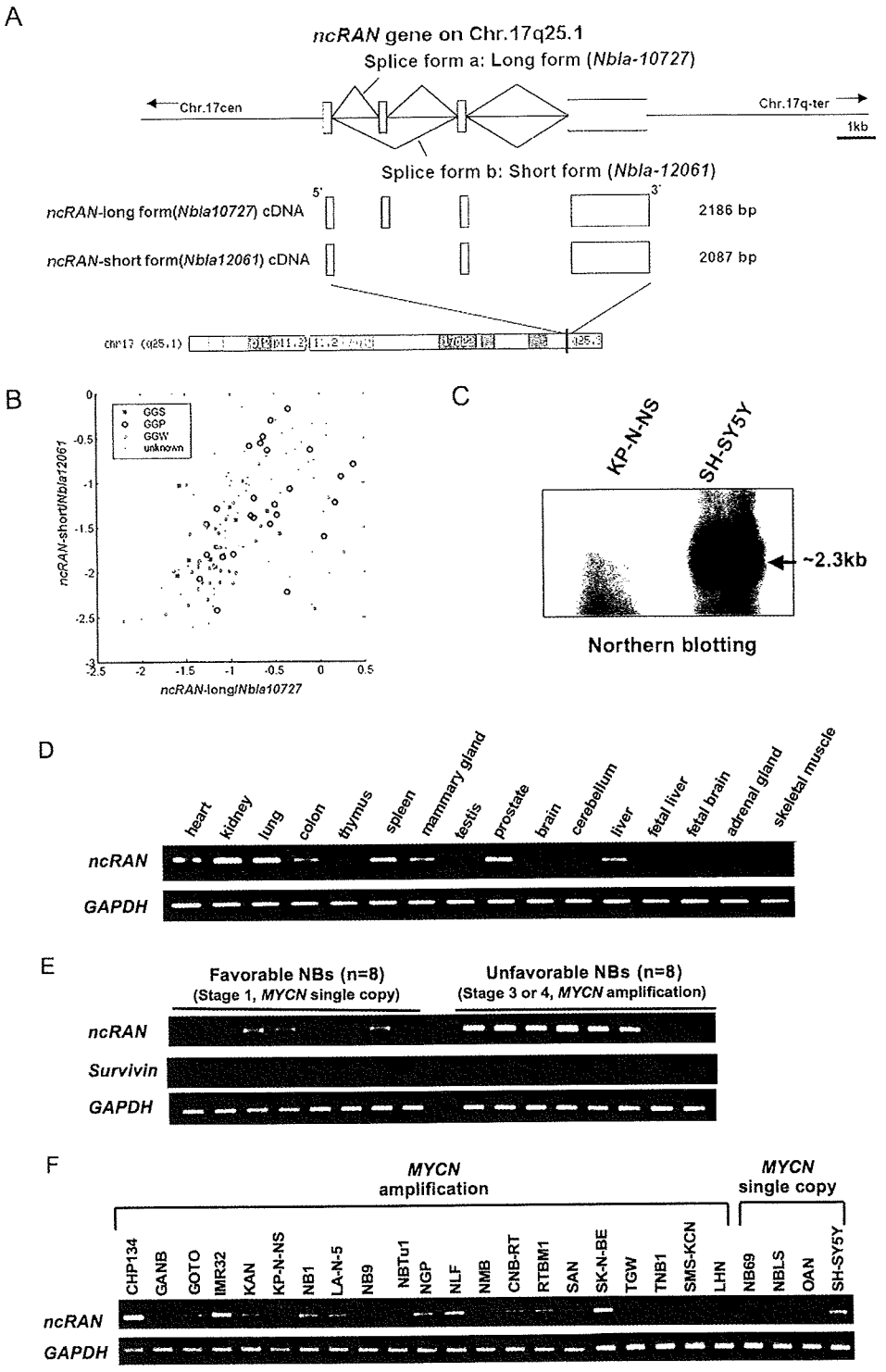

Figure 1. *ncRAN* is mapped to the 17q gain region. A, Genomic structure of *ncRAN* region on chromosome 17q25.1. Splicing variants, whose sequences were contained in cDNAs as *ncRAN-long/Nbla10727* and *ncRAN-short/Nbla12061*, are schematically shown. These are transcribed from a single gene, *ncRAN* (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.

