ERAP140/Nbla10993 is a novel favorable prognostic indicator for neuroblastoma induced in response to retinoic acid

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Received October 24, 2007; Accepted December 3, 2007

Abstract. In the present study, we identified a gene termed Nbla10993 whose expression levels are higher in favorable neuroblastomas versus unfavorable ones. Structural analysis showed that Nbla10993 is a novel splicing variant of the ER-associated protein of 140 kDa (ERAP140), which lacks the central acidic as well as the COOH-terminal Cys/His-rich domain. Similarly, ERAP140 was preferentially expressed in favorable neuroblastomas relative to unfavorable ones. During the all-trans-retinoic acid (ATRA)-mediated neuronal differentiation in neuroblastoma-derived RTBM1 cells, the expression levels of ERAP140/Nbla10993 increased at the mRNA level. Consistent with these observations, the luciferase reporter analysis demonstrated that the ERAP140/Nbla10993 promoter responds to ATRA. In addition, the immunoprecipitation/immunoblotting experiments showed that ERAP140 forms a stable complex with RARα but not with RXRα in cells, suggesting that ERAP140 is involved in RAR-mediated transcriptional regulation. Furthermore, the quantitative real-time PCR analysis using 109 primary neuroblastoma samples demonstrated that the expression levels of ERAP140/Nbla10993 significantly correlate with a better clinical outcome of neuroblastomas. Taken together, our present findings indicate that ERAP140/Nbla10993 plays an important role in the regulation of ATRA-mediated neuronal differentiation, and is a novel member of prognostic indicators for neuroblastoma.

Introduction

Neuroblastoma arising from the sympathoadrenal lineage of the neural crest, is one of the most common early childhood solid tumors (1) and displays distinct biological properties in different prognostic subsets (2). For example, neuroblastomas found in patients <1 year old usually undergo spontaneous regression, which may be in part due to the neuronal differentiation and/or apoptotic cell death. They have no MYCN gene amplification and exhibit better clinical outcome. In marked contrast, neuroblastomas found in patients >1 year old, who usually carry the MYCN gene amplification and allelic loss in the distal part of the short arm of chromosome 1 and gain of chromosome 17q, are often aggressive with an unfavorable prognosis in spite of intensive multimodal therapy (3-5). Extensive efforts to identify novel prognostic indicators for neuroblastoma demonstrated that a high-affinity receptor for the nerve growth factor (NGF) gene, termed TrkA, and a receptor for the brain-derived neurotrophic factor (BDNF) as well as the neurotrophin-4/5 (NT-4/5) gene, termed TrkB, are favorable and unfavorable prognostic indicators, respectively (6-8). However, the precise molecular mechanisms underlying the progression of aggressive neuroblastomas and the spontaneous regression of favorable ones are still elusive. From a clinical point of view, it is necessary to identify as yet unknown prognostic indicator(s) for neuroblastoma.

As previously described (9), we have generated full-length enriched oligo-capping cDNA libraries, derived from favorable and unfavorable neuroblastoma tissues, and cloned a large number of genes. Among them, we have identified several new prognostic indicators for neuroblastoma. Kato et al found that the tubulin tyrosine ligase gene (TTL/Nbla0660) is expressed at a higher level in favorable neuroblastomas versus unfavorable ones and its expression level increases in neuroblastoma-derived cell lines in response to all-trans-retinoic acid (ATRA) (10). Similarly, the expression levels of the BMCC1/Nbla00219 gene were associated with a favorable prognosis in neuroblastomas (11). In contrast, Aoyama et al reported that LMO3/Nbla3267 gene expression correlates with a poor prognosis of neuroblastomas (12). These findings may provide novel insight into understanding the genesis and progression of neuroblastomas.

In the present study, we found that the Nbla10993 gene, which encodes a novel splicing variant of the ER-associated protein of 140 kDa, Nbla10993, neuroblastoma.

