ncRAN* were easily detectable at mRNA level (Fig. 4A). Coincident with the above observation, the *ncRAN* protein product could not be detected using [^{35}S]-methionine-labeling system *in vitro* (Fig. 4C).

Third, we performed sequence comparison of the *ncRAN* gene with genome sequences of other species and found it has high similarity (>90% identity in nucleotides) with primates including orangutan, chimpanzee and rhesus, but not those with mice and rat (Fig. 5). We also searched for the possible long open reading frames of *ncRAN* homologs in these highly similar species, resulting in failure. The highly conserved sequence similarity only with primates may

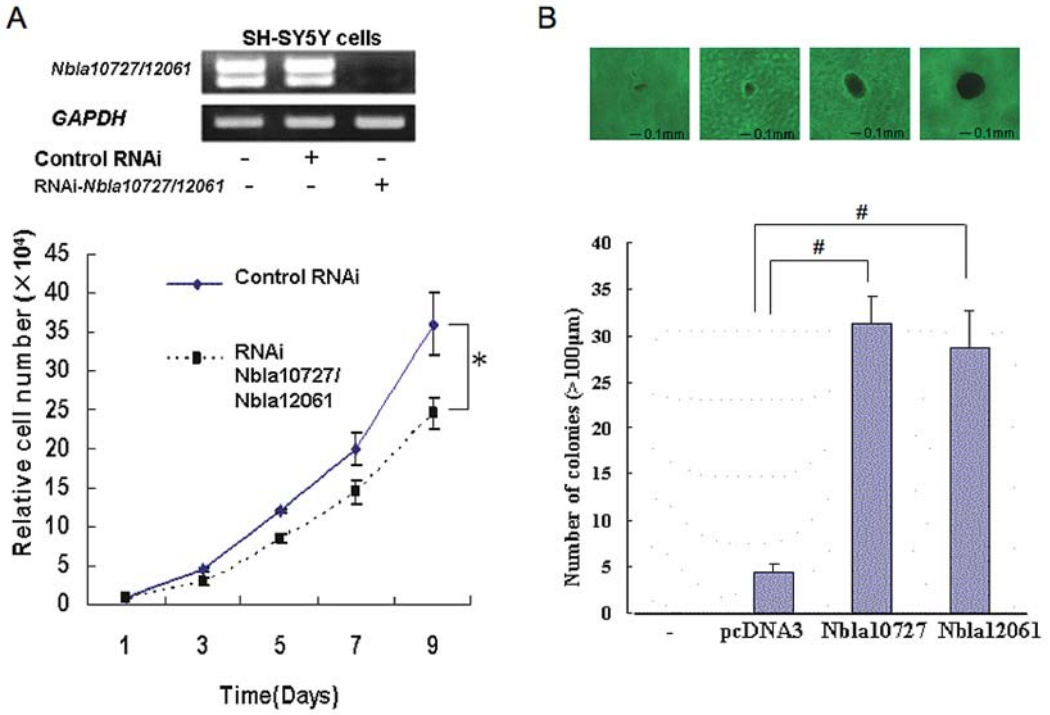

Figure 3. *ncRAN* is involved in inducing enhancement of cell growth in neuroblastoma cells and transformation of NIH3T3 cells. A, Knockdown of *ncRAN* suppress cell growth in SH-SY5Y neuroblastoma cells. SH-SY5Y cells were transfected with expression plasmid for siRNA against *ncRAN* termed pMuni-si*Nbla10727* or with the empty plasmid. On day 2, total RNA was prepared from the cells and subjected to RT-PCR. The expression of two splicing variants of *ncRAN* was knocked-down. At the same time, transfected cells were spread onto 24-well plates and the numbers of the cells at indicated time points were counted using hemocytometer and expressed as the mean ± SEM (n=3). $^{*}p<0.05$. B, Overexpression of *ncRAN* promotes the malignant transformation of NIH3T3 cells. NIH3T3 cells transfected with pcDNA3, pcDNA3-*Nbla10727* and pcDNA3-*Nbla12061* were used to carry out the soft-agar assay as described in Materials and methods. Blank and mock-transfected NIH3T3 cells served as negative controls. $^{\#}p<0.01$.

