

In a sharp contrast to p53, coexpression of MDM2 with p73 resulted in an increase in the amount of p73, although p73 transcriptionally activated the expression of *MDM2* and its function such as transcriptional activation, and proapoptotic activity was significantly impaired through the direct interaction of the NH₂-terminal portion of p73 with MDM2 (Zeng *et al.*, 1999). Recently, Leng *et al.* (2003) reported that, like MDM2, a p53-inducible E3 ubiquitin protein ligase Pirh2 regulates the steady-state level of p53 and it participates in a negative autoregulatory feedback loop with p53. Pirh2 bound to p73 (Leng *et al.*, 2003), but did not promote the p73 degradation (Wu *et al.*, 2004). On the other hand, Rossi *et al.* (2005) found that a HECT-type E3 ubiquitin protein ligase Itch ubiquitinates p73 but not p53 and promotes the proteasome-dependent degradation of p73. These findings clearly implicate that p73 stability is regulated through a pathway distinct from that used for p53. Osada *et al.* (2001) found that the NH₂-terminally truncated forms of p63 (Δ Np63) appeared to be much more stable than wild-type p63, suggesting that the NH₂-terminal transactivation domain of p63 regulates its stability through a pathway that is sensitive to MG-132. Intriguingly, Wu *et al.* (2004) described that the half-life of Δ Np73 α is extended as compared with that of wild-type p73 α , and suggested that the transcriptional activity of p73 is required for its degradation. It is therefore likely that one or more transcriptional targets of p73 might have an ability to target p73 for ubiquitination-mediated degradation by proteasome.

In addition to the ubiquitination-mediated proteasome system, p73 degradation is regulated in a ubiquitination-independent manner. Recently, Ohtsuka *et al.* (2003) demonstrated that p53-inducible cyclin G, which is also transcriptionally activated by p73, significantly reduced their levels in a ubiquitination-independent manner, however, the detailed molecular mechanism underlying the cyclin G-mediated degradation of p53 and p73 remains to be defined. Based on our present results, UFD2a mediated the proteasomal turnover of p73 α without affecting its ubiquitination levels, and the enzymatic activity of UFD2a was not required for the downregulation of p73 α . It is becoming clear that some cellular proteins are subjected to the ubiquitination-independent proteasomal turnover. For example, it has been shown that ornithine decarboxylase (ODC) is degraded by the proteasome independently of ubiquitin modification (Murakami *et al.*, 1992). In this case, ODC was recruited to the proteasome through the physical interaction with antizyme 1 (AZ1) (Coffino, 2001). Additionally, Sheaff *et al.* (2000) found that the direct ubiquitination of p21^{WAF1} is not required for its degradation by proteasome. Recently, Jin *et al.* (2003) described that MDM2 binds to p21^{WAF1} and induces its proteasomal turnover without affecting the ubiquitination levels of p21^{WAF1}. This might be due to the MDM2-mediated recruitment of p21^{WAF1} to the proteasome. Considering that various E3 ubiquitin protein ligases associate with the proteasomal subunits, either directly or through specific adaptor proteins (Xie and Varshavsky,

2002), it is possible that p73 α might be recruited to the proteasome through its binding to UFD2a.

According to previous studies (Jost *et al.*, 1997; De Laurenzi *et al.*, 1998; Ueda *et al.*, 1999), the ability of p73 β to transactivate the p53/p73-responsive promoters as well as to limit cell growth was stronger than p73 α . These different functional properties between them might be attributed at least partly to the COOH-terminal inhibitory region, which is present in p73 α but not in p73 β (Ozaki *et al.*, 1999; Ueda *et al.*, 1999). Recently, Serber *et al.* (2002) found that the p63 α COOH-terminal inhibitory domain binds to its NH₂-terminal transactivation domain, thereby masking critical residues for transactivation. Based on the structural similarity between the COOH-termini of p73 α and p63 α , it is possible that the direct intramolecular folding regulates the transactivation function of p73 α . In addition to this intramolecular inhibition, the COOH-terminal region of p73 might modulate its protein stability. Lee and La Thangue (1999) reported that p73 β is considerably more stable than p73 α . According to our results, UFD2a bound to p73 α but not to p73 β and this interaction was mediated by a COOH-terminal extension of p73 α containing SAM domain. In contrast to p73 α , UFD2a had a negligible effect on the stability and function of p73 β . Thus, it is likely that the UFD2a-dependent degradation pathway contributes to the splicing isoform-specific regulation of p73.

The endogenous UFD2a in SH-SY5Y cells was significantly reduced at a protein level in response to cisplatin. During this apoptotic process, p73 α accumulated at protein level in association with a remarkable induction of its target p21^{WAF1}. It has been shown that the endogenous p73 is stabilized and activated in response to cisplatin treatment in a pathway mediated by nuclear nonreceptor tyrosine kinase c-Abl (Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999). Alternatively, Ren *et al.* (2002) described that protein kinase C δ catalytic fragment phosphorylates p73, and increases its stability, suggesting that post-translational modification such as phosphorylation might contribute to the regulation of p73 stability. It is worth noting that the NH₂-terminal region of UFD2a (amino-acid residues 1–123) is required for its enzymatic activity, and certain apoptotic stimuli, including treatment with staurosporine and ultraviolet irradiation, lead to UFD2a cleavage within the tetrapeptide (120-VDVD-123) by caspase 6 or granzyme B to generate a 110 kDa fragment lacking its NH₂-terminal region (Mahoney *et al.*, 2002). Since our polyclonal anti-UFD2a antibody was raised against the NH₂-terminal polypeptide of UFD2a (amino-acid residues 2–149), it did not recognize the cleaved 110 kDa product. However, we could detect immunoreactive small fragment (~15 kDa) in whole lysates prepared from SH-SY5Y cells exposed to cisplatin (data not shown), indicating that cisplatin treatment causes the cleavage of UFD2a. If this interpretation is correct, the downregulation of UFD2a detected by immunoblotting analysis might be due to the cleavage of UFD2a in response to cisplatin. In preliminary experiments, we have observed that the

NH₂-terminal regulatory region of UFD2a (amino-acid residues 1–149) binds to p73 α , whereas the COOH-terminal catalytic domain (amino-acid residues 1231–1293) failed to interact with p73 α (data not shown). Thus, it is likely that the cisplatin-induced apoptosis might be associated with the cleavage of UFD2a, thereby leading to the dissociation of free active p73 α from the UFD2a/p73 α complex.

As described previously (Stiewe *et al.*, 2002), steady-state levels of p73 protein were kept extremely low in various cancer cell lines and normal human diploid fibroblasts. On the other hand, UFD2a was abundantly expressed at mRNA and protein levels in various cancer cell lines (Mahoney *et al.*, 2002). Given that UFD2a has an ability to promote the degradation of p73 α , UFD2a might keep this proapoptotic protein at low levels, thereby preventing cells from apoptotic cell death in response to DNA damage. Thus, it is conceivable that specific inhibition of UFD2a might provide a novel strategy for anticancer treatment.

Materials and methods

Cell cultures and transfection

Human osteosarcoma cell lines (SAOS-2 and U2OS), human embryonic kidney cell line (HEK293T), human neuroglioma cell line (H4), human lung adenocarcinoma cell line (A549), mouse neuroblastoma cell line (Neuro2a) and monkey embryonic kidney cell line (COS7) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin. Human lung carcinoma cell line (H1299) and human neuroblastoma cell lines (NB-C201 and SH-SY5Y) were grown in RPMI 1640 medium containing 10% heat-inactivated FBS and antibiotics. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. For transfection, H1299 and U2OS cells were transfected with the indicated expression plasmids using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's recommendations. Transient transfection of COS7 cells was performed by FuGENE6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) in accordance with the manufacturer's specifications.

Northern blot analysis

Human UFD2a cDNA was radiolabeled with [α -³²P]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by using the random prime labeling kit (Takara Bio Inc., Ohtsu, Japan). A human MTN Blot (Clontech Laboratories, Palo Alto, CA, USA) was hybridized with the radiolabeled probe DNA overnight at 65°C in a solution containing 6 \times SSC, 1% *N*-lauroyl sarcosine, 7.5% dextran sulfate and 200 μ g heat-denatured salmon sperm DNA/ml. After hybridization, nonspecific probe was removed by a series of washes. RNA loading was verified by stripping the membrane and reprobing it with a probe specific for β -actin.

RT-PCR

SH-SY5Y cells were exposed to cisplatin (20 μ M), and incubated for 0–24 h as indicated. At the indicated time points

after the treatment with cisplatin, total RNA was prepared using the RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The cDNA was generated from each RNA sample (5 μ g) using reagents from SuperScript II (Invitrogen), and then used for PCR-based amplification. Specific primers used are as follows: (a) for p73, 5'-CTTTGAGGGCCGCATCTG-3' (forward primer), 5'-GCCCCAGGTTGGGTGTAG-3' (reverse primer); (b) for p21^{WAF1}, 5'-ATGAAATTCACCCCCTTTCC-3' (forward primer), 5'-CCCTAGGCTGTGCTCACTTC-3' (reverse primer); (c) for UFD2a, 5'-AAAGCGAGCCTCAGTGATA-3' (forward primer), 5'-CTGGATTGGAGCCAGTGTTC-3' (reverse primer); (d) for GAPDH, 5'-ACCTGACCTGCCGTCTA GAA-3' (forward primer), 5'-TCCACCACCCTGTTGCTG TA-3' (reverse primer).

Antibody production

The rabbit polyclonal antibody specific for human UFD2a was raised against a bacterially expressed GST fusion protein containing the NH₂-terminal polypeptide of UFD2a (amino-acid residues 2–149). The specificity of the affinity-purified antibody was assayed by immunoblotting analysis.

Cell viability assay

Cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) assay. In brief, SH-SY5Y cells were seeded in 96-well microtiter plates (5 \times 10³ cells/well). The next day, the medium was changed and cells were treated with cisplatin for 24 h. For the MTT assay, 10 μ l of MTT solution was added to each well for 3 h at 37°C. The absorbance readings for each well were carried out at 570 nm using the microplate reader (Model 450, Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblot analysis

Cells were lysed in lysis buffer containing 25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100, 1 mM PMSF and protease inhibitor mixture (Sigma Chemical Co., St Louis, MO, USA). The protein concentration of the lysates was determined by Bradford protein assay (Bio-Rad Laboratories). Equal amounts of protein were mixed with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT and 0.01% bromophenol blue), run on 8% SDS-PAGE and electroblotted onto PVDF membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA). The membrane was blocked with TBS supplemented with 0.1% Tween 20 and 5% nonfat milk for 1 h at room temperature, and then probed with monoclonal anti-p73 (Ab-4, NeoMarkers Inc., Fremont, CA, USA), monoclonal anti-FLAG (M2, Sigma Chemical Co.), monoclonal anti-HA (12CA5, Roche Molecular Biochemicals), monoclonal anti-p53 (DO-1, Oncogene Research Products, Cambridge, MA, USA), polyclonal anti-p21^{WAF1} (H164, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal anti-UFD2a antibody for 1 h at room temperature. After three washes, the membrane was incubated with a horseradish peroxidase-conjugated appropriate secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. Signals from immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions. Equal loading was checked by reprobing the membrane with polyclonal anti-actin antibody (20–33, Sigma Chemical Co.).

Subcellular fractionation

Cells were washed twice in PBS and lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) containing 0.5% NP-40 supplemented with a protease inhibitor mix (Sigma Chemical Co.). The samples were then incubated for 30 min at 4°C. Cell fractionation was performed as described previously (Nakamura *et al.*, 1997). The nuclear and cytoplasmic fractions were subjected to immunoblot analysis using the anti-UFD2a, monoclonal anti-Lamin B (Ab-1, Oncogene Research Products) or monoclonal anti-Ras (RASK-3, Seikagaku Co., Tokyo, Japan) antibody.

Immunoprecipitation analysis

Whole lysates prepared from transfected cells were precleared by incubation with protein G-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. The supernatant was collected after brief centrifugation, and incubated with the indicated primary antibodies for 2 h at 4°C. The immune complexes were precipitated with protein G-Sepharose beads for 1 h at 4°C, and the nonspecific bound proteins were removed by washing the beads with the lysis buffer three times at 4°C. The immunoprecipitated proteins were eluted by boiling in Laemmli sample buffer, separated on 8% SDS-PAGE and analysed by immunoblotting with anti-p73, anti-HA or anti-UFD2a antibody.

Ubiquitination assay

U2OS cells were transiently cotransfected with the expression plasmid for His-tagged ubiquitin, and HA-p73 α together with or without increasing amounts of the expression plasmid for FLAG-UFD2a as indicated. Total amounts of transfected DNA were kept constant by pcDNA3 (Invitrogen) per transfection. At 48 h after transfection, cells were exposed to the proteasomal inhibitor MG-132 (10 μ M) for 6 h and then lysed in lysis buffer containing 6 M guanidine-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄ and 10 mM imidazole, pH 8.0. Ubiquitinated proteins were recovered by Ni-NTA resin (Qiagen) and analysed by immunoblotting with the anti-HA antibody.

Luciferase reporter assay

H1299 cells were transiently cotransfected with 100 ng of the indicated p53/p73-responsive luciferase reporter plasmid (*BAX* or *p21^{WAF1}*), 10 ng of pRL-TK *Renilla* luciferase cDNA and 25 ng of the indicated expression plasmid (p53 or HA-p73 α) in the presence or absence of 200 ng of the antisense *UFD2a* expression plasmid. The total amounts of DNA were kept constant (510 ng) with pcDNA3. At 48 h after transfection,

cells were lysed and luciferase activity was measured by using the dual-luciferase reporter assay system (Promega Corp.) according to the manufacturer's instructions. The normalized luciferase activities represent the firefly luciferase activity corrected for *Renilla* luciferase as a measure of transfection efficiency.

Protein decay rate analysis

U2OS cells were cotransfected with the indicated combinations of the expression plasmids. At 24 h after transfection, cycloheximide (Sigma Chemical Co.) was added to the culture medium at a final concentration of 20 μ g/ml and cells were harvested at the indicated time points. Equal amounts of whole lysates (50 μ g) were subjected to immunoblot analysis with the anti-p73 antibody.

