

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

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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 RAR α but not with RXR α 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

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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 μ g/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 μ M. 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 μ g) 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'-GGAAAC CAGGACACCCTAAAC-3' (forward) and 5'-GCATCTGG CAAAAAGGA-3' (reverse); *ERAP140*, 5'-GAGGGCAA TAAAGAGCCAGA-3' (forward) and 5'-GCTGATCGAAC CCAGTCTTT-3' (reverse); *GAPDH*, 5'-ACCTGACCTGCC GTCTAGAA-3' (forward) and 5'-TCCACCACCCTGTT GCTGTA-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-carboxytetramethylrhodamine (TAMRA), were as follows: *ERAP140/Nbla10993*, 5'-CACTGAAATTTAACATCACT CCAAT-3' (forward), 5'-GTTGGCATCTGGCACAA AAA-3' (reverse) and 5'-ATCATGTTTGACCCTCATAAA TCTGATCCTCTGGT-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-GAP34 (9-1E21, Chemicon, Temecula, CA, USA), polyclonal anti-ERAP140 or with polyclonal anti-actin (20-33, Sigma) 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. Immunoreactive 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-RAR α (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with polyclonal anti-RXR α (D-20, Santa Cruz Biotechnology) antibody. The immunoprecipitates were analyzed by immunoblotting with the indicated antibodies.

Luciferase reporter assay. The genomic fragment containing 1 kbp of the 5'-upstream region of the *ERAP140/Nbla10993* gene was amplified from human placenta genomic DNA by PCR-based strategy and subcloned into pGL2-basic luciferase

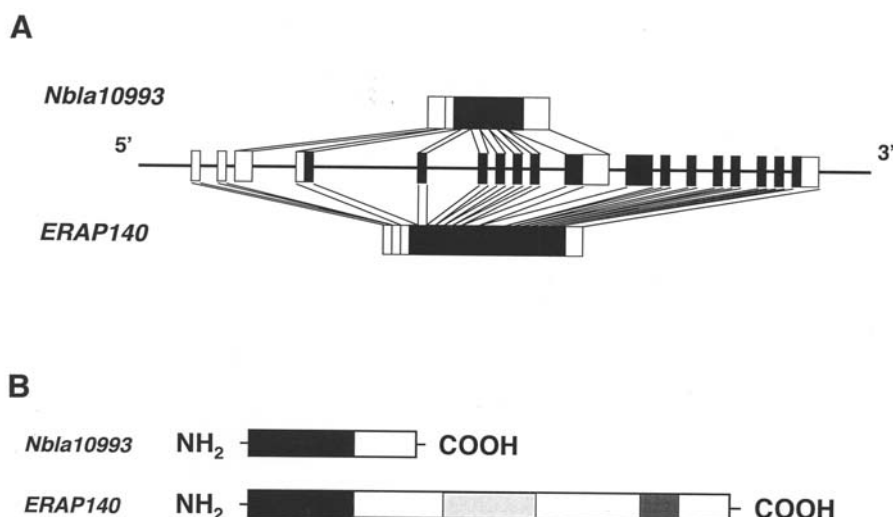


Figure 1. The structure of *ERAP140/Nbla10993*. (A) The genomic structure of *ERAP140/Nbla10993*. The open and solid boxes indicate non-coding and coding exons, respectively. (B) A schematic drawing of *ERAP140/Nbla10993*. The NH₂-terminal basic region, central acidic region and COOH-terminal Cys/His-rich domain are indicated.