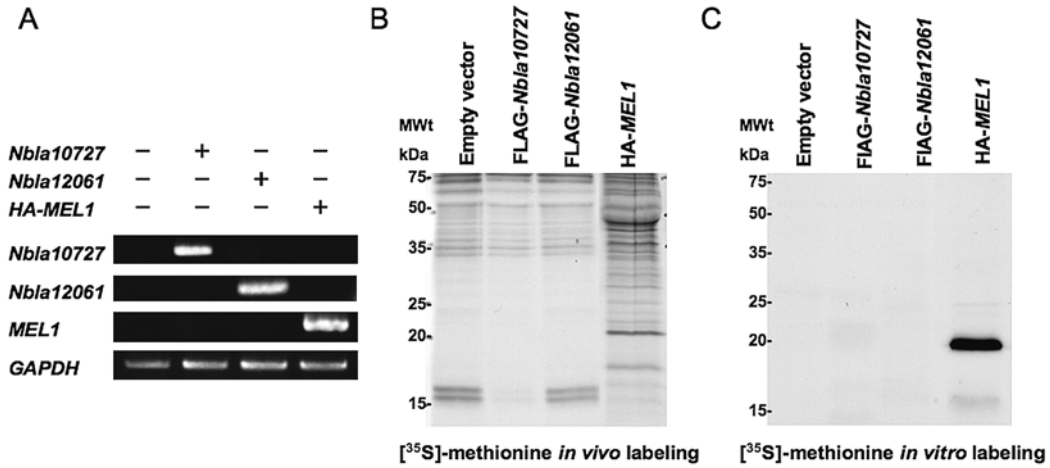

Figure 4. *ncRAN* is a non-protein-coding RNA. A, Ectopic expression of *ncRAN* transcripts in COS7 cells. The *ncRAN* expression vectors were transfected into COS7 cells and total RNA was subjected to RT-PCR. pcDNA3-HA-*MEL1* was used as a positive control. B, *In vivo* [^{35}S]-methionine labeling experiment. COS7 cells transfected with the indicated expression vectors were maintained in fresh growth media without methionine for 2 h and then cultured in the media containing [^{35}S]-methionine overnight. Cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody. Immune complex was washed extensively, resolved by SDS-PAGE and detected by autoradiography. Cell lysate prepared from COS7 cells transfected with pcDNA3-HA-*MEL1* were immunopricipitated with anti-HA antibody. C, *In vitro* translation assay. *In vitro* translation was performed in the presence of [^{35}S]-methionine according to the manufacturer's instructions. pcDNA3-HA-*MEL1* was used as a positive control.

suggest that *ncRAN* might be an evolutionally developed non-coding RNA.

Finally, previous studies have shown that certain large non-coding RNAs are relevant to host RNAs that harbor small RNAs such as microRNA (miRNA) (18). Therefore, we made a search for sequences of known miRNAs in conserved regions within the *ncRAN* locus, but none were identified. These results inferred that the *ncRAN* transcript might not be

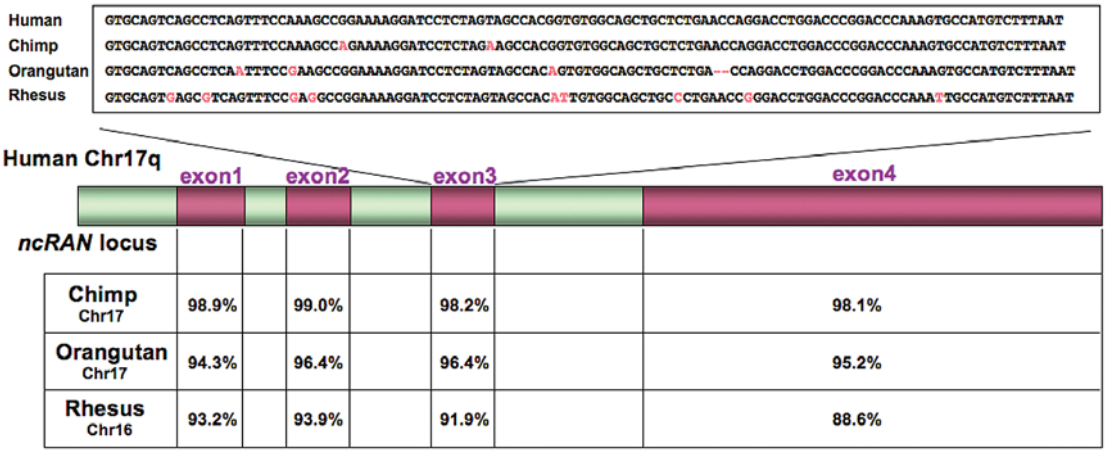

Figure 5. Schematic representation of *ncRAN* sequence conservation in primates. Sequence conservation in *ncRAN* gene locus among human and primates is indicated. Nucleotide sequences of exon3 of *ncRAN* in primates are indicated by numbers in brackets. Genomic sequences within the highly conserved sequence are marked black; mismatches are marked pink. % identities to humans are shown below for each exon. Other lower species, such as mouse, rat, dog, cow, horse, zebrafish, or *C. elegans*, do not have *ncRAN* in their genomes.

Table II. Multivariate analyses of *ncRAN/Nbla10727* mRNA expression as well as other prognostic factors in primary neuroblastomas.