Apoptosis assay

H1299 cells were transiently cotransfected with 0.2 μ g of the GFP expression plasmid and 0.5 μ g of the expression plasmid for HA-p73 α or p53 together with or without 1.5 μ g of the FLAG-UFD2a expression plasmid. At 48 h after transfection, transfected cells were identified by the presence of green fluorescence. Cell nucleus was stained with DAPI to reveal nuclear condensation and fragmentation. The number of GFP-positive cells with apoptotic nuclei was scored.

Colony formation assay

H1299 cells were cotransfected with 200 ng of the expression plasmid encoding HA-p73 α or HA-p73 β together with or without increasing amounts of the expression plasmid for antisense *UFD2a* (200, 400 or 800 ng). At 48 h post-transfection, cells were maintained in the culture medium containing G418 (400 μ g/ml). After 2 weeks of selection, the plates were stained with Giemsa's solution and numbers of drug-resistant colonies were scored.

Acknowledgements

We are grateful to Dr KI Nakayama for providing us with mammalian expression plasmid for FLAG-tagged mouse UFD2a and Ms Y Nakamura for assistance with DNA sequencing. 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 Japan Society for the Promotion of Science.

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CpG Island Methylator Phenotype Is a Strong Determinant of Poor Prognosis in Neuroblastomas

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Abstract

Neuroblastoma, one of the most common pediatric solid tumors, is characterized by two extreme disease courses, spontaneous regression and life-threatening progression. Here, we conducted a genome-wide search for differences in DNA methylation that distinguish between neuroblastomas of the two types. Three CpG islands (CGI) and two groups of CGIs were found to be methylated specifically in neuroblastomas with a poor prognosis. By quantitative analysis of 140 independent cases, methylation of all the five CGI (groups) was shown to be closely associated with each other, conforming to the CpG island methylator phenotype (CIMP) concept. The presence of CIMP was sensitively detected by methylation of the *PCDHB* CGIs and associated with significantly poor survival (hazard ratio, 22.1; 95% confidence interval, 5.3-93.4; $P < 0.0001$). Almost all cases with *N-myc* amplification (37 of 38 cases) exhibited CIMP. Even in 102 cases without *N-myc* amplification, the presence of CIMP (30 cases) strongly predicted poor survival (hazard ratio, 12.4; 95% confidence interval, 2.6-58.9; $P = 0.002$). Methylation of *PCDHB* CGIs, located in their gene bodies, did not suppress gene expression or induce histone modifications. However, CIMP was significantly associated with methylation of promoter CGIs of the *RASSF1A* and *BLU* tumor suppressor genes. The results showed that neuroblastomas with CIMP have a poor prognosis and suggested induction of silencing of important genes as an underlying mechanism. (Cancer Res 2005; 65(3): 828-34)

Introduction

Epigenetic abnormalities, especially alterations in DNA methylation, are intimately involved in development of various human tumors (1). Aberrant methylation of promoter CpG islands (CGI) causes inactivation of tumor suppressor genes. Genomic instability is caused by genomic hypomethylation and is associated with hypermethylation (2, 3). Identification of epigenetic abnormalities in human cancers is expected to lead not only to discovery of novel disease mechanisms but also to development of new diagnostic markers. Therefore, we previously developed a genome-wide scanning method, methylation-sensitive representational difference analysis (MS-RDA), for detecting differences in DNA methylation (4, 5). This technique analyzes

unmethylated, CpG-rich regions of the genome and has already identified genes silenced in human lung, stomach, breast, and pancreatic cancers (6-9).

Neuroblastoma derived from primitive cells of the sympathetic nervous system is one of the most common solid tumors in childhood, characterized by two extreme disease courses, spontaneous regression, and life-threatening progression (10, 11). The clinical outcome is associated with disease stage, age at diagnosis, histologic classification, *N-myc* amplification, DNA ploidy, and *TrkA* overexpression (10-12). These characteristics are therefore used to classify cases into low-, intermediate-, and high-risk groups. However, especially in the cases with intermediate risk, prediction of prognosis and therapeutic decision-making are still difficult, and development of new markers is an urgent priority. Moreover, the molecular bases underlying the two distinct clinical courses are still unknown, and their clarification is needed to allow development of novel therapeutics.

In the present study, considering the major involvement of epigenetic machinery in embryonic development (13, 14), we searched for differences in DNA methylation between neuroblastomas with a good prognosis and counterparts with a poor prognosis by MS-RDA.

Materials and Methods

Tissue Samples and Cell Lines. Tumor samples were obtained from 145 nonrecurrent cases between 1995 and 1999 and were used under approval of institutional review boards. The mean age at initial diagnosis was 27 months (range, 0-216 months). Their clinical stages were determined according to the International Neuroblastoma Staging System, and 40, 17, 20, 60, and 8 cases belonged to stages I, II, III, IV, and IVS, respectively. Normal adrenal medulla tissue was collected from a case undergoing nephrectomy for a renal cancer. Neuroblastoma cell lines were obtained from the American Type Culture Collection (Manassas, VA), the Japanese Collection of Research Bioresources (Tokyo, Japan), and the RIKEN Bio Resource Center (Tsukuba, Japan). GANB was established by A.N. and normal human bronchial epithelial cells were purchased from Cambrex (East Rutherford, NJ). High molecular weight DNA and total RNA were extracted as previously described (7). Total RNAs of brain and adrenal glands were purchased from Clontech (Palo Alto, CA).

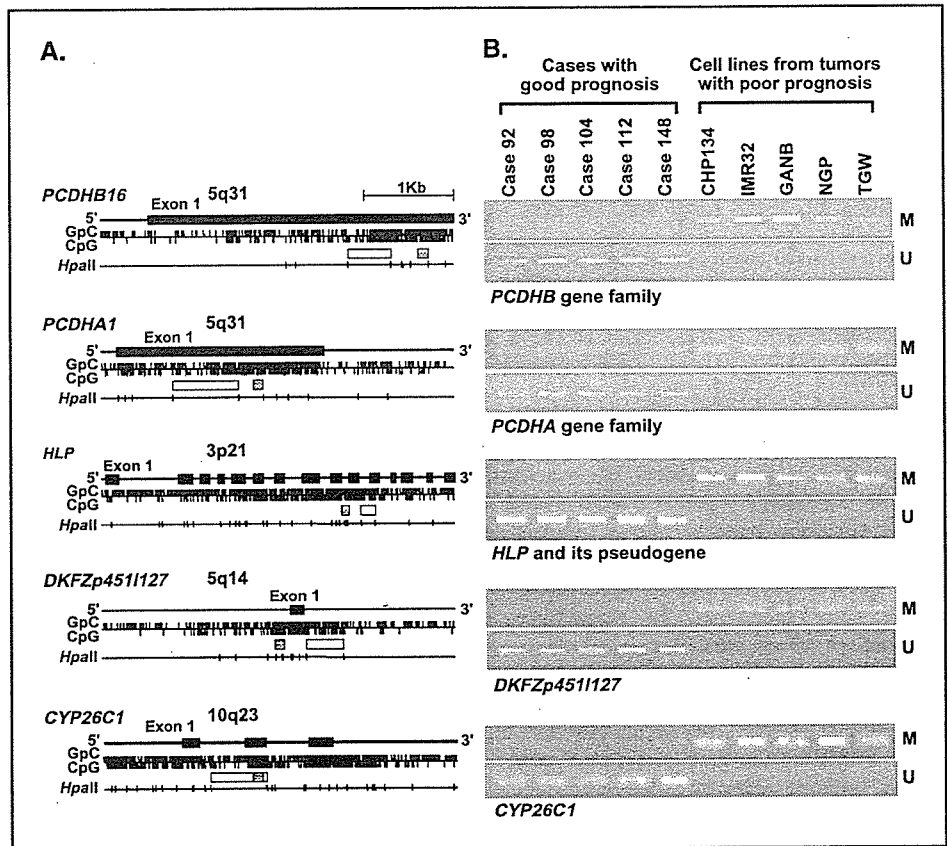
MS-RDA and Database Search. MS-RDA was done as previously described (4, 5). Genomic DNA of primary neuroblastomas with a good prognosis (cases 92, 98, 104, 112, and 148) and neuroblastoma cell lines established from cases with a poor prognosis (CHP134, IMR32, GANB, NGP, and TGW) were digested with *HpaII*, and then two pooled DNA samples were prepared. Although use of cell lines is highly recommended for MS-RDA (5), no cell lines were available for neuroblastomas with a good prognosis, and therefore we used the primary samples. To isolate CGIs that were hypermethylated in the latter, the cell line pool was used as the tester, and the primary tumor pool as the driver. MS-RDA in the opposite direction

Note: Supplementary data for this article are available at Cancer Research online (<http://cancerres.aacrjournals.org/>).

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Figure 1. Five CGIs isolated by MS-RDA and their methylation statuses in the samples used for MS-RDA. **A**, genomic structures of the five CGIs. GpC, CpG, and *Hpa*II recognition sites (5'-CCGG-3') are shown by ticks. Closed boxes, exons; open boxes, clones isolated by MS-RDA; shaded boxes, regions analyzed by MSP. **B**, methylation statuses analyzed by MSP. *M*, MSP using primers specific to methylated DNA; *U*, MSP using primers specific to unmethylated DNA. All the five CGIs were found to be differentially methylated between the two groups used for MS-RDA.



was also done. For each series of MS-RDA, 96 clones were analyzed for redundancy, and nonredundant clones were sequenced. Their genomic origins were examined using BLASTN software (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Sodium Bisulfite Modification and Methylation-Specific PCR. One microgram of DNA underwent sodium bisulfite modification (15), and was suspended in 20 μ L of TE buffer. For methylation-specific PCR (MSP), 1 μ L of the solution was used for PCR with primers specific to methylated or unmethylated sequences. Using DNA from normal human bronchial epithelial and DNA methylated with *Sss*I methylase, annealing temperatures specific for methylated and unmethylated primers were determined. Quantitative MSP was done separately for methylated DNA molecules and for unmethylated DNA molecules. Standard DNA was prepared by cloning PCR products amplified by methylated and unmethylated primers into a vector, respectively. The numbers of methylated and unmethylated molecules in a test sample were determined by comparing their amplification with those of standard samples containing 10 to 10^6 molecules. The "methylation index" was calculated as the fraction of methylated molecules in the total DNA molecules (no. methylated molecules + no. unmethylated molecules). Each sample was analyzed twice, blind to clinical information, and high reproducibility was confirmed (correlation coefficient = 0.98).

The *protocadherin* β (*PCDHB*) family consists of 16 genes with single exons and three pseudogenes on 5q31, and their CGIs are located in the gene bodies. MSP primers were designed to recognize 17 of the 19 members (all except for the *PCDHB1* gene and the *PCDHB19* pseudogene). The *protocadherin* α (*PCDHA*) family consists of 15 genes and one pseudogene having unique first exons and shared exons 2 to 4 on 5q31, and their CGIs are located in exon 1. MSP primers were designed to recognize 13 of the 16 members (all except for the *PCDHAC1* and *PCDHAC2* genes and the *PCDHA14* pseudogene). The *hepatocyte growth factor-like protein* (*HLP*/*MSP*/*MST1*) gene is highly homologous to *macrophage stimulating*,

pseudogene 9 (*MSTP9*), and MSP primers were designed to recognize both of these. For *DKFZp4511127*, *FLJ37440*, *Zinc finger protein 297* (*ZNF297*), and *Cytochrome p450 CYP26C1* (*CYP26C1*), MSP primers were designed to recognize each of them specifically. The primers and PCR conditions are shown in Supplementary Table 1.

Semiquantitative and Quantitative Reverse Transcription-PCR. cDNA was synthesized from 3 μ g of total RNA treated with DNase using a Superscript II kit (Invitrogen Co., Carlsbad, CA). For semiquantitative reverse transcription-PCR (*PCDHB1-PCDHB15*), multiple cycles of PCR were tested for each gene, and numbers giving a wide dynamic range were determined. The primers and PCR conditions are shown in Supplementary Table 2. For quantitative reverse transcription-PCR (*PCDHB16*), the number of cDNA molecules was determined by quantitative PCR, as in quantitative MSP, and the copy number was normalized to that of *GAPDH*.

Chromatin Immunoprecipitation Assay. From 1×10^6 cells, DNA/histone complexes were immunoprecipitated, and DNA was eluted in 30 μ L of TE after reversing cross-linking. Copy numbers of DNA molecules of the *PCDHB16* exon, *RASSF1A* promoter, and *GAPDH* promoter in 1 L of the eluate were determined by quantitative PCR (primer sequences in Supplementary Table 3), and normalized to the copy numbers in the input. Anti-acetyl-histone H3 antibody (AcH3) and anti-dimethylated-histone H3 (lysine 9; Meth3K9) were purchased from Cell Signalling (Beverly, MA).

Statistical Analysis. Associations between methylation levels among CGI groups were examined using the Pearson correlation coefficient and Fisher's exact test. Survival time was measured from the date of initial diagnosis to the date of death or last contact. Kaplan-Meier analysis and log-rank tests were done to compare survival between the groups defined by methylation levels. Hazard ratio (HR) between groups and dose-response relationships between methylation levels and survival were estimated by the Cox proportional hazard model. Kaplan-Meier curves were drawn with the help of Aabel software (Gigawiz. Ltd. Co., Tulsa, OK) and other analyses were conducted using SAS version 8.2 (SAS Institute, Inc., Cary, NC).

Results

Genome-Scanning for Differentially Methylated CpG Islands. MS-RDA was done using five primary neuroblastomas with a good prognosis and five neuroblastoma cell lines established from cases with a poor prognosis. Seven DNA fragments, derived from CGIs of *PCDHB16*, *PCDHAI*, *HLP*, *DKFZp4511127*, *FLJ37440*, *ZNF297*, and *CYP26C1*, were isolated as methylated in the latter samples. No DNA fragments were isolated as methylated in the former samples. Methylation statuses of (i) 17 CGIs of the *PCDHB* family (detailed structure in Supplementary Fig. 1), (ii) 13 CGIs of the *PCDHA* family, (iii) *HLP* and its pseudogene, and (iv) other four unique CGIs were examined by MSP. This revealed that the *PCDHB* family (5q31), the *PCDHA* family (5q31), *HLP* (3p21) and its pseudogene (1p36), *DKFZp4511127* (5q14), and *CYP26C1* (10q23) were specifically methylated in the latter samples (Fig. 1A and B).