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 5×10^4 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 μ M 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 neuroblastoma. To confirm the preferential expression of *ERAP140/Nbla10993* in favorable neuro-

blastomas 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

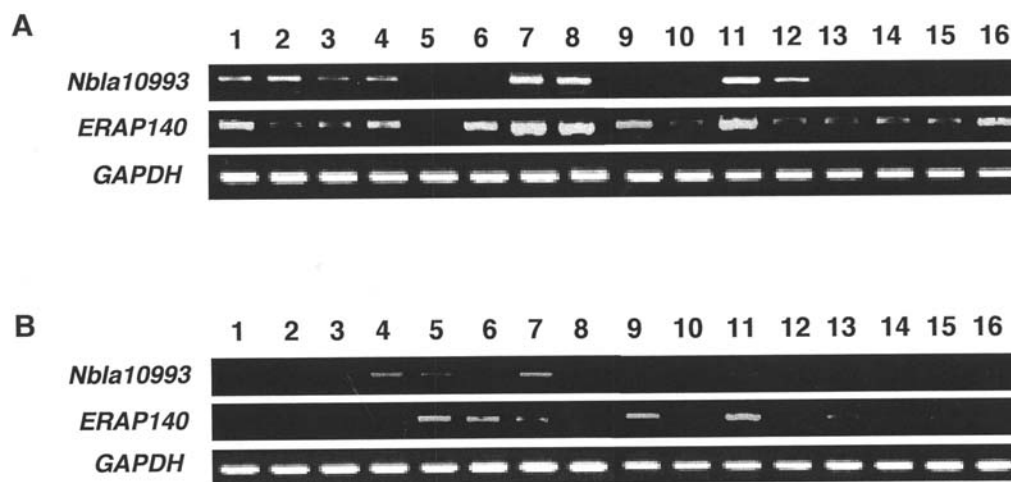


Figure 2. *ERAP140/Nbla10993* is highly expressed in favorable neuroblastomas versus unfavorable ones. Total RNA prepared from 16 favorable neuroblastomas (A) and unfavorable ones (B) was analyzed for the expression levels of *Nbla10993* and *ERAP140* by semi-quantitative RT-PCR. *GAPDH* was used as an internal control.

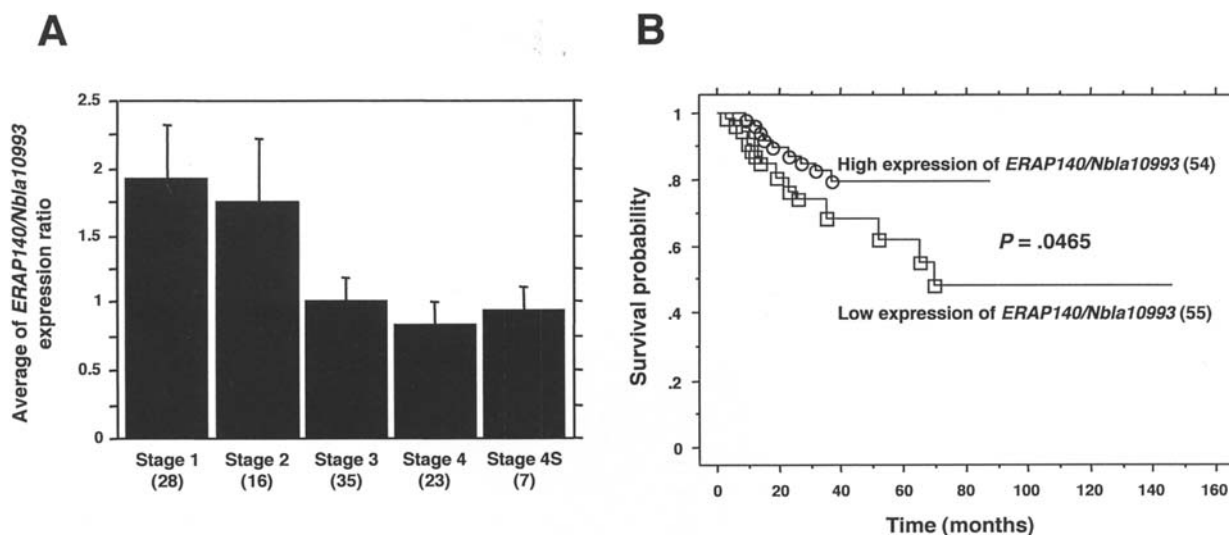


Figure 3. Real-time PCR analysis for the expression of *ERAP140/Nbla10993* in 109 primary neuroblastomas. (A) The expression levels of *ERAP140/Nbla10993* in 109 neuroblastoma primary samples categorized, based on the patient clinical stages, were examined by a quantitative real-time PCR. The relative expression levels of *ERAP140/Nbla10993* were determined by calculating the ratio between *ERAP140/Nbla10993* and *GAPDH*. (B) Kaplan-Meier survival curves of patients with neuroblastomas based on high (open circle) or low expression (open square) of *ERAP140/Nbla10993*.