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Abbreviations: ATRA, all-trans-retinoic acid; ER, estrogen receptor; ERAP140, ER-associated protein of 140 kDa; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor

Key words: all-trans-retinoic acid, differentiation, ER-associated protein of 140 kDa, Nbla10993, neuroblastoma
protein of 140 kDa (ERAP140), is preferentially expressed in favorable neuroblastomas relative to unfavorable ones. The ATRA-mediated neuronal differentiation of neuroblastoma-derived cell lines was associated with the up-regulation of ERAP140/Nbla10993, suggesting that ERAP140/Nbla10993 is involved in neuroblastoma differentiation and is a novel prognostic indicator for favorable neuroblastoma.

Materials and methods

Patient population. Tumors were staged following the International neuroblastoma staging system criteria (13). The patients were treated according to the protocols proposed by the Japanese infantile neuroblastoma cooperative study (14) and the study group of Japan for the treatment of advanced neuroblastoma (15). All patients agreed to participate and provided written informed consent. Our present study was approved by the institutional ethics review committee. For neuroblastoma tissues used in the present study, 28 tumors were stage 1, 16 stage 2, 35 stage 3, 23 stage 4 and 7 stage 4S.

Cell culture and transient transfection. Human neuroblastoma-derived RTBM1 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), penicillin (50 U/ml) and streptomycin (50 μg/ml). Mouse neuroblastoma-derived Neuro2a and African green monkey embryonic kidney-derived COS7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotic mixture. Cells were cultured at 37˚C in a humidified atmosphere of 5% CO2. Where indicated, RTBM1 cells were exposed to all-trans-retinoic acid (ATRA, Sigma, St. Louis, MO, USA) at a final concentration of 5 μM. For transient transfection, cells were transfected with the indicated expression plasmids by using a Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s recommendations.

Preparation of RNA and RT-PCR. Total RNA was isolated from fresh-frozen tissues of primary neuroblastomas and neuroblastoma-derived RTBM1 cells treated with ATRA by using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Total RNA (5 μg) was reverse transcribed by using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The resultant cDNAs were subjected to a PCR-based amplification. The oligonucleotide primer sets used were as follows: Nbla10993, 5’-TTCTTGAGCCTACAAC-3’ (forward) and 5’-GCATCTGGCACAAAAAGGA-3’ (reverse); ERAP140, 5’-GAGGGCAGGCCAAGACATC-3’ (forward) and 5’-GCTGATCGAACGTCTGTA-3’ (reverse). PCR products were separated on 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Real-time PCR for the quantification of the gene expression. Total RNA was prepared from 109 primary neuroblastoma tissues and reverse transcribed using the TaqMan reverse transcription reagent kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The expression of Nbla10993 gene was quantified as previously described (11,12). Expression levels were calculated as a ratio of the mRNA level for a given gene relative to the mRNA level for GAPDH in the same cDNA. The oligonucleotide primers and TaqMan probes, labeled at the 5’-end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3’-end with 6-carboxy-4,7-dimethoxy-2-methylbenzimidazolylrhodamine (TAMRA), were as follows: ERAP140/Nbla10993, 5’-CAGGACACCCTAAAC-3’ (forward), 5’-GTGAGGTCACTGACGACA-3’ (reverse) and 5’-ATCATGTTTGACCCCTCATAAA TCTGATCCTCCTGTG-3’ (probe).

The generation of a specific antibody against ERAP140/Nbla10993. Anti-ERAP140/Nbla10993 polyclonal antibody was raised in rabbits against GST-ERAP140 fusion protein encoding amino acid residues 2 to 295 (Protein Express, Kisarazu, Japan).

Immunoblotting. Cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in 1X SDS sample buffer containing 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS and 62.5 mM Tris-HCl, pH 6.8 and then boiled for 5 min. The protein concentration of the whole cell lysates was determined by using a Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of whole cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 0.3% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 at 4˚C overnight and then incubated with monoclonal anti-GAPDH (9-1E21, Chemicon, Temecula, CA, USA), polyclonal anti-ERAP140 or with polyclonal anti-actin (C-20, Santa Cruz Biotechnology) antibody at room temperature for 1 h, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Beverly, MA, USA) at room temperature for 1 h. Immuno-reactive bands were visualized by the ECL system (Amersham Bioscience, Uppsala, Sweden) according to the manufacturer’s protocols.