Close Association between Methylation and Poor Prognosis in 140 Independent Primary Samples. To analyze the significance of the differential methylation of the above five CGI (groups) in primary neuroblastomas, 140 primary samples, all different from the initial five samples, were analyzed by quantitative MSP. When distributions of methylation indices were analyzed (Fig. 2), a clear bimodal distribution was observed for (i) the CGI group in the *PCDHB* family (17 CGIs), (ii) the CGIs of *HLP* and its pseudogene, and (iii) the *CYP26C1* CGI. The results thus indicated that the cases could be classified into two groups, one with high methylation and the other with low methylation. The dose-response relationships between high *PCDHB* methylation and poor prognosis were analyzed by the

Cox proportional model using the methylation index as a continuous value, and the association was confirmed with a trend $P < 0.0001$. Normal adrenal medulla had a methylation index of 4%.

According to the bimodal distribution, the effect of high methylation was assessed by dichotomous groups. For the *PCDHB* family, cutoff values of 30%, 40%, 50%, 60%, 70%, and 80% were tested, and HRs of 16.8 [95% confidence interval (95% CI), 4.0-70.9], 22.1 (95% CI, 5.3-93.4; Fig. 3), 13.1 (95% CI, 4.5-37.9), 9.1 (95% CI, 3.8-23.4), 7.0 (95% CI, 3.1-15.8), and 7.8 (95% CI, 3.4-17.6), respectively, were obtained ($P < 0.001$ for all cutoff values). This showed that cases can be classified into two groups with distinct prognoses, and we adopted a cutoff value of 40%, which gave the highest HR, for convenience in the following analysis.

The dose-response relationships were also confirmed for other four CGI (groups), *PCDHA* ($P = 0.004$), *HLP* ($P < 0.0001$), *DKFZp4511127* ($P = 0.02$), and *CYP26C1* ($P < 0.0001$). Cutoff values were similarly tested, and those for *PCDHA*, *HLP*, *DKFZp4511127*, and *CYP26C1* were set at 80%, 10%, 20%, and 70%, respectively, with HRs of 5.7 (95%CI, 1.4-24.0; $P = 0.07$), 21.7 (95% CI, 5.1-91.4; $P < 0.0001$), 3.2 (95% CI, 1.0-10.5; $P = 0.045$), and 8.7 (95% CI, 4.1-18.1; $P < 0.0001$), respectively (Fig. 3).

Existence of the CpG Island Methylator Phenotype in Neuroblastomas. Methylation of the different CGI (groups) had shown close associations with each other (Table 1). When correlation was analyzed as a continuous value, Pearson correlation coefficients between *PCDHB* and *PCDHA*, *HLP*, *DKFZp4511127* and *CYP26C1* were 0.55, 0.70, 0.26 and 0.77, respectively. This showed that multiple CGIs were simultaneously methylated in

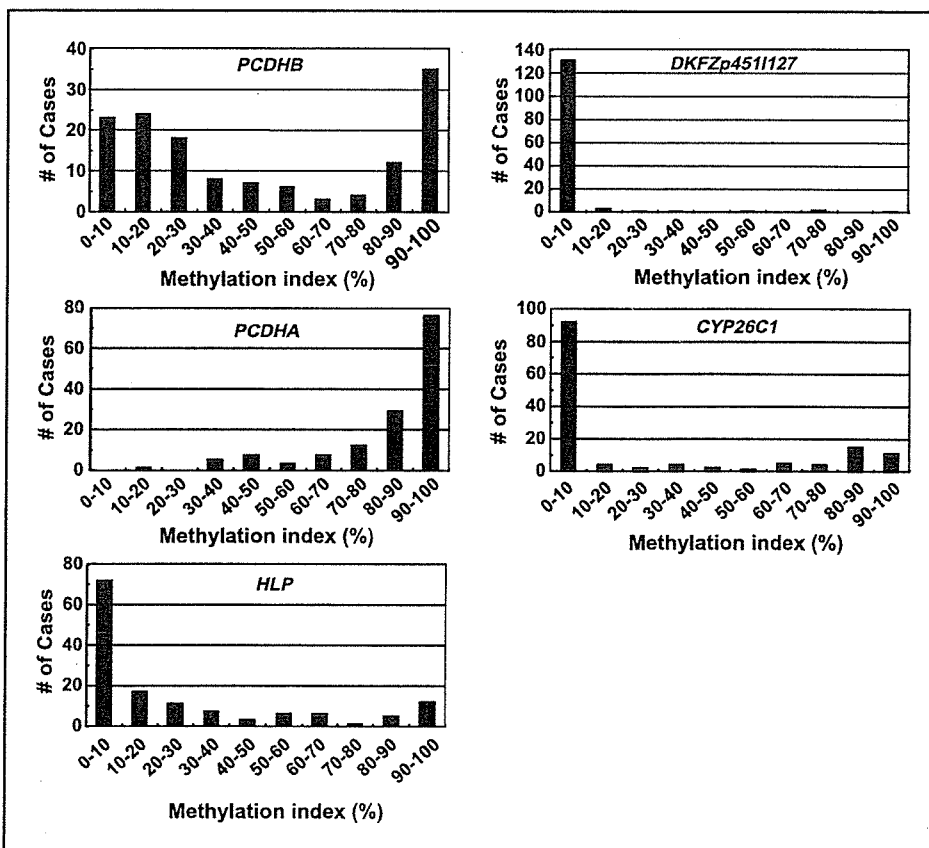


Figure 2. The distribution of methylation indices among the 140 cases analyzed: (i) 17 CGIs of the *PCDHB* family, (ii) 13 CGIs of the *PCDHA* family, (iii) CGIs of *HLP* and its pseudogene, (iv) *DKFZp4511127*, and (v) *CYP26C1*.

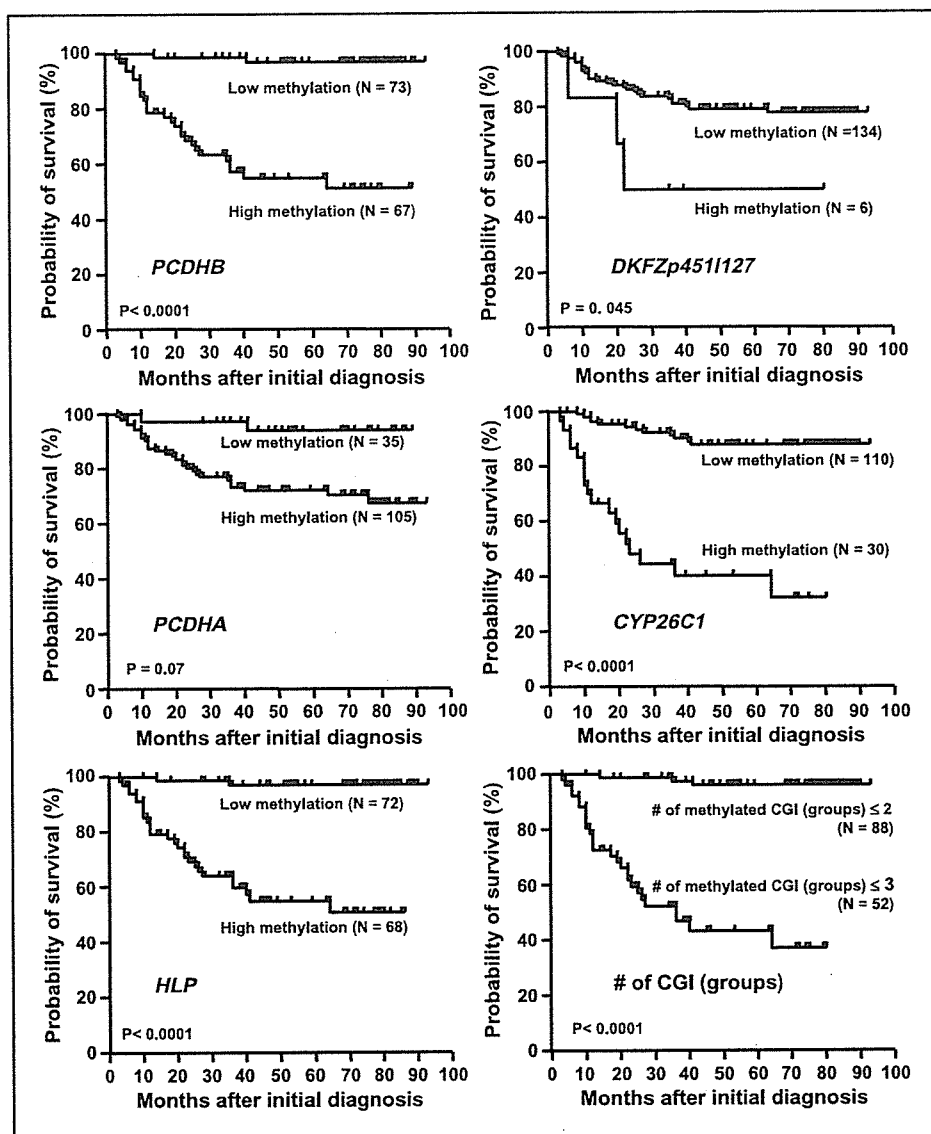


Figure 3. Predictive powers of methylation of the five CGI (groups) identified, and their multiple methylation: (i) 17 CGIs of the *PCDHB* family, (ii) 13 CGIs of the *PCDHA* family, (iii) CGIs of *HLP* and its pseudogene, (iv) *DKFZp4511127*, (v) *CYP26C1*, and (vi) methylation of three of these or more were analyzed by the Kaplan-Meier method using 140 primary samples. The *PCDHB* family, *HLP*, *DKFZp4511127*, *CYP26C1*, and methylation of multiple CGI (groups) had significant influence on survival.

neuroblastomas with a poor prognosis (Supplementary Fig. 2A). The simultaneous methylation of (i) 17 CGIs of the *PCDHB* family, (ii) 13 CGIs of the *PCDHA* family, (iii) CGIs of *HLP* and its pseudogene, (iv) *DKFZp4511127* CGI, and (v) *CYP26C1* CGI conformed with the concept of the CpG island methylator phenotype (CIMP; ref. 16).

Associations between CIMP and poor prognosis were examined by defining CIMP as cases with methylation of two CGI (groups) or more, those with three or more, those with four or five, and those with five. When CIMP was defined as cases with methylation of three CGI (groups) or more, the largest association with poor prognosis was observed, with a HR of 25.4 (95% CI, 7.6-84.5; Fig. 3). However, the HR (22.1) given by 17 CGIs of the *PCDHB* gene family approximated to this, and the *PCDHB* methylation level closely correlated with the number of methylated CGI (groups; Supplementary Fig. 2B). Therefore, for simplicity of analysis, we defined CIMP in neuroblastomas on the basis of high methylation of the *PCDHB* family, tentatively with a cutoff value of 40%.

Predictive Power of CIMP, Compared with Known Prognostic Factors. Univariate analyses showed that *N-myc* amplification, low *TrkA* expression, DNA diploidy, and an age no younger than 1 year gave HRs of 9.5 (95% CI, 4.4-20.5), 3.9 (95% CI, 1.7-9.3), 4.2 (95% CI, 1.65-10.8), and 12.3 (95% CI, 3.7-41.7). Cases were stratified by these known factors (Table 2). In those without *N-myc* amplification, CIMP also showed an influence with a HR of 12.4 (95% CI, 2.6-58.9), but almost all cases with *N-myc* amplification (37 of the 38 cases) showed CIMP. It was suggested that cases with *N-myc* amplification were contained in the cases with CIMP. CIMP was independent from *TrkA* overexpression, DNA ploidy, and age at diagnosis. Stage seemed to be a stronger prognostic factor. Notably, even when limited to cases in stages III and IV without *N-myc* amplification, which are classified into the intermediate risk group and clinically important, CIMP gave a HR of 4.8 (95% CI, 1.0-23.0; $P = 0.048$).

Multivariate analyses were finally done taking all the five known prognostic factors into account. Although CIMP gave a HR of 5.0 (95% CI, 0.47-52.7), it was not significant ($P = 0.18$), possibly due to limitation in the number of cases.

Table 1. Association between the *PCDHB* methylation and methylation of other CGIs

Variables	Methylation level of <i>PCDHB</i> family gene		P*
	High (≥40%)	Low (<40%)	
No. cases (n = 140)	67	73	
Methylation of CGIs outside promoter regions (n = 140)			
<i>PCDHA</i> gene family (exon 1) [†]	65/67	41/73	<0.0001
<i>HLP</i> (exons 2-13) [‡]	52/67	16/73	<0.0001
<i>CYP26C1</i> (exon 2) [§]	30/67	0/73	<0.0001
<i>p41Arc</i> (intron 8)	1/67	1/73	0.48
<i>SIM2</i> (exon 2)	0/67	0/73	
Methylation of CGIs in promoter regions (n = 140)			
<i>DKFZp4511127</i>	6/67	0/73	0.011
<i>RASSF1A</i>	51/67	10/73	<0.0001
<i>BLU</i>	25/67	3/73	<0.0001
<i>p16</i>	0/67	0/73	
<i>hMLH1</i>	0/67	0/73	
<i>PCDHB1</i>	0/67	0/73	
<i>TAF7</i>	0/67	0/73	
<i>p41Arc</i>	0/67	0/73	
<i>SIM2</i>	0/67	0/73	

*Fisher's exact test.

[†]Boundaries for high methylation and low methylation of *PCDHA* gene family were set at 80% of the methylation index.[‡]Boundaries for high methylation and low methylation of *HLP* were set at 10% of the methylation index.[§]Boundaries for high methylation and low methylation of *CYP26C1* were set at 70% of the methylation index.^{||}Boundaries for high methylation and low methylation of *DKFZ-p4511127* were set at 20% of the methylation index.**Effects of *PCDHB* Methylation on Gene Expression and Chromatin Structure.**

The CGIs of the *PCDHB* family were located in their gene bodies, whose methylation generally does not block gene transcription (17). The actual effects of methylation on expression were examined for 16 genes of the *PCDHB* family using 10 primary neuroblastomas with low methylation and five primary neuroblastomas with high methyl-

ation. The methylation was not associated with loss of expression (a representative result is shown in Fig. 4A). The effect of methylation of the *PCDHB16* CGI on the histone modification was further examined by chromatin immunoprecipitation assay. It was found that DNA methylation of the *PCDHB16* CGI did not induce histone H3 lysine 9 methylation or histone H3 deacetylation (data not shown).