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 μ M 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 μ M 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

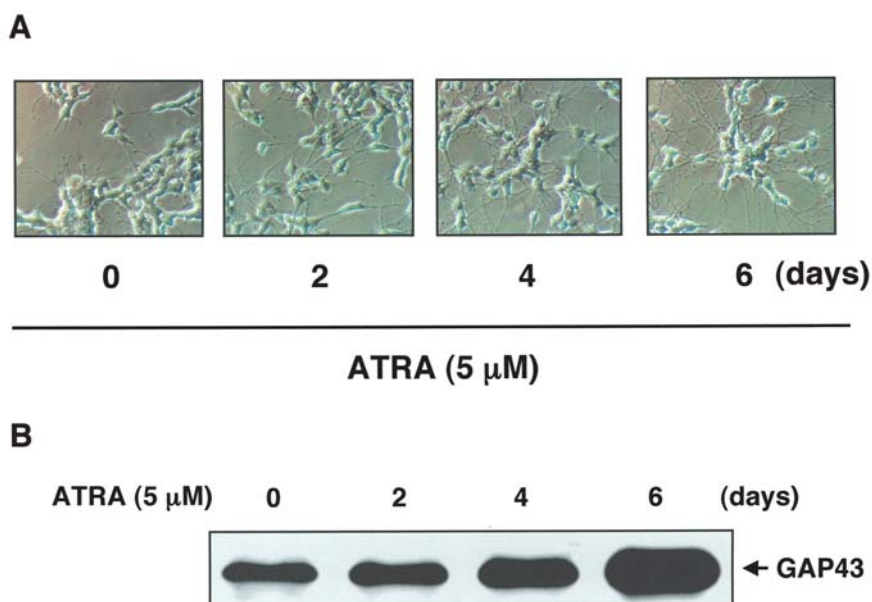


Figure 4. The neurite extension of neuroblastoma-derived RTBM1 cells in response to ATRA. (A) RTBM1 cells were treated with 5 μ M ATRA. At the indicated points after treatment with ATRA, neurite outgrowth was examined with a phase-contrast microscope. (B) Immunoblotting. RTBM1 cells were treated as in (A). At the indicated time points after treatment with ATRA, whole cell lysates were prepared and subjected to immunoblotting with monoclonal anti-GAP43 antibody.