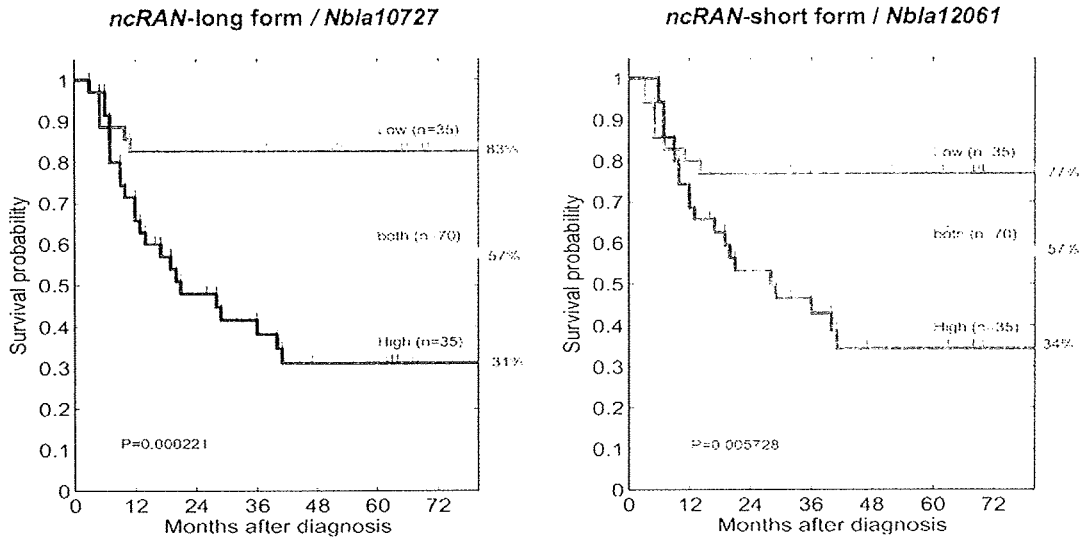

Figure 2. The high expression of *ncRAN/Nbla10727/12061* mRNA is a prognostic indicator of unfavorable neuroblastomas. The Kaplan-Meier survival curves were drawn from the results of the cDNA microarray data of 70 sporadic neuroblastomas (log-rank test, p=0.000221 and p=0.005728, respectively).

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

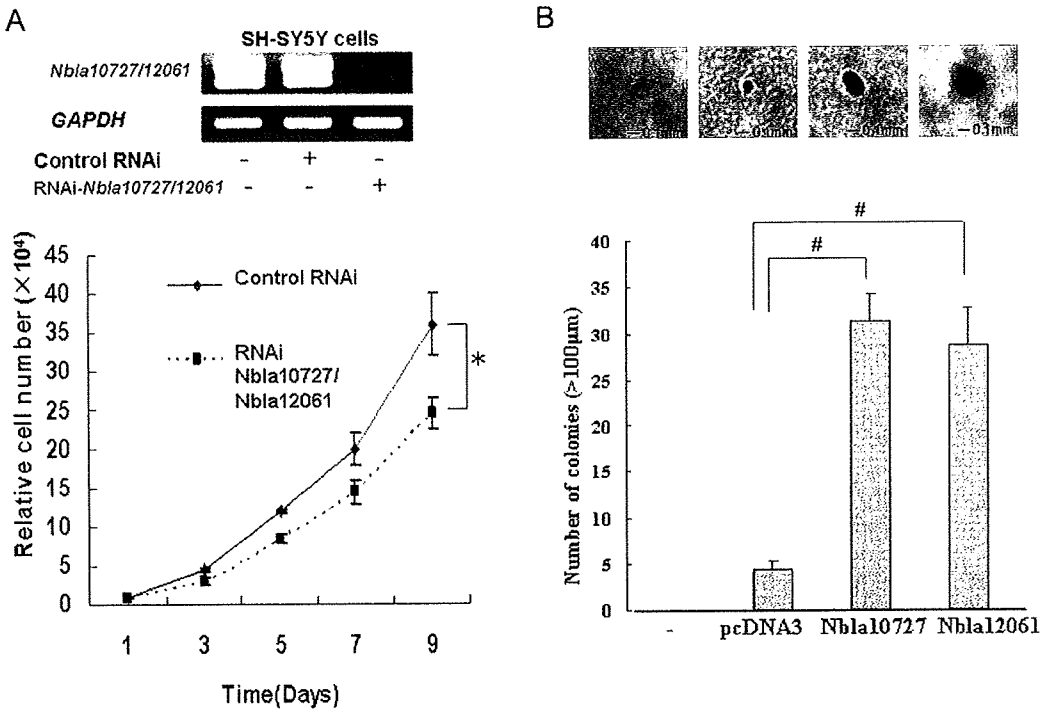

Figure 3. *ncRAN* is involved in inducing enhancement of cell growth in neuroblastoma cells and transformation of NIH3T3 cells. A, Knockdown of *ncRAN* suppress cell growth in SH-SY5Y neuroblastoma cells. SH-SY5Y cells were transfected with expression plasmid for siRNA against *ncRAN* termed pMuni-si*Nbla10727* 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. $^{\#}p<0.01$.