Association between CIMP and Promoter Methylation. High methylation of *PCDHB* CGIs, a sensitive surrogate marker of CIMP in neuroblastomas, did not repress gene expression or induce histone modification. This indicated that CIMP is involved in the poor prognosis of neuroblastomas by causing methylation of promoter CGIs, although it is known that promoter CGIs are resistant to *de novo* methylation (18, 19).

Among the five CGI (groups) identified in this study, only that of *DKFZp4511127* was located in a promoter region. Although its methylation was infrequent, the methylation was observed only in neuroblastomas with CIMP (Table 1), and was associated with expression loss (Fig. 4B). To make the association clearer, methylation statuses were analyzed for eight additional CGIs in promoter regions. It was shown that methylation of promoter CGIs of *RASSF1A* (3p21) and *BLU* (3p21) was far more frequently observed in neuroblastomas with CIMP (Table 1, $P < 0.0001$). At the same time, there was a preference for CGIs affected by CIMP among CGIs in promoter regions, and also among those outside promoter regions (Table 2).

Discussion

Extensive methylation of multiple CGIs, conforming with the concept of CIMP, was here found specifically present in neuroblastomas with a poor prognosis and could be sensitively detected by focusing on the *PCDHB* family. *PCDHB* methylation did not suppress gene expression or induce histone modification. However, CIMP was associated with promoter methylation of *RASSF1A* and *BLU* genes and one of the mechanisms underlying the poor prognosis of neuroblastomas seemed to be silencing of these and possibly other tumor suppressor genes and genes important for differentiation.

CIMP was originally identified in colon cancers (16), but there has been some dispute over its presence (20). The clear correlation between CIMP and a poor prognosis found here for neuroblastomas was unequivocal and presumably reflects an intrinsic tendency for methylation of CGIs. This is because, first, neuroblastomas have a much shorter history than colon cancers, and the accumulated number of methylated CGIs in neuroblastomas is expected to parallel the speed of occurrence of

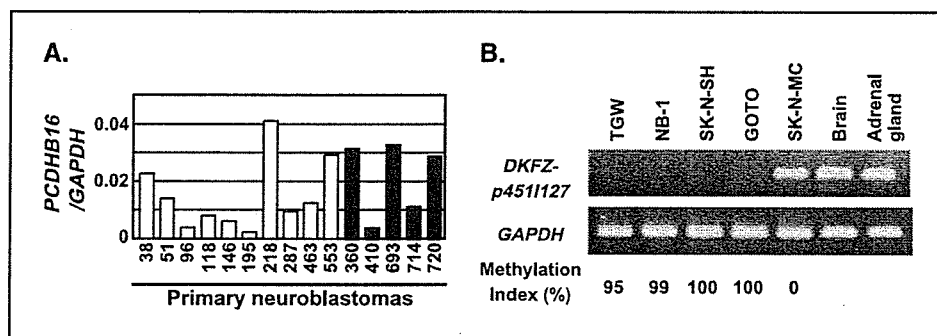


Figure 4. Effects of methylation of the *PCDHB* family and *DKFZp4511127* on gene expression. **A**, *PCDHB16* expression was analyzed by quantitative RT-PCR in 10 primary samples with low methylation (*open columns*) and five primary samples with high methylation (*closed columns*), and no difference was observed between the two groups. **B**, silencing of *DKFZp4511127* by methylation of its promoter CGI. The CGI was methylated in four cell lines, TGW, NB-1, SK-N-SH, and GOTO, whereas it was unmethylated in one cell line, SK-N-MC. *DKFZp4511127* was expressed in SK-N-MC, but not expressed at all in the four cell lines with the promoter methylation.

Table 2. HRs of death by *PCDHB* methylation status in subgroup of known prognostic factors

Stratified by		<i>PCDHB</i> methylation	No. cases	No. deaths	HR* (95% CI)	P [†]
Overall (n = 140)		High	67	1	22.1 (5.3-93.4)	< 0.0001
		Low	73	2	1	
N- <i>myc</i> amplification (n = 140)	No	High	30	8	12.4 (2.6-58.9)	0.002
		Low	72	2	1	
	Yes	High	37	20	NE	—
		Low	1	0		
<i>TrkA</i> overexpression (n = 130)	Yes	High	20	6	18.3 (2.2-152.6)	0.007
		Low	49	1	1	
	No	High	40	19	NE	
		Low	21	0		
DNA ploidy (n = 125)	Aneuploid	High	17	5	18.3 (2.1-156.7)	0.008
		Low	49	1	1	
	Diploid	High	38	17	NE	
		Low	21	0		
Clinical stages (n = 140)	Stages I, II, and IVS	High	8	0	NE	—
		Low	52	0		
	Stages III and IV	High	59	28	7.4 (1.8-31.3)	0.006
		Low	21	2	1	
Age at diagnosis (n = 140)	<1	High	11	3	NE	
		Low	59	0		
	≥1	High	56	25	4.5 (1.1-18.9)	0.043
		Low	14	2	1	

*HR of death for a case with high *PCDHB* methylation compared with a case with low methylation. NE shows not estimable due to no events in at least one category.

†Significance level for a high *PCDHB* methylation to low methylation using Cox proportional model.

methylation. Second, methylation of the *PCDHB* family did not affect gene expression, and there should have been no selection of cells with the *PCDHB* methylation, in contrast to the case of promoter methylation of tumor suppressor genes. Investigation into the mechanism of the intrinsic tendency for methylation of multiple CGIs is necessary. Furthermore, alleviation of the intrinsic tendency could block progression of neuroblastomas and have potential therapeutic value.

Among the six CGI (groups) outside promoter regions analyzed here, CIMP in neuroblastomas preferentially affected four CGI (groups); those of the *PCDHB* family, the *PCDHA* family, *HLP*, and *CYP26C1*. Unexpectedly, three CGIs that are known to be frequently methylated in human colon cancers with CIMP, *MINT1*, *MINT2*, and *MINT17* (16) were not methylated in neuroblastoma cell lines (data not shown). Among the nine CGIs in promoter regions analyzed, CIMP in neuroblastomas affected only three, those of *RASSF1A*, *BLU*, and *DKFZp4511127*. The nine CGIs were selected based upon previous reports as tumor suppressor genes (*RASSF1A*, *BLU*, *p16*, and *hMLH1*; refs. 21-23), the chromosomal location flanking the *PCDHB* family (*PCDHB1*

and *TAF7*), our previous report on the fidelity in inheriting methylation patterns (*p41Arc* and *SLM2*; ref. 19), and the findings here (*DKFZp4511127*). Because gene expression and possibly chromatin structures affect the frequency of *de novo* methylation (24, 25), the available data suggest that CGIs useful to sensitively detect CIMP might vary according to the tumor type.

The influence of CIMP on prognosis was here found to be comparable to that of the currently most reliable marker, *N-myc* amplification, and stronger than *TrkA* overexpression and DNA ploidy on univariate analysis. Subgroup analysis showed that the influence was independent of *TrkA* overexpression, DNA ploidy and age at diagnosis and CIMP had influence even in cases without *N-myc* amplification and in advanced stages. These points strongly indicated CIMP to be a promising new prognostic marker. However, the cutoff values adopted here are tentative, and the HRs obtained could have been overestimated. A validation study using independent samples is necessary for further evaluation. The fact that cases with CIMP contained almost all the cases with *N-myc* amplification suggested that a common molecular mechanism caused both alterations, or that CIMP may lead to *N-myc*

amplification. Whatever the case, the findings might provide clues to molecular mechanisms of neuroblastoma development.

In summary, the present study showed that CIMP is present specifically in neuroblastomas with poor prognosis and that can be sensitively detected by focusing on *PCDHB* methylation. CIMP seems to be a promising new prognostic marker, and its evaluation and investigations into the mechanisms underlying CIMP in neuroblastomas seem warranted.

Acknowledgments

Received 7/27/2004; revised 11/14/2004; accepted 11/24/2004.

Grant support: Grant-in-aid for the Third-term Cancer Control Strategy Program from the Ministry of Health, Labour, and Welfare, Japan and Research Resident Fellowship from the Foundation for Promotion of Cancer Research (M. Abe).

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We thank Drs. E. Okochi-Takada and G. S. Goldberg for critical reading of the article and the institutions for participation in the collection of clinical materials.

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LMO3 Interacts with Neuronal Transcription Factor, HEN2, and Acts as an Oncogene in Neuroblastoma

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Abstract

LIM-only proteins (LMO), which consist of LMO1, LMO2, LMO3, and LMO4, are involved in cell fate determination and differentiation during embryonic development. Accumulating evidence suggests that LMO1 and LMO2 act as oncogenic proteins in T-cell acute lymphoblastic leukemia, whereas LMO4 has recently been implicated in the genesis of breast cancer. However, little is known about the role of LMO3 in either tumorigenesis or development. In the present study, we have identified LMO3 and HEN2, which encodes a neuronal basic helix-loop-helix protein, as genes whose expression levels were higher in unfavorable neuroblastomas compared with those of favorable tumors. Immunoprecipitation and immunostaining experiments showed that LMO3 was associated with HEN2 in mammalian cell nucleus. Human neuroblastoma SH-SY5Y cells stably overexpressing LMO3 showed a marked increase in cell growth, a promotion of colony formation in soft agar medium, and a rapid tumor growth in nude mice compared with the control transfectants. More importantly, the increased expression of LMO3 and HEN2 was significantly associated with a poor prognosis in 87 primary neuroblastomas. These results suggest that the deregulated expression of neuronal-specific LMO3 and HEN2 contributes to the genesis and progression of human neuroblastoma in a lineage-specific manner. (Cancer Res 2005; 65(11): 4587-97)

Introduction

The LIM domain-containing proteins are important regulators in determining cell fate and controlling cell growth and differentiation during embryonic development (1). The LIM domain is a highly conserved cysteine-rich zinc finger-like motif found in a variety of nuclear and cytoplasmic proteins and acts as a docking site for the assembly of multiprotein complexes (2-4). However, the precise role of the LIM domain is still unclear. Several distinct subgroups of the LIM domain-containing proteins are defined and some of them also possess a functionally divergent domain, including a DNA-binding homeodomain or a protein kinase domain (1, 2).

The LIM-only proteins (LMO) are one of the families of the LIM domain-containing proteins and possess only two tandem LIM domains. They consist of four members, including LMO1, LMO2, LMO3, and LMO4 (2, 4). *LMO1* and *LMO2* have been identified as the genes that are activated in human acute T-cell leukemia (T-cell ALL) by tumor-specific chromosomal trans-

locations (4). Transgenic mice overexpressing LMO1 or LMO2 developed immature and aggressive T-cell leukemia, suggesting that these proteins act as T-cell oncoproteins (5-7). On the other hand, LMO4 has been identified as a nuclear protein that interacts with the adaptor protein Ldb1 (8). It has been shown recently that LMO4 is highly expressed in primary human breast cancers, and overexpression of LMO4 inhibits differentiation of mammary epithelial cells, suggesting that deregulated expression of LMO4 contributes to the breast carcinogenesis (9). LMO4 has also been reported to be associated with BRCA1 to repress its transcriptional activity (10). Thus, LMO1, LMO2, and LMO4 have been implicated in tumorigenesis. However, to date, little is known about the oncogenic function of LMO3, which has been discovered based on sequence homology with LMO1 (11).

The nuclear LMO proteins, which lack intrinsic DNA-binding activity, have been considered to be involved in transcriptional regulation (2), raising a possibility that they alter the transcription of target genes by forming a complex with other transcription factors with DNA-binding activity. Indeed, in T-cell acute lymphoblastic leukemia in children, a basic helix-loop-helix transcription factor, TAL1, is physically associated with LMO1 or LMO2 and enhances their oncogenic activities (12, 13). Interestingly, the neuronal-specific basic helix-loop-helix transcription factors, HEN1 and HEN2, were identified based on cross-hybridization with TAL1 (14, 15). Their expression was restricted to the developing nervous system and a human neuroblastoma cell line. However, the role of HEN1 and HEN2 in tumorigenesis has long been elusive.

Neuroblastoma is one of the most common childhood cancers and is originated from sympathoadrenal lineage of the neural crest (16). It is clinically and cytogenetically divided into two major subgroups with favorable and unfavorable prognosis (17). The recent molecular and cellular analyses have revealed that amplification of *MYCN* and *DDX1* as well as loss of heterozygosity at the region of chromosome 1p36 are strongly associated with a poor outcome, whereas high levels of expression of the neurotrophin receptors *TrkA*, *CD44*, and *Fyn*, are well correlated with favorable prognosis (16-23). However, we still do not know many other genes that play important roles in the genesis and progression of neuroblastoma. To identify the other genes closely involved in neuroblastoma, we have constructed several cDNA libraries from different subsets of neuroblastoma and randomly cloned 4,200 genes (24). Screening of the genes differentially expressed between favorable and unfavorable subsets of the tumor has identified *Nbla3267* as one of the genes expressed at higher levels in unfavorable than favorable neuroblastomas (25).

In the present study, we found that *Nbla3267* encoded the human LMO, LMO3, and that high expression of *LMO3* as well as *HEN2* was strongly associated with a poor prognosis of neuroblastoma. Furthermore, LMO3 interacted with HEN2 in mammalian

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cell nucleus, and enforced expression of LMO3 in human neuroblastoma-derived cell line SH-SY5Y markedly enhanced tumor growth in nude mice, supporting the oncogenic role of LMO3 in neuroblastoma.

Materials and Methods

Patient population. The RNA samples obtained from 87 patients with neuroblastoma were subjected to semiquantitative and quantitative real-time reverse transcription-PCR (RT-PCR) analyses. All patients were diagnosed clinically as well as pathologically and tested for DNA ploidy, MYCN amplification, and TrkA expression. Tumors were staged according to the International Neuroblastoma Staging System criteria (26). Thirty-four patients were stage I, 14 were stage II, 8 were stage III, 26 were stage IV, and 5 were stage IVS. Stages I, II, and IVS were considered as favorable and stages III and IV as unfavorable. The patients were treated following the protocols proposed by the Japanese Infantile Neuroblastoma Cooperative Study (27) and the Study Group of Japan for Treatment of Advanced Neuroblastoma (28). The clinical follow-up ranged from 4 to 58 months, with a median of 36 months. We have a precise list of patient characteristics, including age, stage, and clinical follow-up time, and this list will be provided upon request.