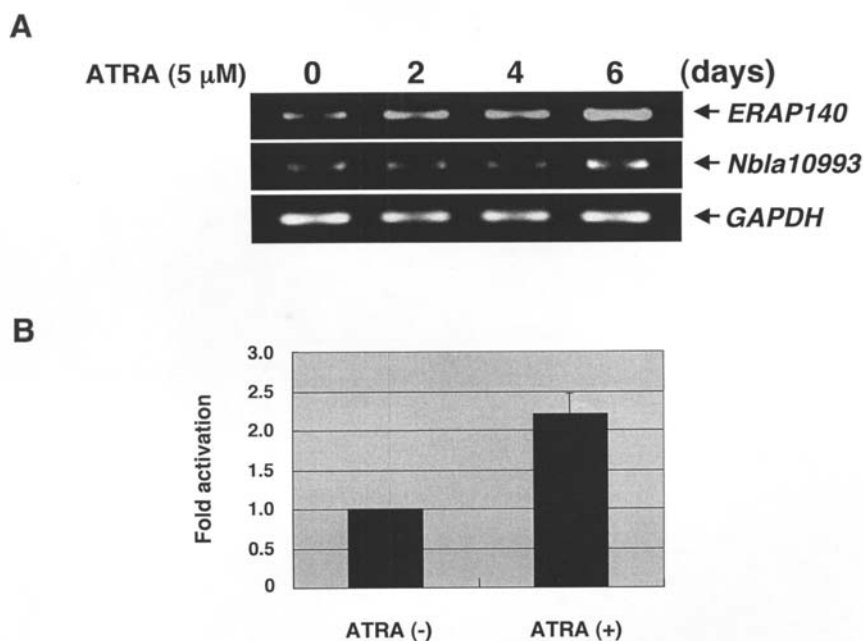


Figure 5. ATRA-mediated transcriptional activation of *ERAP140/Nbla10993* in neuroblastoma cells. (A) RT-PCR. At the indicated time periods after treatment with 5 μ M ATRA, total RNA was prepared from RTBM1 cells and subjected to semi-quantitative RT-PCR. *GAPDH* was used as an internal control. (B) Luciferase reporter assay. Mouse neuroblastoma-derived Neuro2a cells were transiently co-transfected with the constant amount of luciferase reporter plasmid driven by an *ERAP140/Nbla10993* promoter (pluc-*ERAP140*) and *Renilla* luciferase reporter plasmid (pRL-TK). Twenty-four hours after transfection, the cells were transferred into a fresh medium with or without 5 μ M ATRA. Forty-eight hours after treatment, the cells were lysed and their luciferase activities were measured. The firefly luciferase activities were normalized to the *Renilla* luciferase activities.

antibody against the NH₂-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,

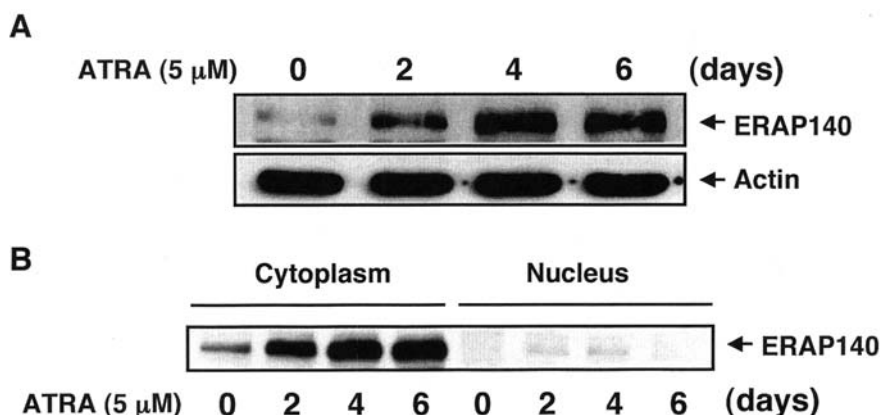


Figure 6. ATRA-mediated induction of ERAP140 in response to ATRA. (A) RTBM1 cells were exposed to 5 μ M ATRA. At the indicated time points after ATRA treatment, whole cell lysates were prepared and processed for immunoblotting with anti-ERAP140 antibody. Actin served as a loading control. (B) The cytoplasmic induction of ERAP140 in response to ATRA. RTBM1 cells were treated with ATRA as in (A). At the indicated time periods after treatment with ATRA, cells were fractionated into cytoplasmic and nuclear fractions and subjected to immunoblotting with polyclonal anti-ERAP140 antibody.