Immunoprecipitation. COS7 cells were transiently co-transfected with the indicated combinations of the expression plasmids. Forty-eight hours after transfection, the cells were washed in ice-cold PBS, lysed in lysis buffer containing 25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100 supplemented with protease inhibitor mixture (Sigma) for 30 min at 4˚C and centrifuged at 15,000 rpm for 15 min at 4˚C to remove insoluble materials. Equal amounts of whole cell lysates were immunoprecipitated with normal rabbit serum (NMS), polyclonal anti-RARα (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with polyclonal anti-RXRα (D-20, Santa Cruz Biotechnology) antibody. The immunoprecipitates were analyzed by immunoblotting with the indicated antibodies.

Luciferase reporter assay. The genomic fragment containing 1 kb of the 5’-upstream region of the ERAP140/Nbla10933 gene was amplified from human placenta genomic DNA by PCR-based strategy and subcloned into pGL2-basic luciferase
reporter plasmid (Promega, Madison, WI, USA) to give pLuc-ERAP140. The luciferase reporter construct was verified by restriction digestion and DNA sequencing (Applied Biosystems, Piscataway, NJ, USA). For the luciferase reporter assay, Neuro2a cells were seeded at a density of 5x10⁴ cells/12-well tissue culture dishes and allowed to attach overnight. Cells were then transiently co-transfected with 100 ng pLuc-ERAP140 and 10 ng Renilla luciferase reporter plasmid (pRL-TK, Promega). A total amount of plasmid DNA per transfection was kept constant (500 ng) with pcDNA3 (Invitrogen). Twenty-four hours after transfection, the cells were treated with 5 μM ATRA or left untreated. Forty-eight hours after treatment, the cells were lysed in passive lysis buffer (Promega) and their luciferase activities were measured by the dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions. The firefly luminescence signal was normalized based on the Renilla luminescence signal.

Results

Identification of the Nbla10993 gene. As previously described (9), we have constructed oligo-capping cDNA libraries derived from different subsets of primary neuroblastomas. Based on the expression analysis of genes which were randomly picked up from these cDNA libraries, we identified the Nbla10993 gene which is preferentially expressed in favorable neuroblastomas (stage 1) versus unfavorable ones (stage 3 or 4) (see below). A sequence analysis and homology search of the Nbla10993 gene showed that Nbla10993 is a novel alternative splicing variant of the ER-associated protein of 140 kDa (ERAP140) (16), which encodes NH₂-terminal 295 amino acid residues of ERAP140 and lacks a central acidic region and COOH-terminal Cys/His-rich domain (Fig. 1A and B).

The preferential expression of ERAP140/Nbla10993 in favorable neuroblastomas versus unfavorable ones, we analyzed the expression levels of ERAP140/Nbla10993 in 16 favorable (stage 1) and 16 unfavorable neuroblastoma samples (stage 3 and 4) by semi-quantitative RT-PCR. Although the expression levels of Nbla10993 and ERAP140 varied among those neuroblastoma tissues that we examined, the two mRNAs were expressed at higher levels in favorable neuroblastomas relative to unfavorable ones (Fig. 2A and B). To further examine the expression levels of ERAP140/Nbla10993 and its clinical significance in primary neuroblastomas, we performed a quantitative real-time PCR analysis. For this purpose, total RNA was extracted from 109 primary neuroblastomas including 28 (stage 1), 16 (stage 2), 35 (stage 3), 23 (stage 4) and 7 (stage 4S) tumors. In these expression analyses, we used a primer set which amplifies the region encoding the NH₂-terminal portion of ERAP140. As shown in Fig. 3A and B, the quantitative real-time PCR analysis clearly demonstrated that the higher levels of ERAP140/Nbla10993 expression are observed in favorable neuroblastomas (stage 1 and 2) and closely correlate with a better clinical outcome as shown in the Kaplan-Meier cumulative survival curves, indicating that the ERAP140/Nbla10993 gene may be a novel prognostic indicator for favorable neuroblastomas.

The transcriptional up-regulation of ERAP140/Nbla10993 during the retinoic acid-mediated neuronal differentiation. Since ERAP140/Nbla10993 was expressed at higher levels in favorable neuroblastomas versus unfavorable ones and its expression levels correlated with a better clinical outcome of patients with neuroblastomas, it is likely that ERAP140/Nbla10993 is closely involved in the regulation of neuronal differentiation and/or apoptotic cell death. As previously described (17), neuroblastoma-derived RTBM1 cells underwent neuronal differentiation as judged by the extensive neurite outgrowth in response to the all-trans-retinoic acid (ATRA) (Fig. 4A). In support of these observations, the expression levels of GAP43 which is one of the markers for neuronal differentiation (18) increased in response to ATRA.