Cloning of human LMO3, HEN1, and HEN2. To obtain a complete human LMO3 cDNA, a cDNA library derived from human fetal brain (Stratagene, La Jolla, CA) was screened with a ³²P-labeled *Nbla3267* cDNA. Plaques showing positive signals were picked up and rescreened twice. To construct the expression plasmid for hemagglutinin (HA)-tagged LMO3-A, the cDNA fragment encoding the entire LMO3-A protein was amplified by PCR from the phage clone as a template using the primers designed to add a synthetic linker encoding the HA epitope on the NH₂-terminal side of LMO3-A (forward 5'-GGTACCATGGCTTACCCATACGATGTTCCA-GATTACGCTAGCCTCTCAGTCCAGCCAGACAC-3' and reverse 5'-TCAGATATCATTAGATCAGCGAACCTGGG-3'). The PCR product was digested with *KpnI* and *EcoRV* and subcloned into the identical restriction sites of pcDNA3 expression plasmid to give pcDNA3-HA-LMO3-A. cDNA encoding human HEN1 (amino acid residues 1-133) or HEN2 (amino acid residues 1-135) was generated by reverse transcribing total RNA isolated from neuroblastoma cell line, IMR32, using a forward primer (5'-AAGGAATTCATGCTCAACTCAGACACCATG-3') and a reverse primer (5'-ATAAGAATGCGGCCGCTCAGACGT-3') for HEN1 and a forward primer (5'-AAGGAATTCATGCTGAGTCCGGACCAAGCA-3') and a reverse primer (5'-ATAAGAATGCGGCCGCTACACGTCAGGACGTGGTT-3') for HEN2. The amplified PCR products were digested with *EcoRI* and *NotI* and subcloned into the identical restriction sites of pcDNA3-FLAG expression plasmid to give pcDNA-FLAG-HEN1 and pcDNA3-FLAG-HEN2.

Generation of a polyclonal anti-LMO3 antibody. The polyclonal anti-LMO3 and anti-HEN2 antibodies were raised against a peptide "Cys" plus containing the amino acid sequence between positions 127 and 145 of LMO3 and the amino acid sequence between positions 1 and 19 of HEN2, respectively. The peptides and the polyclonal antibodies were produced by Biologica Co. (Nagoya, Japan).

Cell culture and transfection. Human neuroblastoma (SK-N-AS, SH-SY5Y, NB69, OAN, SK-N-BE, NGR, NLF, IMR32, NB1, and KP-N-NS), ALL (RPMI, KOPT, HSB, and MOLT), osteosarcoma (OST, SAOS-2, and U2OS), rhabdomyosarcoma (RMS-MK), colon cancer (COLO-320), breast cancer (MCF-7 and MDA-MB-453), melanoma (G361, G32TG, and A875), thyroid cancer (TTC11), small cell lung carcinoma (H1299), and cervical cancer (HeLa) cell lines and COS7 cells were maintained in RPMI 1640 or DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in an atmosphere of 5% CO₂ in the air. For transient transfection, COS7 cells were transfected with the indicated expression plasmids using FuGene 6 transfection reagent as recommended by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). Stable transfections of SH-SY5Y cells were done with the empty plasmid (pcDNA3, Invitrogen, Carlsbad, CA) or with the expression plasmid for FLAG-tagged LMO3-A using LipofectAMINE Plus transfection reagent according to the manufacturer's

instructions (Invitrogen). The transfected cells were cultured in the presence of G418 at a final concentration of 400 µg/mL (Sigma Chemical Co., St. Louis, MO). Thereafter, the selection medium was replaced every 3 days. Three weeks after the selection in G418, drug-resistant clones were isolated and allowed to proliferate in medium containing G418.

Reverse transcription-PCR analysis. Total RNA was prepared from cultured cells and human tissues by using Trizol reagent (Life Technologies, Grand Island, NY) or the RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcription was carried out using random primers and SuperScript II (Invitrogen). Following the reverse transcription, the resultant cDNA was subjected to PCR-based amplification. Oligonucleotides used to amplify LMO3-A, LMO3-B, LMO1, LMO2, LMO4, *Ldb1*, *Ldb2*, *TALI*, *HEN1*, *HEN2*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs were as follows: LMO3-A: forward 5'-ACTGTGCTTACTGAACGGCCCT-3' and reverse 5'-CCGGTCCTTGATCTTTCGGTTG-3'; LMO3-B: forward 5'-TGCAACTCAGACACGCTAAG-3' and reverse 5'-CCGGTCCTTGATCTTTCGGTTG-3'; LMO1: forward 5'-GCTCCACCCTTACACCAAG-3' and reverse 5'-CTGCCCTTCCTCATAGTCCA-3'; LMO2: forward 5'-AATGCGGGTGAAAGACAAAG-3' and reverse 5'-CCCCAAGTGCCTAAGAGTG-3'; LMO4: forward 5'-GCAAGGCAATGTGTATCATCT-3' and reverse 5'-GCATTCTGCAT-TACTCTGACC-3'; *Ldb1*: forward 5'-CCAGGAGCAGAGACAGAA-3' and reverse 5'-AGAGGCCAGGTTCCAAG-3'; *Ldb2*: forward 5'-TAGCC-CAAGTGTGAAACAA-3' and reverse 5'-TAAACTGCCACAAACCAA-3'; *TALI*: forward 5'-GTTCTTAGGCTGTGGGATG-3' and reverse 5'-GATTTGG-GACTGAGGGAAGA-3'; *HEN1*: forward 5'-AGAGACTGAGTCGGGCTTCA-3' and reverse 5'-CAGGCGCAGAACTCAATCT-3'; *HEN2*: forward 5'-CCCCAAGGGTTGTGGTTT-3' and reverse 5'-TCTGAACTTCTGCCCT-CATTCTT-3'; and *GAPDH*: forward 5'-ACCTGACCTGCCGTCTAGAA-3' and reverse 5'-TCCACCACCTGTGTCTGTA-3'. Amplified products were electrophoretically separated on agarose gels and visualized by ethidium bromide staining. The gels were photographed under UV illumination.

Northern analysis. A human MTN blot (Clontech, Palo Alto, CA), a nylon membrane on which poly(A)⁺ RNAs extracted from various human normal tissues were blotted, was used for analysis of the distribution of LMO3 expression in human normal tissues. ³²P-labeled probe was prepared by random priming of the 2.5-kb restriction fragment of LMO3 cDNA. The membrane was hybridized overnight at 65°C in a solution containing 7.5% dextran sulfate, 1 mol/L NaCl, 1% *N*-lauroyl sarcosine, 100 µg/mL heat-denatured salmon sperm DNA, and the radiolabeled probe. The membrane was washed twice in 0.5 × SSC/0.1% *N*-lauroyl sarcosine at 50°C. Specific signals were obtained by autoradiography.

Section in situ hybridization. Section *in situ* hybridization was done as described previously (29). A riboprobe was synthesized with digoxigenin-UTP and T3 or T7 polymerase (Roche Molecular Biochemicals). The alkaline phosphatase reaction was done with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals). The riboprobe used for the section *in situ* hybridization were transcripts of the human cDNA fragments of the LMO3 gene.

Immunohistochemistry. Neuroblastoma tissues were stained with immunoperoxidase method using anti-HEN2 antibody. They included unfavorable neuroblastomas with MYCN gene amplification and favorable neuroblastomas with a single copy of MYCN gene. Neuroblastoma specimens were fixed in 10% buffered formalin and embedded in paraffin, and 3 µm sections were applied to the immunostaining. Before incubation with anti-HEN2 antibody, the sections were treated with 0.05% Pronase in 0.05 mol/L Tris-HCl (pH 7.6) for 5 minutes. The sections were incubated with anti-HEN2 antibody, which was diluted to 1:200 at 4°C overnight. The biotin-streptavidin method (Nichirei, Tokyo, Japan) was done, and the sections were visualized with diaminobenzidine solution. The nuclei were counterstained with hematoxylin.

Immunofluorescent staining. COS7 cells were doubly transfected with the expression plasmids for HA-LMO3-A and FLAG-HEN2. Forty-eight hours after transfection, cells were fixed for 30 minutes with 3.7% formaldehyde in PBS and permeabilized with 0.2% Triton X-100 for 5 minutes, and nonspecific epitopes were blocked for 1 hour in PBS containing 3% bovine serum albumin. The cells were then incubated with a polyclonal anti-HA antibody (1:200 dilution, Medical and Biological Laboratories, Nagoya,

Japan) and a monoclonal anti-FLAG antibody (1:50, M2, Sigma Chemical). After three washes with PBS, cells were stained with a FITC- or a rhodamine-conjugated secondary antibody (1:200, Invitrogen). The coverslips were mounted onto glass slides, and the stained cells were viewed using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Western blot analysis and immunoprecipitation. After transfection, cells were rinsed twice with ice-cold PBS and then lysed immediately with SDS sample buffer. Equal amounts of proteins were separated under denaturing conditions by electrophoresis in 15% polyacrylamide gel containing SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). After blocking in a solution containing 5% skim milk, the membrane was incubated with a monoclonal anti-FLAG, a polyclonal anti-HA, a polyclonal anti-LMO3, or a polyclonal anti-actin antibody (20-33, Sigma Chemical) and then incubated with a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Protein bands were visualized with an enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). For immunoprecipitation, transfected cells were lysed in EBC buffer [50 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride] containing protease inhibitor mixture (Sigma Chemical). The precleared soluble supernatants were mixed with a polyclonal anti-HA or a monoclonal anti-FLAG antibody and incubated for 2 hours at 4°C. Protein A-Sepharose

beads were then added to the reaction mixtures and incubated for 1 hour at 4°C. The immune complexes were washed with the lysis buffer thrice at 4°C. The bound proteins were resuspended in SDS sample buffer, resolved by SDS-PAGE, and analyzed by Western blotting.

Cell proliferation and soft agar assay. Cells were seeded in triplicate in 24-well plates (5×10^3 per well) in culture medium containing 10% or 1% FBS. Cells were allowed to adhere to the bottom of the cell culture dish for 24 hours. At the indicated times, cells were trypsinized and cell counting was carried out using a Coulter Counter (Coulter Electronics Ltd., Hialeah, Finland). For soft agar assay, 2.5×10^3 cells of the stable transfectants or the parental SH-SY5Y cells were seeded in triplicate in 35-mm cell culture plates containing 0.2% agar and RPMI 1640 supplemented with 10% FBS. After 21 days, colonies with diameters $>300 \mu\text{m}$ were scored as positive.

Tumor formation in nude mice. For tumor formation, 6-week-old female athymic *nu/nu* mice (Charles River Laboratory, Sulzfeld, Germany) were injected into the femur with 5×10^6 parental SH-SY5Y cells or SH-SY5Y cells transfected with the empty plasmid or with the expression plasmid encoding LMO3-A suspended in 100 μL PBS. Tumor size and body weight were measured once weekly and mice were sacrificed 7 weeks after injection. For histologic examinations, tumor tissues were fixed in fresh 10% buffered formalin and embedded in paraffin. The handling of animals was in accordance with the guidelines of the Chiba Cancer Center Research Institute (Chiba, Japan).

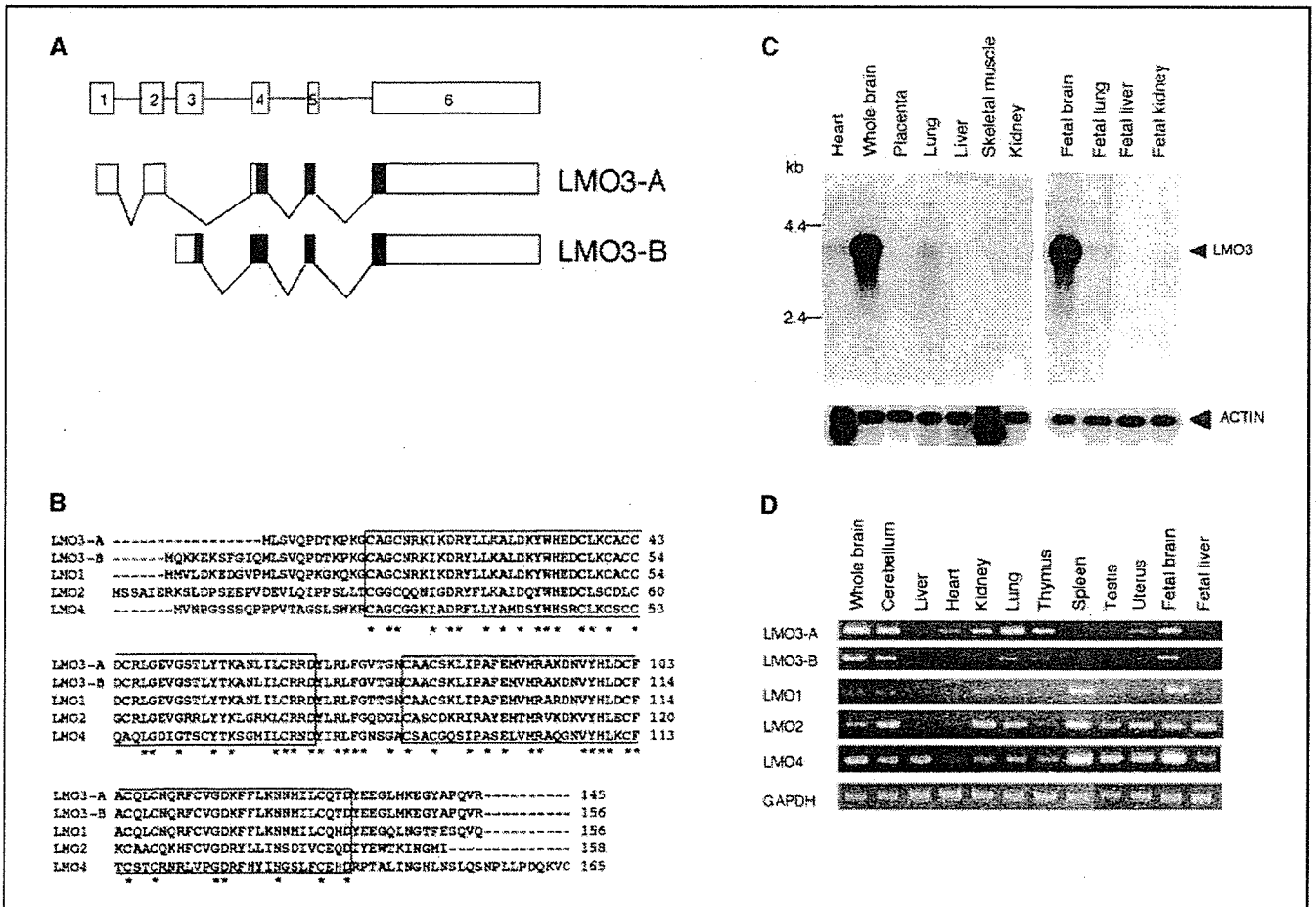


Figure 1. Identification of human LMO3-A and LMO3-B and their relation to the other LMO family members. *A*, schematic representation of the exons of human *LMO3* gene. *Solid* and *open boxes*, coding and untranslated regions, respectively. *B*, deduced amino acid sequences of human LMO3-A and LMO3-B and their alignments with those of human LMO1, LMO2, and LMO4. *Asterisks*, identical amino acid residues. Two LIM domains are *boxed*. *C*, tissue-specific expression of *LMO3*. Human multiple tissue Northern blots containing poly(A)⁺ RNA were hybridized with a radiolabeled human *LMO3* cDNA (*top*) or with a radioactive probe derived from human β -actin cDNA (*bottom*). β -actin was used as a control for equal loading. The 2-kb band was hybridized ubiquitously, and an additional 1.8-kb band was hybridized in heart and skeletal muscle with the β -actin probe. *D*, coordinated expression of *LMO3-A* and *LMO3-B* in various human tissues. Total RNA isolated from the indicated human tissues was subjected to RT-PCR analysis to examine the expression levels of *LMO3-A*, *LMO3-B*, *LMO1*, *LMO2*, and *LMO4*. *GAPDH* expression is shown as an internal control.