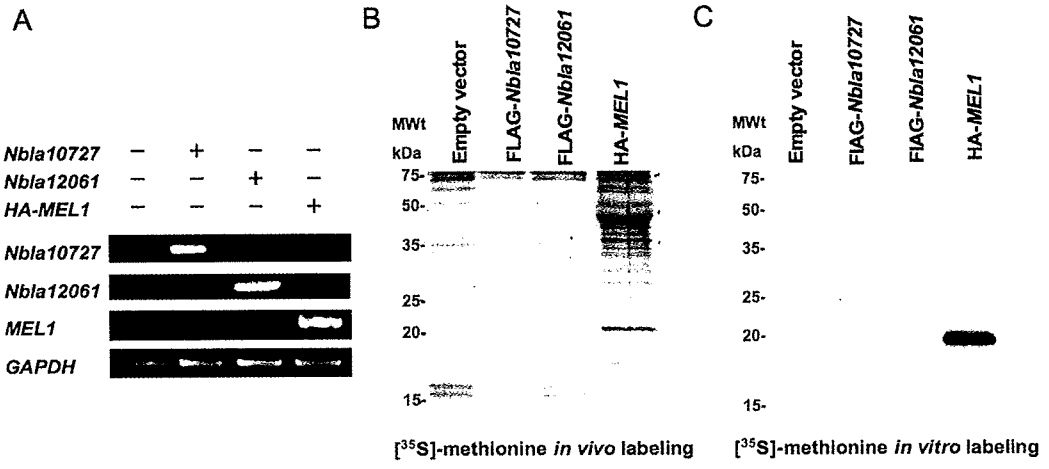

Figure 4. *ncRAN* is a non-protein-coding RNA. A, Ectopic expression of *ncRAN* transcripts in COS7 cells. The *ncRAN* expression vectors were transfected into COS7 cells and total RNA was subjected to RT-PCR. pcDNA3-HA-*MEL1* was used as a positive control. B, *In vivo* [^{35}S]-methionine labeling 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

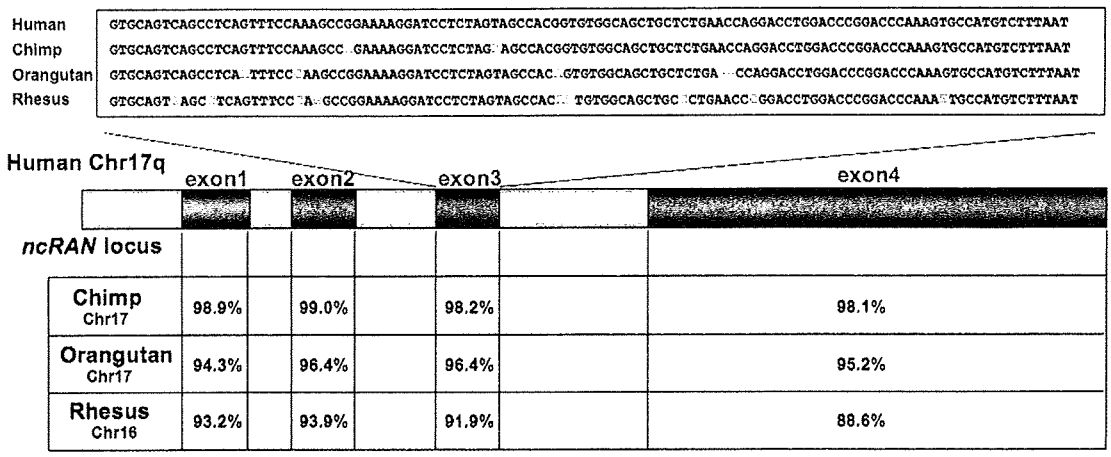

Figure 5. Schematic representation of *ncRAN* sequence conservation in primates. Sequence conservation in *ncRAN* gene locus among human and primates is indicated. Nucleotide sequences of exon3 of *ncRAN* in primates are indicated by numbers in brackets. Genomic sequences within the highly conserved 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.

Acknowledgements

We are grateful to the hospitals and institutions that provided us with surgical specimens. We also thank Atsushi Kawasaki, Osamu Shimozato, Youquan Bu, Yusuke Suenaga for their valuable suggestions; Shin Ishii for help with microarray statistical analyses; Takehiko Kamijo for providing a *MEL1* plasmid construct; Chengguo Yu for critical reading of the manuscript and Natsue Kitabayashi, Yuki Nakamura, Akane Sada for their technical assistances. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan for the Third-Term Comprehensive Control Research for Cancer.

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厚生労働科学研究費補助金
がん臨床研究事業
「小児がんに対する標準治療・診断確立のための研究」

平成21年度

平成22年4月発行
発行者：堀部敬三（研究代表者）
事務局：独立行政法人国立病院機構
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〒460-0001　名古屋市中区三の丸4丁目1番1号
TEL:052-951-1111　　FAX:052-963-5503
印刷所：サカイ印刷株式会社