Factor	n	p-value	q-value	H.R.	C.I.
Age (>12-month vs. <12-month)	45 vs. 25	0.0096		3.4	(1.2-9.9)
ncRAN expression	n=70	0.0015	0.0281	3.6	(1.7-7.9)
Age (>18-month vs. <18-month)	40 vs. 30	0.0150		2.9	(1.2-7.1)
ncRAN expression	n=70	0.0023	0.0361	3.5	(1.6-7.8)
Stage (1, 2, 4s vs. 3, 4)	42 vs. 28	<0.0001		8.0	(2.9-14)
ncRAN expression	n=70	0.0457	0.3151	2.4	(1.0-5.6)
Origin (adrenal vs. non-adrenal)	27 vs. 43	<0.0001		9.1	(2.6-33)
ncRAN expression	n=70	0.0107	0.1335	2.8	(1.3-6.1)
MYCN expression	n=70	0.0003		2.0	(1.4-2.8)
ncRAN expression	n=70	0.0035	0.0470	3.3	(1.5-7.3)

n, number of samples; H.R., hazard ratio; C.I., confidence interval. The q-value denotes estimated false discovery rate if all genes whose p-values are equal to or smaller than that of *ncRAN* are discovered as significant (17).

processed to one or more small RNAs. In addition, database search did not identify genes with anti-direction to *ncRAN*, excluding the possibility that *ncRAN* is an antisense gene for certain known genes. Collectively, these results strongly suggested that the *ncRAN* transcript functions as a novel large non-coding RNA.

Discussion

In the present study, we used the combination of array-CGH (5) and gene expression profiling by using an in-house neuroblastoma-proper cDNA microarray (10) to identify genes that strongly correlate with chromosome 17q gain in aggressive neuroblastoma. Our array CGH analysis demonstrated three major genomic groups of chromosomal aberrations such as silent (GGS), partial gains and/or losses (GGP), and whole gains and/or losses (GGW). Correlation analysis revealed that the global feature of the aberrations was maximally correlated with the gain of the long arm of chromosome 17 and with the gain of a whole chromosome 17, therefore the genomic groups GGP and GGW were defined by the status of aberration, by 17q gain and 17 whole chromosomal gain occurred in chromosome 17, respectively (5). Survival analysis for each genetic group suggested that 17q gain was a characteristic and prognosis-related event in primary neuroblastomas. Therefore, we searched for genes that were expressed significantly higher in primary neuroblastomas of GGP compared to that of GGS and GGW and finally found a novel gene *ncRAN* mapped on 17q25.1. The level of its mRNA expression was strongly correlated with the status of chromosome 17 (Table I and Fig. 1B) as well as with patient survival (Table II and Fig. 2).

To our surprise, our results suggested that *ncRAN* is a large non-coding RNA. Non-coding RNA is a general term for functional and untranslatable RNAs. Increasing evidence has shown that they play important roles in a variety of biological events such as transcriptional and translational gene regulation, RNA processing and protein transport (18,19). Recently, the numerous miRNAs, a class of small non-coding RNAs, have been identified, and miRNA-expression profiling of the human tumors has identified signatures in relation to diagnosis, staging, progression, prognosis and response to treatment (19). On the other hand, another class of non-coding RNAs named as the large non-coding RNA, which are usually produced by RNA polymerase II and lack significant and utilized open reading frame, receives relatively little attention. However, recently, increasing number of studies have provided evidence that large non-coding RNAs also play important roles in certain biological processes of the cancers, such as acquisition of drug resistance, transformation, promoting metastasis and inhibition of tumor development (19). In addition, certain candidate non-coding RNAs were isolated from the tissue- and stage-specific libraries, suggesting a possible involvement of non-coding RNAs in development and tumor cell differentiation (20). Given that *ncRAN* was identified from the cDNA libraries generated from different subsets of primary neuroblastomas, it is possible that *ncRAN* might be involved in carcinogenic processes as well as development and differentiation of normal neurons.

In conclusion, we identified a novel large non-coding RNA transcript, *ncRAN*, mapped to the region of 17q gain frequently observed in aggressive neuroblastomas. The levels of *ncRAN* expression are relatively low in normal nerve tissues including adrenal gland, whereas they are upregulated in advanced neuroblastomas with gain of chromosome 17q. From our functional analyses, *ncRAN* appears to act like an oncogene. Notably, knockdown of *ncRAN* with siRNA was able to significantly repress the cell growth in SH-SY5Y neuroblastoma cells with 17q gain as well as high endogenous level of *ncRAN*. Considering emerging evidence on the large non-coding RNAs regulating transcription of other genes (19), the present results not only contribute to further understanding of the molecular and biological mechanism of neuroblastoma genesis, but also provide a potential target for new diagnostic and therapeutic intervention in the future.

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