Figure 1. The structure of ERAP140/Nbla10993. (A) The genomic structure of ERAP140/Nbla10993. The open and solid boxes indicate non-coding and coding exons, respectively. (B) A schematic drawing of ERAP140/Nbla10993. The NH₂-terminal basic region, central acidic region and COOH-terminal Cys/His-rich domain are indicated.
under our experimental conditions (Fig. 4B). These observations prompted us to examine the expression levels of ERAP140/Nbla10993 in response to ATRA. To this end, RTBM1 cells were exposed to 5 μM ATRA. At the indicated time points after treatment with ATRA, total RNA was prepared and subjected to semi-quantitative RT-PCR. As shown in Fig. 5A, ATRA treatment resulted in an increase in the expression levels of ERAP140 in a time-dependent manner. On the other hand, the expression levels of Nbla10993 remained almost unchanged up to 4 days after treatment with ATRA, whereas its expression levels markedly increased at 6 days after exposure to ATRA.

To further confirm the ATRA-mediated transcriptional up-regulation of ERAP140/Nbla10993, we generated a luciferase reporter plasmid carrying the 5'-upstream region of this gene termed pluc-ERAP140. Mouse neuroblastoma-derived Neuro2a cells which displayed quite a high transfection efficiency (data not shown), were transiently co-transfected with the constant amount of pluc-ERAP140 and Renilla luciferase reporter plasmid (pRL-TK). Twenty-four hours after transfection, the cells were treated with 5 μM ATRA or left untreated. Forty-eight hours after treatment, the cells were lysed and their luciferase activities were measured. As shown in Fig. 5B, a significant increase in the luciferase activity was observed in the cells exposed to ATRA as compared with those left untreated, suggesting that ATRA treatment enhances the promoter activity of the ERAP140/Nbla10993 gene.

ATRA-mediated induction of the ERAP140 protein. To detect the ERAP140/Nbla10993 protein, we generated a polyclonal
antibody against the NH$_2$-terminal portion of ERAP140.

According to the results obtained by semi-quantitative RT-PCR, immunoblotting showed that the expression of ERAP140 is up-regulated at the protein level in response to ATRA (Fig. 6A). Similar results were also obtained in neuroblastoma-derived LA-N-5 and NB-39-nu cell lines (data not shown). Unfortunately, we could not detect the endogenous Nbla10993 protein under our experimental conditions, which may be due to a quality of our specific antibody and/or quite low expression level of the Nbla10993 protein. To address whether ERAP140 could be induced in cytoplasm and/or the cell nucleus in response to ATRA,
ATRA-treated RTBM1 cells were fractionated into cytoplasmic and nuclear fractions and subjected to immunoblotting. Lamin B and tubulin-α were used as nuclear and cytoplasmic markers, respectively (data not shown). As shown in Fig. 6B, cytoplasmic ERAP140 was significantly induced in response to ATRA, whereas ERAP140 was barely detectable in the cell nucleus regardless of ATRA treatment.

ERAP140 is associated with RARα but not with RXRα in cells. It has been shown that ATRA treatment in neuroblastoma cells leads to neuronal differentiation, cell cycle arrest and/or apoptotic cell death through nuclear receptors such as retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (19-21). As previously described (16), ERAP140 was associated with RARα but not with RXRα as examined with an in vitro pull-down assay. To further examine whether ERAP140 could form a complex with RARα and/or with RXRα in cells, the COS7 cells were transiently co-transfected with the expression plasmid for ERAP140 together with the expression plasmid encoding RARα or RXRα. Forty-eight hours after transfection, whole cell lysates were prepared and immunoprecipitated with normal rabbit serum (NRS), polyclonal anti-RARα or with polyclonal anti-RXRα antibody followed by immunoblotting with the indicated antibodies.