Quantitative real-time PCR. Total RNA prepared from primary neuroblastomas was reverse transcribed into cDNA (SuperScript II kit) and subjected to the real-time PCR. The expression level of *GAPDH* was measured in all samples to normalize *LMO3* and *HEN2* expression according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Oligonucleotide primers and TaqMan probes, which were labeled at the 5' end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3' end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA), were as follows: *LMO3*: forward 5'-TCTGAGGCTCTT-TGGTGTAAACG-3', reverse 5'-CCAGGTGGTAAACATGTCCTTG-3', and probe 5'-FAM-AAACTGCGCTGCCTGTAGTAAGCTCATCC-TAMRA-3' and *HEN2*: forward 5'-CCCAAGGGTTGTGTTT-3', reverse 5'-TCTGAACCTCTGCCTCATCTTT-3', and probe 5'-FAM-TTGAATTCCTCC-TACATTCATCCGCCACAA-TAMRA-3'. Amplification and detection were done using the ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Statistical analysis. Student's *t* tests were used to explore possible associations between *LMO3* expression and other factors. Because the values of the *LMO3* expression were skewed, a log transformation was used to achieve the normality in the analyses using *t* test and Cox regression. The distinction between high and low levels of *LMO3* expression was based on the median value (low, *LMO3* < 0.2493 e.u.; high, *LMO3* > 0.2493 e.u.) regardless of tumor stage, *MYCN* copy number, or survival. The distinction between high and low levels of *HEN2* expression was based on the distribution of the values (low, undetectable; high, detectable). χ^2 tests were

used to examine possible associations between *HEN2* expression and other factors, such as tumor stage. Kaplan-Meier survival curves were calculated, and survival distributions were compared using the log-rank test. Cox regression models were used to explore associations among *LMO3* expression, *HEN2* expression, age, *MYCN* amplification, mass screening, origin, and survival. Statistical significance was declared if *P* < 0.05. The statistical analysis was done using Stata Statistical Software Release 7.0 (Stata Corp., College Station, TX, 2001).

Results

Identification of the human *LMO3* gene. To identify the genes specifically involved in the genesis and progression of neuroblastoma, we have previously constructed cDNA libraries from the primary neuroblastomas and screened for the differentially expressed genes between the tumors with good and poor clinical outcome (25). One of the cDNA clones, *Nbla3267*, significantly overexpressed in the poor prognostic neuroblastomas contained a partial nucleotide sequence encoding a LMO family protein, LMO3. To obtain the missing 5' part of the *LMO3* cDNA, we screened a cDNA library derived from human fetal brain. From ~6 × 10⁵ recombinant phage clones, 10 independent phage clones were isolated. Sequence analysis

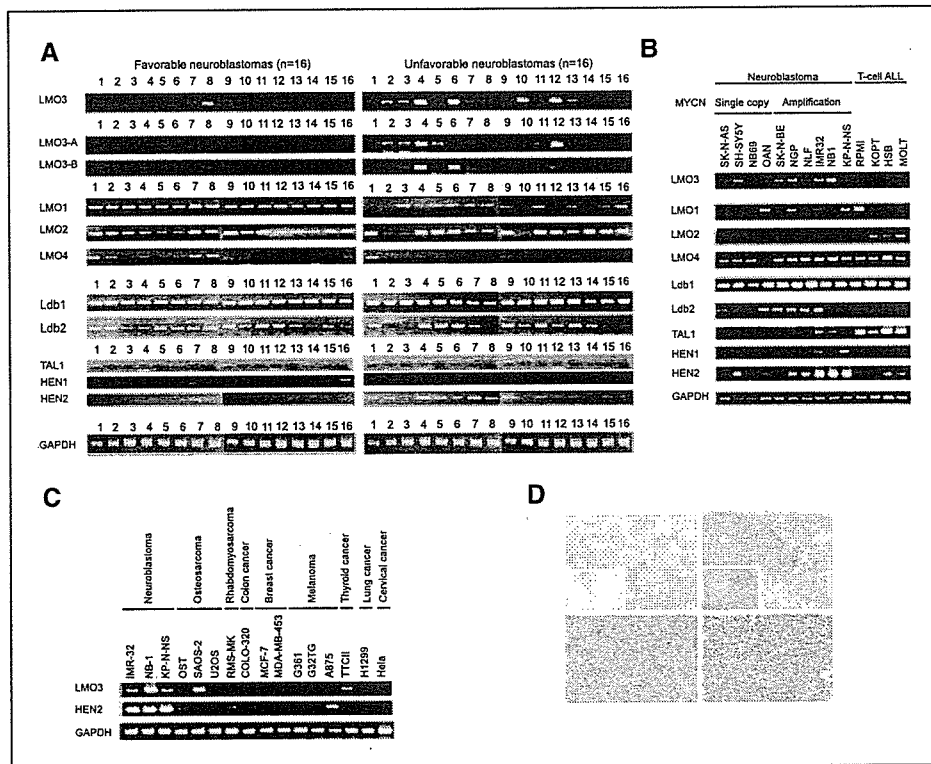


Figure 2. Increased expression of *LMO3* and *HEN2* in unfavorable neuroblastomas and neuroblastoma-derived cell lines. **A**, expression of *LMO3* and *LMO*-related genes in primary neuroblastomas with favorable (stage I, a single copy of *MYCN* and high expression of *TrkA*) and unfavorable (stages III and IV, *MYCN* amplification and decreased expression of *TrkA*) characteristics. Total RNA was isolated from the indicated neuroblastoma tissues, reverse transcribed, and amplified by PCR to examine the expression levels of *LMO3*, *LMO3-A*, *LMO3-B*, *LMO1*, *LMO2*, *LMO4*, *Ldb1*, *Ldb2*, *TAL1*, *HEN1*, and *HEN2*. Expression of *GAPDH* serves as an internal control. PCR products were visualized by ethidium bromide staining. **B**, expression of *LMO3* and *LMO*-related genes in neuroblastoma cell lines without *MYCN* amplification (SK-N-AS, SH-SY5Y, NB69, and OAN), neuroblastoma cell lines with *MYCN* amplification (SK-N-BE, NGP, NLF, IMR32, NB1, and KP-N-NS), and ALL cell lines (RPMI, KOPT, HSB, and MOLT). Total RNA prepared from the indicated cultured cells was subjected to RT-PCR analysis. Expression of *GAPDH* serves as an internal control. **C**, expression of *LMO3* and *HEN2* in various tumor-derived cell lines. Total RNA prepared from the indicated culture cells was subjected to RT-PCR analysis as described above. **D**, section *in situ* hybridization of neuroblastoma with the *LMO3* probe. Serial sections of the favorable neuroblastoma tissue (*top left* and *inset*) or the unfavorable one with *MYCN* amplification (*top right* and *inset*) were prepared, and expression of the *LMO3* gene was examined by section *in situ* hybridization. The *LMO3* transcripts are positive in unfavorable neuroblastoma. Immunohistochemical staining of *HEN2* in primary neuroblastoma tissues. *HEN2* is strongly positive in the nucleus of most tumor cells with *MYCN* amplification (*bottom right*), whereas it is negative in the favorable neuroblastoma tissue (*bottom left*).

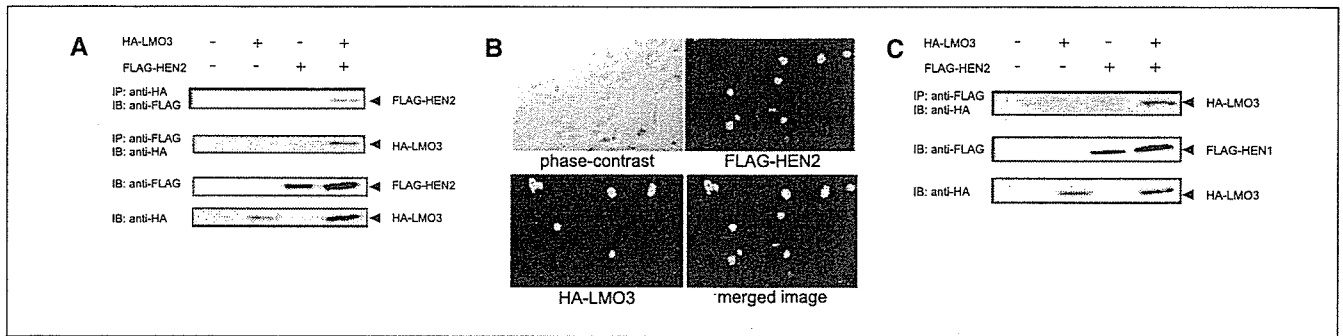
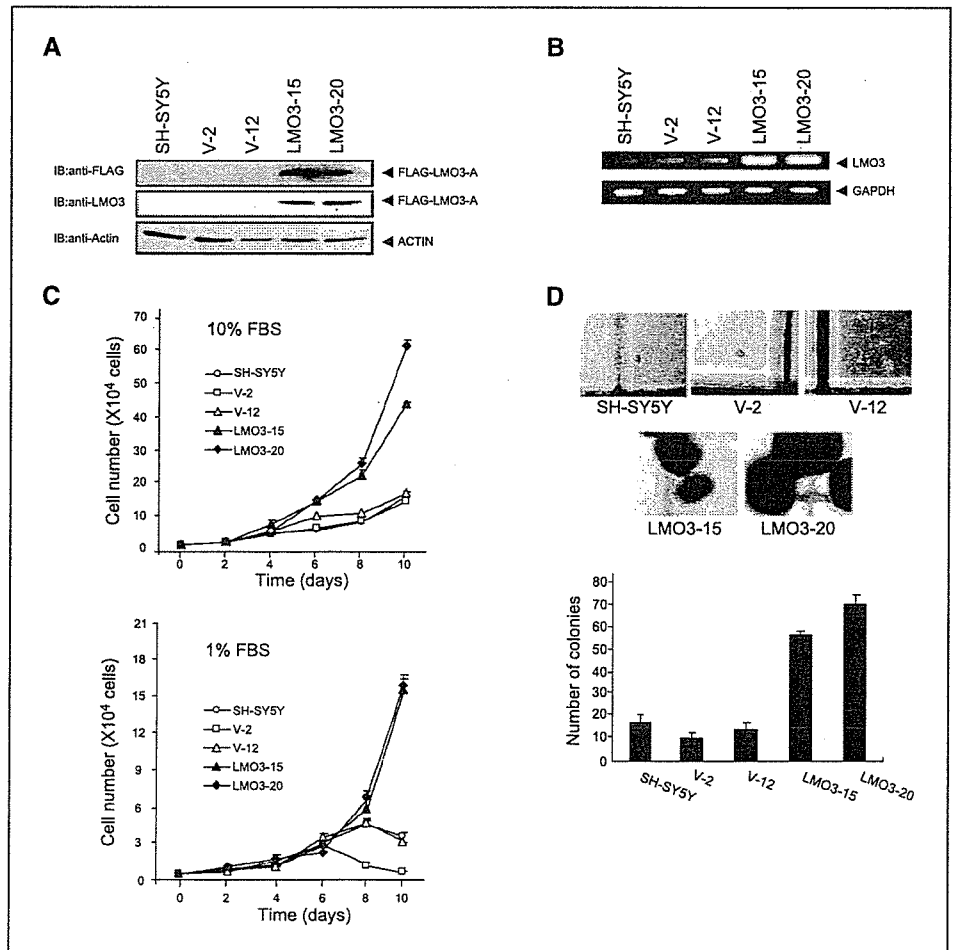


Figure 3. LMO3 interacts with HEN2 in mammalian cells. *A*, coimmunoprecipitation analysis. COS7 cells were transfected with the indicated expression plasmids. Forty-eight hours after transfection, whole cell lysates were prepared and subjected to the immunoprecipitation/Western analysis (*top* and *top middle*). Whole cell lysates were monitored on immunoblot for the expression of FLAG-HEN2 (*bottom middle*) and HA-LMO3-A (*bottom*). *B*, nuclear colocalization of LMO3 and HEN2 in cultured cells. COS7 cells were cotransfected with the expression plasmids for HA-LMO3-A and FLAG-HEN2. Forty-eight hours after transfection, cells were fixed and incubated with the polyclonal anti-HA and monoclonal anti-FLAG antibodies. Cells were then processed for double immunofluorescence using the FITC-conjugated anti-rabbit IgG (*green*) and with the rhodamine-conjugated anti-mouse IgG (*red*). The merged images (*yellow*) suggest the nuclear colocalization of LMO3 and HEN2. The phase-contrast images are also shown. *C*, coimmunoprecipitation of FLAG-HEN1 and HA-LMO3. Whole cell lysates prepared from COS7 cells transfected with the indicated combinations of the expression plasmids were immunoprecipitated with the anti-FLAG antibody followed by immunoblotting with the anti-HA antibody (*top*). Levels of FLAG-HEN1 and HA-LMO3 were also examined by immunoblotting with the anti-FLAG antibody (*middle*) and with the anti-HA antibody (*bottom*), respectively.