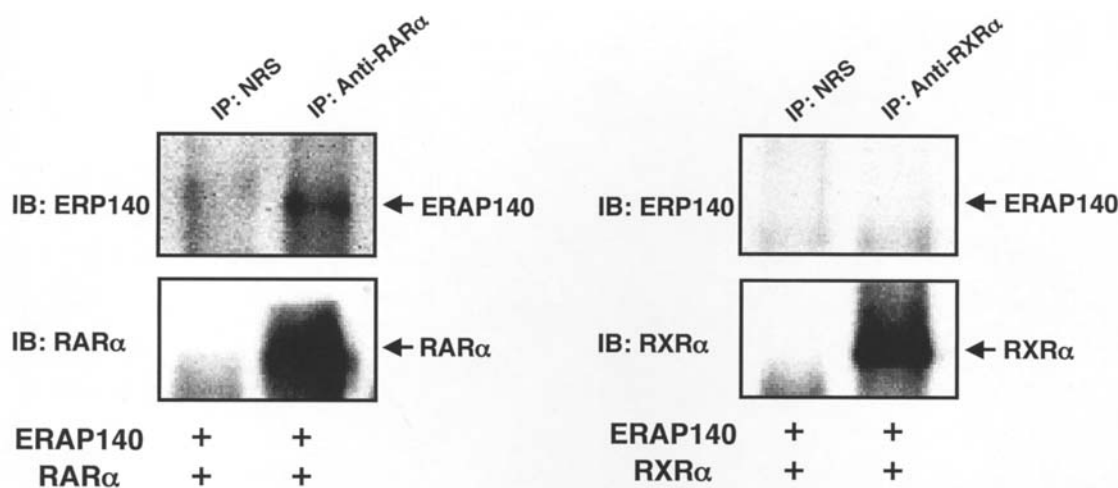


Figure 7. ERAP140 interacts with RAR α but not with RXR α in cells. The COS7 cells were transiently co-transfected with the expression plasmids for ERAP140 and RAR α (left panel) or with the expression plasmids encoding ERAP140 and RXR α (right panel). Forty-eight hours after transfection, whole cell lysates were prepared and immunoprecipitated with normal rabbit serum (NRS), polyclonal anti-RAR α or with polyclonal anti-RXR α antibody followed by immunoblotting with the indicated antibodies.

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 RAR α but not with RXR α 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 RAR α but not with RXR α as examined with an *in vitro* pull-down assay. To further examine whether ERAP140 could form a complex with RAR α and/or with RXR α in cells, the COS7 cells were transiently co-transfected with the expression plasmid for ERAP140 together with the

expression plasmid encoding RAR α or RXR α . Forty-eight hours after transfection, whole cell lysates were prepared and immunoprecipitated with normal rabbit serum (NRS), polyclonal anti-RAR α or polyclonal anti-RXR α antibody. The immunoprecipitates were analyzed by immunoblotting with polyclonal anti-ERAP140 antibody. Consistent with previous observations (16), the anti-RAR α immunoprecipitates contained ERAP140, whereas ERAP140 was not co-immunoprecipitated with RXR α (Fig. 7). Since ERAP140 was expressed largely in the cytoplasm, ERAP140 may interact with RAR α in cytoplasm and modulate its function.

Discussion

It has been described that ERAP140 is identified as one of the binding partners of ER α 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



for the interaction with the COOH-terminal ligand domain (LBD) of ER α . Moreover, ERAP140 bound to RAR α but not to RXR α *in vitro*. Since ERAP140 had the ability to enhance the transcriptional activity of ER α as well as RAR α , 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 neuroblastomas, we have identified a large number of genes which are expressed differentially in favorable and unfavorable neuroblastomas (9). Our experimental approaches may contribute to the efficient and correct prediction of the clinical outcome of neuroblastomas (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 neuroblastomas versus unfavorable ones. As expected, the expression levels of *ERAP140/Nbla10993* were closely associated with a better prognosis of neuroblastoma, suggesting that *ERAP140/Nbla10993* may be a new member of prognostic indicators for neuroblastoma.

Our additional new finding in this study was that *Nbla10993* as well as *ERAP140* are up-regulated in neuroblastoma-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 neuroblastoma 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 ER α . In

addition, ERAP140 also enhanced the promoter activity of the target gene of ATRA in collaboration with RAR α . Our immunoprecipitation/immunoblotting experiments showed that ERAP140 forms a complex with RAR α but not with RXR α 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 RAR α 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 RAR α remains unclear. Further studies are necessary to address this issue.

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