Discussion

It has been described that ERAP140 is identified as one of the binding partners of ERα by using the far-Western screening procedure and is highly expressed in the human brain (16). According to results, the central region of ERAP140 (amino acid residues 489-559) which contains a short α-helix was
required for the interaction with the COOH-terminal ligand binding domain (LBD) of ERα. Moreover, ERAP140 bound to RARα but not to RXRα in vitro. Since ERAP140 had the ability to enhance the transcriptional activity of ERs as well as RARα, it is likely that ERAP140 acts as a tissue-specific nuclear receptor co-activator. Based on a large-scale differential screening of cDNA libraries generated from primary neuroblastosas, we have identified a large number of genes that are expressed differentially in favorable and unfavorable neuroblastosas (9). Our experimental approaches may contribute to the efficient and correct prediction of the clinical outcome of neuroblastosas (22). In the present study, we focused our attention on the Nbla10993 gene. The structural analysis showed that Nbla10993 encodes a novel splicing variant of ERAP140. Expression studies demonstrated that Nbla10993 as well as ERAP140 are expressed preferentially in favorable neuroblastosas versus unfavorable ones. As expected, the expression levels of ERAP140/Nbla10993 were closely associated with a better prognosis of neuroblasto, suggesting that ERAP140/Nbla10993 may be a new member of prognostic indicators for neuroblasto.

Our additional new finding in this study was that Nbla10993 as well as ERAP140 are up-regulated in neuroblasto-derived RTBM1 cells exposed to ATRA. According to these results, the Nbla10993/ERAP140 promoter responded to ATRA as examined by the luciferase reporter assay. We have discovered a putative retinoic acid responsive element (RARE) within the promoter region of the Nbla10993/ERAP140 gene (data not shown). In marked contrast to ATRA, estrogen treatment had an undetectable effect on the expression levels of ERAP140 (16). Notably, semi-quantitative RT-PCR analysis showed that the kinetics of the ATRA-mediated up-regulation of ERAP140 is distinct from that of Nbla10993. The expression levels of ERAP140 increased in response to ATRA in a time-dependent manner, whereas the increased expression of Nbla10993 was detectable at 6 days after ATRA treatment, suggesting that ERAP140 and Nbla10993 play an important role in the regulation of neuroblasto differentiation in the early and late phases of the differentiation process, respectively. To further confirm this notion, it is necessary to examine the expression pattern of ERAP140 and Nbla10993 in response to ATRA. To this end, we have generated a polyclonal antibody against the NH2-terminal portion of ERAP140. Our specific antibody clearly detected an increase in the expression levels of ERAP140 in RTBM1 cells treated with ATRA, which was consistent with the results obtained by semi-quantitative RT-PCR. Unfortunately, our antibody could not recognize the estimated size of the Nbla10993 protein, which may be attributed to a quality of our antibody and/or quite low expression of the Nbla10993 protein. Shao et al described that immunoblotting with their own antibody against ERAP140 detects multiple bands corresponding to ERAP140 arising from alternative splicing and/or post-translational modifications (16).

According to previous observations (16), ERAP140 contains a nuclear localization signal (NLS) at its NH2-terminus and is primarily localized in the cell nucleus. They also described that ERAP140 is recruited onto the promoter region of the ER-target gene through interaction with ERα. In addition, ERAP140 also enhanced the promoter activity of the target gene of ATRA in collaboration with RARα. Our immunoprecipitation/immunoblotting experiments showed that ERAP140 forms a complex with RARα but not with RXRα in cells. Immunoblotting using nuclear and cytoplasmic fractions demonstrated that ERAP140 is largely expressed in cytoplasm and ATRA treatment results in a remarkable increase in cytoplasmic ERAP140, indicating that a complex formation between ERAP140 and RARα may occur in cytoplasm. Although these discrepancies may be in part due to different cell systems, the functional significance of the cytoplasmic interaction of ERAP140 with RARα remains unclear. Further studies are necessary to address this issue.

Acknowledgements

We thank Mr. H. Kageyama for valuable discussion. This work was supported in part by a Grant-in-Aid from the Ministry of Health, Labour and Welfare for Third Term Comprehensive Control Research for Cancer, a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Uehara Memorial Foundation and Hisamitsu Pharmaceutical Co.

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