revealed that they were divided into two types, designated LMO3-A (145 amino acids) and LMO3-B (156 amino acids), with the different translation initiation sites. The NH₂-terminal region of LMO3-A was identical to that of the previously reported

LMO3 protein (11). As shown in Fig. 1A, the putative translation initiation sites of LMO3-A and LMO3-B were located within exons 4 and 3, respectively. Because *LMO3* is a single gene, it is likely that LMO3-A and LMO3-B arise from differential splicing

Figure 4. Growth-promoting activity of LMO3 in SH-SY5Y cells. *A*, stable SH-SY5Y transfectants expressing exogenous FLAG-LMO3-A. SH-SY5Y cells were stably transfected with the empty plasmid or with the expression plasmid for FLAG-LMO3-A and maintained in the presence of G418 (at a final concentration of 400 μ g/mL) for 3 weeks. Whole cell lysates prepared from the indicated drug-resistant cell clones in addition to the parental SH-SY5Y cells were subjected to Western blot analysis using the anti-FLAG (*top*), anti-LMO3 (*middle*), or anti-actin (*bottom*) antibody. *B*, RT-PCR analysis of LMO3 in the indicated stable transfectants along with the parental SH-SY5Y cells. Expression of GAPDH serves as an internal control. *C*, effects of LMO3 overexpression on cell growth in SH-SY5Y cells. SH-SY5Y cells and the indicated transfectants were grown in the culture medium containing 10% (*top*) or 1% (*bottom*) FBS. Cells were harvested at 48-hour time intervals and number of cells was counted in triplicate. Points, means from three independent experiments; bars, SE. *D*, anchorage-independent growth of LMO3-overexpressing transfectants. The parental SH-SY5Y cells and the indicated transfectants (2.5×10^3 cells per dish) were grown in soft agar medium. After 3 weeks of culture, cells were examined by phase-contrast microscopy (*top*), and the numbers of colonies with a diameter of $>300 \mu$ m were counted (*bottom*). Columns, means from three independent experiments; bars, SE.



or alternative promoter usage. Amino acid sequence alignment of LMO3 with the other LMO family proteins (LMO1, LMO2, and LMO4) showed a significant homology among them (Fig. 1B). LIM domains of LMO3 presented 98%, 60%, and 55% amino acid homology with those of LMO1, LMO2, and LMO4, respectively.

To determine the expression pattern of human *LMO3* mRNA, we did Northern blot analysis on a human multiple tissues blot using β -actin as a control. As shown in Fig. 1C, *LMO3* mRNA (~4 kb) was abundantly expressed in brain and at relatively low levels in the heart and lung but not in the other tissues examined. Similar to the adult tissues, *LMO3* mRNA was expressed predominantly in fetal brain, with a lower level in fetal lung. We then compared the tissue distribution of *LMO3-A* expression with those of *LMO3-B* and the other *LMO* family gene expression in various human adult and fetal tissues by RT-PCR (Fig. 1D). The expression pattern of *LMO3-A* was similar to that of *LMO3-B*, with relatively higher levels in brain, cerebellum, and fetal brain. In contrast, *LMO2* and *LMO4* were expressed ubiquitously in human tissues, and *LMO1* was expressed at higher levels in spleen and fetal brain.

Expression of *LMO3* and *HEN2* in aggressive neuroblastomas. As described previously, LMO family protein interacts with the nuclear LIM domain-binding protein 1 and 2 (Ldb1 and Ldb2), which act as adaptors for several LIM domain-containing proteins (30–32), and also binds to the basic helix-loop-helix transcription factor, TAL1, to regulate its transcriptional activity (12, 33, 34). Of interest, HEN1 and HEN2 were previously identified based on their homology with TAL1, and it was shown that LMO3 was associated with HEN1 (35). Furthermore, TAL1 was coexpressed with LMO1 or LMO2 in T-cell ALL (36), and double transgenic mice overexpressing TAL1 and LMO1 or LMO2 developed leukemia (37). As shown in Fig. 2A, *LMO3* (A and B) and *HEN2* were expressed at higher levels in unfavorable neuroblastomas compared with favorable tumors, whereas the levels of *LMO1* expression were predominantly high in the favorable tumors. No significant changes in the expression levels of *LMO2*, *Ldb1*, and *Ldb2* were detected between unfavorable and favorable neuroblastomas. *LMO4*, *TAL1*, and *HEN1* showed extremely low levels of expression in both types of neuroblastoma. We then studied the expression of these genes in 10 neuroblastoma and 4 T-cell ALL cell lines to examine the presence or absence of the lineage specificity, neuronal or hematopoietic. Consistent with the previous reports (36), *LMO2* and *TAL1* were coexpressed in T-cell ALL-derived cell lines (RPMI, KOPT, HSB, and MOLT; Fig. 2B). However, of interest, *LMO3* and *Ldb2* were expressed predominantly in neuroblastoma cell lines compared with the leukemia-derived lines. In addition, *HEN2* tended to be less highly expressed in leukemia cells compared with neuroblastoma cells. *HEN1* expression was also restricted to neuroblastoma but limited to only a few cell lines. On the other hand, there was no difference in the expression of *LMO4* and *Ldb1* between neuroblastoma-derived and T-cell ALL-derived cell lines. Interestingly, coexpression of *LMO3* and *HEN2* was observed in the majority of neuroblastoma cell lines but not in the other tumor-derived cell lines with different origin (Fig. 2C). These results revealed that only *LMO3* and *HEN2* were expressed at high levels in aggressive neuroblastomas in a neuronal-specific pattern.

Figure 2D shows the results of *in situ* hybridization for *LMO3* in primary neuroblastomas. *LMO3* mRNA was expressed in a

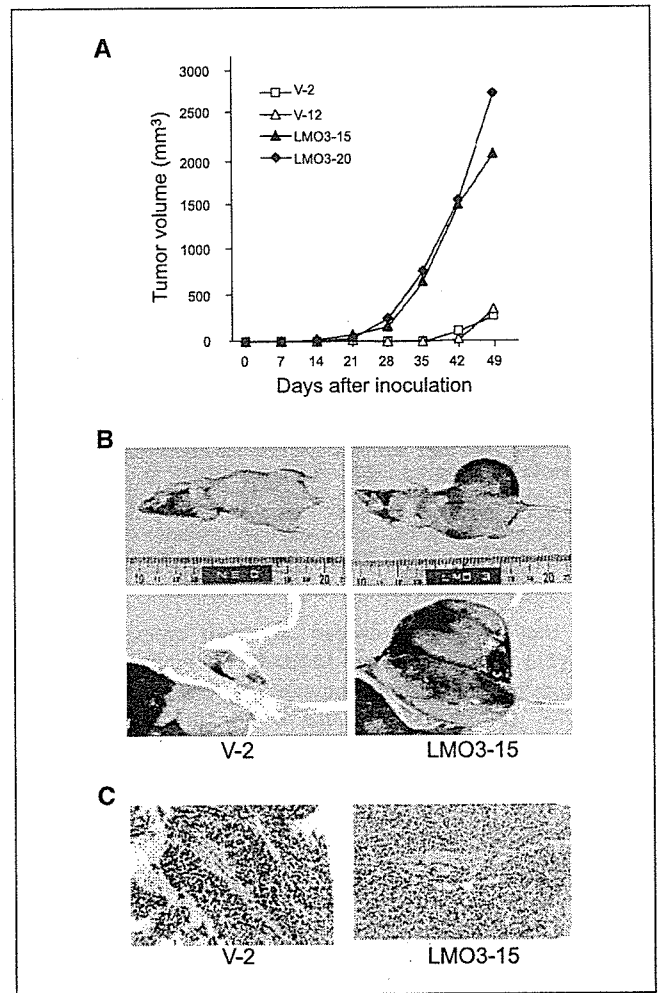


Figure 5. Tumor growth in nude mice. A, nude mice were injected s.c. with 5×10^6 of SH-SY5Y cells or the indicated stable transfectants and tumor volumes were estimated weekly. Points, mean of 8 to 11 independent tumors. B, photographs of the tumors 49 days after s.c. injection of V-2 (left) and LMO3-15 cells (right) into nude mice. C, paraffin sections of the tumors arising from V-2 (left) and LMO3-15 cells (right) were stained with H&E.

stage IV neuroblastoma with *MYCN* amplification, whereas it was negative in a stage I tumor with a single copy of *MYCN* and high expression of *TrkA*. Unfortunately, our antibody raised against human LMO3 protein did not work for the immunohistochemical analysis. The immunostaining of HEN2 was also strongly positive in the nuclei of most tumor cells in *MYCN*-amplified neuroblastoma, albeit it was negative in favorable subset of the tumor (Fig. 2D).

LMO3 physically interacts with HEN2. Because LMO3 and HEN2 were coexpressed in the majority of unfavorable neuroblastomas as well as neuroblastoma cell lines, we examined whether LMO3 could interact with HEN2 in mammalian cells. Whole cell lysates prepared from COS7 cells transfected with the expression plasmids for HA-tagged LMO3 and FLAG-tagged HEN2 were immunoprecipitated with the anti-HA or with the anti-FLAG antibody followed by immunoblotting with the anti-FLAG or with the anti-HA antibody, respectively. As shown in Fig. 3A, FLAG-HEN2 was coimmunoprecipitated with HA-LMO3. We then examined the subcellular distribution of LMO3 and

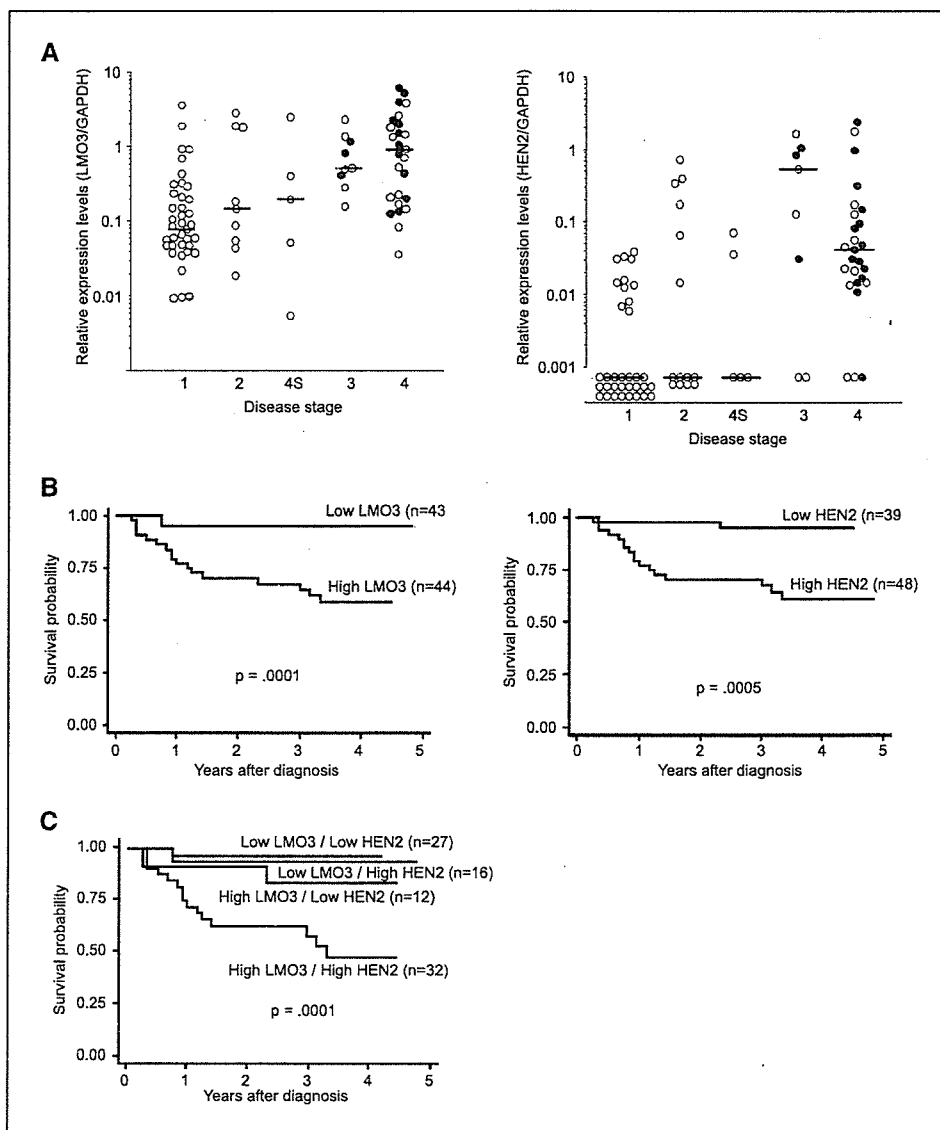
HEN2. COS7 cells were cotransfected with the expression plasmids for HA-LMO3 and FLAG-HEN2 and double stained with anti-HA and anti-FLAG antibodies. As shown in Fig. 3B, LMO3 as well as HEN2 appear exclusively nuclear. On closer inspection by merging two images, these two proteins colocalized in the nucleus. Consistent with the previous reports (35), HA-LMO3 was coimmunoprecipitated with FLAG-HEN1 under our experimental conditions (Fig. 3C).

Overexpression of LMO3 accelerates growth of SH-SY5Y neuroblastoma cells. We addressed the question whether LMO3 could induce cell growth of neuroblastoma. To this end, we transfected the expression plasmid for FLAG-LMO3-A or the empty plasmid into SH-SY5Y neuroblastoma cells and established two stable transfectants overexpressing FLAG-LMO3-A (named as LMO3-15 and LMO3-20). As shown in Fig. 4A, the expression levels of FLAG-LMO3-A were higher in LMO3-15 and LMO3-20 cells than in the parental SH-SY5Y and the control transfectants (V-2 and V-12). LMO3-15 expressed FLAG-LMO3-A at the level comparable with that in LMO3-20. Similar results were also obtained by RT-PCR analysis (Fig. 4B). No obvious morphologic

changes could be observed in LMO3-15 and LMO3-20 cells (data not shown). As shown in Fig. 4C, LMO3-15 and LMO3-20 cells proliferated at a much faster rate than the control transfectants and SH-SY5Y cells in culture medium containing 10% serum. More importantly, LMO3-15 and LMO3-20 cells continued to grow exponentially even in the low serum culture medium, whereas the growth of the vector-transfected cells as well as SH-SY5Y cells was significantly suppressed under this condition.

To examine whether the LMO3-A-overexpressing cells have an ability to grow in soft agar medium, each transfectants were cultured in soft agar medium for 3 weeks. The numbers of colonies with diameters >300 μm formed by each transfectants in soft agar were scored. LMO3-15 and LMO3-20 cells formed large distinct colonies and showed a statistically significant increase in the number of colonies compared with the vector-transfected cells and SH-SY5Y cells (Fig. 4D). These results strongly suggest that overexpression of LMO3 is sufficient to induce malignant transformation in neuroblastoma cells. We also tried to obtain the cells stably transfected with HEN2 but never been successful with unknown reason.

Figure 6. Expression of *LMO3* and *HEN2* mRNA in 87 primary neuroblastomas. *A*, expression levels of *LMO3* (left) and *HEN2* (right) transcripts in 87 primary neuroblastoma samples categorized by the patient's clinical stage were examined by a quantitative real-time RT-PCR. Relative expression levels of *LMO3* or *HEN2* mRNA were determined by calculating the ratio between *GAPDH* and *LMO3* or *HEN2*. Bars, median levels of *LMO3* or *HEN2* expression in each stage; open and closed circles, samples from patients who are alive and dead, respectively. *B* and *C*, Kaplan-Meier survival curves of patients with neuroblastomas based on high or low expression of *LMO3*, *HEN2* (*B*), or *LMO3* and *HEN2* (*C*).



LMO3 induces marked tumor growth in nude mice. SH-SY5Y cells with a single copy of *MYCN* form tumors in nude mice, although the growth rate is slow compared with that of the other neuroblastoma cell lines with *MYCN* amplification (38). To examine whether overexpression of LMO3 in SH-SY5Y cells could affect the tumor growth *in vivo*, we injected the each transfectants into the left flank of athymic nude mice, and the tumor volumes were measured weekly. V-2 and V-12 cells slowly formed tumors with similar kinetics and of similar sizes 35 to 42 days after injection (Fig. 5A). In contrast, the tumors grew rapidly in nude mice implanted with LMO3-15 or LMO3-20 cells. The sizes of the excised tumors from the LMO3-15-implanted mice on day 49 were >10-fold larger than those of control mice (Fig. 5B) and showed histologically undifferentiated neuroblastoma with small round cell shapes and small amounts of stromal components (Fig. 5C).

Expression of LMO3 and HEN2 is associated with a poor outcome of neuroblastoma. To verify whether a significant relationship could be observed between the expression of *LMO3* and/or *HEN2* in primary neuroblastomas and the patients' survival, we quantitatively measured the expression levels of *LMO3* and *HEN2* mRNA in 87 primary tumors by using a quantitative real-time RT-PCR. The values of the levels of *LMO3* and *HEN2* expression were normalized to that of *GAPDH* expression [relative expression values (REV)]. The high level of *LMO3* expression was significantly associated with high expression of *HEN2* (Student's *t* tests, mean \pm SE: 1.43 \pm 0.27 REV, *n* = 48 versus 0.54 \pm 0.17 REV, *n* = 39; *P* = 0.001), older age (\geq 1-year-old: 1.37 \pm 0.29, *n* = 32 versus <1-year-old: 0.84 \pm 0.21, *n* = 55; *P* = 0.008), advanced disease stages (stages III + IV: 1.83 \pm 0.35, *n* = 34 versus stages I + II + IVS: 0.52 \pm 0.14; *P* < 0.00005; Fig. 6A), low levels of *TrkA* expression (low *TrkA*: 1.63 \pm 0.34, *n* = 37 versus high *TrkA*: 0.59 \pm 0.15, *n* = 50; *P* = 0.0003), *MYCN* amplification (amplification: 1.91 \pm 0.44, *n* = 27 versus single copy: 0.64 \pm 0.13, *n* = 60; *P* = 0.0002), and sporadic cases of

Table 2. Multiple Cox regression models using LMO3 expression and dichotomous factors of HEN2 expression, age, MYCN amplification, mass screening, and origin (*n* = 87)

Model	Factor	P	Hazard ratio (95% confidence interval)
A	LMO3 expression (high vs low)	0.005	1.61 (1.16-2.23)
	HEN2 expression (high vs low)	0.029	5.32 (1.19-23.9)
B	LMO3 expression (high vs low)	0.005	1.62 (1.15-2.28)
	Age (>1 vs <1 y)	0.002	5.79 (1.86-18.1)
C	LMO3 expression (high vs low)	0.066	1.36 (0.98-1.89)
	MYCN amplification (1 copy vs >1 copy)	<0.0005	0.075 (0.02-282)
D	LMO3 expression (high vs low)	0.044	1.42 (1.01-2.01)
	Mass screening (+ vs -)	0.005	0.051 (0.007-0.404)
E	LMO3 expression (high vs low)	<0.0005	1.78 (1.31-2.41)
	Origin (adrenal gland vs others)	0.21	2.02 (0.666-6.12)

NOTE: All variables with two categories, except *LMO3* expression (log). Hazard ratio shows the relative risk of death of first category relative to the second.

Table 1. Simple Cox regression models using LMO3 expression and dichotomous factors of HEN2 expression, age, MYCN amplification, mass screening, and origin (*n* = 87)

Model	Factor	P	Hazard ratio (95% confidence interval)
A	LMO3 expression (high vs low)	<0.0005	1.80 (1.32-2.47)
B	HEN2 expression (high vs low)	0.004	8.69 (2.00-37.7)
C	Age (\geq 1 vs <1 y)	<0.0005	8.75 (2.87-26.7)
D	MYCN amplification (1 copy vs >1 copy)	<0.0005	0.049 (0.014-0.171)
E	Mass screening (+ vs -)	0.001	0.032 (0.004-0.237)
F	Origin (adrenal gland vs others)	0.20	2.06 (0.684-6.23)

NOTE: All variables with two categories, except *LMO3* expression (log). Hazard ratio shows the relative risk of death of first category relative to the second. Because all patients with advanced tumor stages and low expression of *TrkA* had died of the tumor, a Cox regression model with the tumor stage or *TrkA* expression was not fitted.

neuroblastoma (sporadic: 1.68 \pm 0.32, *n* = 39 versus mass screening: 0.51 \pm 0.14, *n* = 48; *P* < 0.00005). The high level of *HEN2* expression was also significantly correlated with high expression of *LMO3* (χ^2 tests: *P* = 0.001), older age (*P* < 0.0005), advanced stages (*P* < 0.0005; Fig. 6B), low *TrkA* expression (*P* < 0.0005), *MYCN* amplification (*P* < 0.0005), and sporadic cases of neuroblastoma (*P* < 0.0005). Thus, high expression of *LMO3* and *HEN2* was well associated with conventional markers indicating the poor prognosis of neuroblastoma.

We next tested if expression levels of *LMO3* and *HEN2* could have prognostic significance in primary neuroblastomas. The results for log-rank tests showed that high expression of *LMO3* or *HEN2* was significantly associated with poor survival (*P* = 0.0002 and 0.0005, respectively; Fig. 6C and D). Remarkably, the combination of high expression of both *LMO3* and *HEN2* showed the significantly worse prognosis compared with the other combinations of *LMO3* and *HEN2* expression levels as shown in Fig. 6E. As expected, older patients and the patients with advanced tumors, low expression of *TrkA*, amplified *MYCN*, and the tumors found by mass screening were associated with short time to survival (*P* < 0.00005). However, the adrenal origin of the tumor was not associated with the outcome (*P* = 0.19; data not shown).

The univariate analysis suggested that *LMO3* expression (*P* < 0.0005), *HEN2* expression (*P* = 0.004), age (*P* < 0.0005), *MYCN* amplification (*P* < 0.0005), and mass screening (*P* = 0.001) were of prognostic importance, supporting the results of the log-rank test (Table 1). Furthermore, the multivariate analysis showed that

LMO3 expression was significantly associated with survival after controlling HEN2 expression ($P = 0.005$), age ($P = 0.005$), mass screening ($P = 0.044$), and origin ($P < 0.0005$), suggesting that LMO3 expression was an independent prognostic factor from the other factors (Table 2). LMO3 expression was marginally associated with survival after controlling MYCN amplification ($P = 0.066$). On the other hand, because HEN2 expression was highly associated with age, MYCN amplification, and mass screening, it was not significantly associated with survival after controlling age, MYCN amplification, and mass screening in the corresponding multiple Cox regression models (data not shown).

Discussion

In the present study, we have identified that both LMO3 and HEN2 are expressed at higher levels in aggressive neuroblastomas especially with MYCN amplification than those with favorable prognosis. Coexpression of LMO3 and HEN2 has been observed almost exclusively in neuroblastoma cell lines, not the other lines, suggesting that their expression and function are neuronal specific. Furthermore, LMO3 physically interacted with HEN2 in mammalian cells. The functional significance of LMO3 expression was shown by a stable transfection into SH-SY5Y neuroblastoma cells, colony formation in soft agar, and tumor growth in nude mice, all of which have suggested that LMO3, probably by interacting with endogenous HEN2, markedly promotes the tumor growth. Indeed, the tumors with high expression of both LMO3 and HEN2 have shown the worst prognosis in the analysis of 87 primary neuroblastomas. Thus, our results suggested that, in concert with HEN2, the neuronal specifically expressed LMO3 plays an important role in the tumorigenesis of neuroblastoma. Our observation is strikingly intriguing because that LMO1 or LMO2 is already known to be the oncogene in T-cell acute lymphoblastic leukemia and that LMO4 has recently been implicated in the genesis of breast cancer (4, 9).

We have identified a *Nbla3267/LMO3* clone from the screening of differentially expressed genes between favorable and unfavorable subsets of neuroblastoma. LMO3 was one of the genes expressed at higher levels in the latter than the former (24), like MYCN oncogene and DDX1, a DEAD box gene coamplified with MYCN in aggressive neuroblastomas. In the development of hematopoietic system, LMO1 and LMO2 form a transcriptional complex with Ldb1, a LIM domain-binding protein, and a basic helix-loop-helix protein TAL1, which was identified as an oncogene at the translocation breakpoint in T-cell ALL (4-7). From the analogy with the LMO1 or LMO2 transcriptional machinery in T-cell ALL, we searched for the similar complex in the neuronal system by using the different subsets of primary neuroblastoma and the cell lines in comparison with the T-cell ALL cell lines. As a result, the neuronal-specific pattern of expression was observed in LMO3, Ldb2, HEN1, and HEN2, among which LMO3 and HEN2 were significantly highly expressed in the unfavorable subset of neuroblastomas with MYCN amplification compared with the favorable subset. This result strongly suggested that LMO3 may function in collaboration with HEN2 in advanced stages of neuroblastoma. Indeed, both genes were coexpressed only in neuroblastoma derived-cell lines, not in other tumor-derived ones, suggesting that their expression is lineage specific. Furthermore, LMO3 and HEN2

physically interacted in mammalian cells, albeit with weak interaction between LMO3 and HEN1 (35). Thus, these results also suggest that LMO3 and HEN2 form a neuronal cassette mimicking the hematopoietic complex composed of LMO2 and TAL1 and regulate the growth of neuroblastoma.

The neuronal-specific basic helix-loop-helix transcription factors, HEN1 and HEN2, were originally identified from the cDNA library of a neuroblastoma cell line based on cross-hybridization with TAL1 (14, 15). Their expression was restricted to the developing nervous system and a neuroblastoma cell line. However, their function has long been unclear. Recently, Bao et al. have reported that HEN1 interacts with LMO proteins by yeast two-hybrid screen and that *Xenopus* HEN1, in concert with XLMO3, is a critical regulator of neurogenesis (35). This prompted us to test our hypothesis both *in vitro* and *in vivo*. As the results, we found that the SH-SY5Y neuroblastoma cells stably overexpressing LMO3, presumably by acting with endogenous HEN2, gained rapid cell growth in the culture medium with 10% or 1% serum, in the soft agar medium, and in nude mice. These suggested that LMO3 is a neuronal-specific oncogene in neuroblastoma, without any rearrangement of the LMO3 gene (data not shown). However, we failed to establish a stable SH-SY5Y cell line transfected with HEN2. It is presumed that overexpression of HEN2 might have caused cell death or growth arrest in the cells, albeit the reason is elusive.

The double transgenic mice overexpressing LMO2 and TAL1 displayed a more rapid development of leukemia compared with those overexpressing LMO2 alone, suggesting that LMO2 and TAL1 act synergistically through their complex formation in the development of leukemia (13). Of note, Ono et al. reported that LMO2 and TAL1 act as cofactors for GATA3 to induce the expression of the *retinaldehyde dehydrogenase 2* gene in T-cell ALL (39). On the other hand, a stable complex comprising LMO2, TAL1, and GATA1 was required to promote erythroid differentiation (32). Therefore, LMO3 and HEN2 may also form a nuclear complex, including family members of GATA to regulate cell growth and differentiation in neuroblastoma. Our preliminary data have suggested that GATA2, GATA3, GATA4, and GATA6 are highly expressed in neuroblastoma cell lines, among which GATA4 and GATA6 are predominantly coexpressed in neuroblastoma cell lines compared with T-cell ALL lines. Thus, LMO3 and HEN2, in collaboration with GATA and Ldb families, may play a role in determining cell fate in both neural development and neuroblastoma genesis, although this hypothesis needs to be elucidated. Recently, it has been shown that LMO3 enhanced the ability of HEN1 through the physical interaction to transactivate the expression of *Neurogenin-1* as well as *NeuroD* and thereby induced the neuronal differentiation in frog embryos (35). We tested if this is the case in the neuroblastoma cells. However, our preliminary results suggested that the LMO3/HEN2 complex does not transactivate the *Neurogenin-1* as well as *NeuroD* promoter in neuroblastoma cell lines,⁵ although it is unclear if the complex could work in normal neuronal development. Thus, like LMO2, alterations in the LMO3-containing transcriptional complex might differentially regulate expression of the downstream target genes closely involved in neuronal differentiation or tumor formation.

⁵ Unpublished data.