Two Transcription Factors, E1AF and N-*myc*, Correlate with the Invasiveness of Neuroblastoma Cell Lines

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The *ets* transcription factor E1AF can activate several matrix-degrading metalloproteinase (*MMP*) genes and is implicated in enhancement of tumor cell invasion. Here we compared the invasive activity of five human neuroblastoma cell lines (TGW, GOTO, SK-N-BE, SK-N-SH and SK-N-AS), which exhibit distinct levels of N-*myc* amplification, together with the expression of E1AF. Extracellular matrix-degrading proteases and their inhibitor proteins, which play an important role in local invasion, were also analyzed. The activity to invade through reconstituted basement membrane was high in cells (TGW, GOTO, and SK-N-BE) with N-*myc* amplification, and these cells produced relatively large amounts of E1AF mRNA, correlating with the invasive activities. Of several matrix metalloproteinases (MMPs) and a tissue inhibitor of MMPs (TIMP), only membrane-bound type 1 MMP (MT1-MMP) was specifically detected in N-*myc*-amplified cells, suggesting a role of MT1-MMP in neuroblastoma cell invasion. MMP-2 (72 kD type IV collagenase), TIMP-1 and TIMP-2 were expressed in all five cell lines. Urokinase-type plasminogen activator was undetectable. These findings indicate that the transcription factors E1AF and N-*myc* are related to malignant phenotypes of neuroblastoma.

Key words: E1AF — N-*myc* — Neuroblastoma — Invasion — MMP

Neuroblastoma arises from primitive sympathoblasts in the adrenal gland or sympathetic ganglia and readily invades and metastasizes in progressive stages. The N-*myc* proto-oncogene was isolated as an amplified DNA fragment from human neuroblastomas on the basis of its homology to the v-*myc* and c-*myc* oncogenes.[1, 2] The N-*myc* gene encodes a transcription factor that regulates gene expression during cell differentiation and growth.[3, 4] N-*myc* amplification in neuroblastoma is clinically significant due to its correlation with advanced disease stages, poor prognosis and proliferation *in vitro* as an established cell line.[5–8]

It was suggested that the amplification of N-*myc* is associated with the capacity to invade surrounding organs or tissues.[6] Neuroblastoma with N-*myc* amplification is more invasive, and the amplification is correlated with the histologic grade of differentiation.[9] *In vitro* studies revealed that morphologic differentiation of human neuroblastoma cells ensues after a reduction in N-*myc* expression induced by retinoic acid treatment.[10] These findings suggest an important regulatory role of N-*myc* amplification or its product in tumor growth and expression. However, little is known about the mechanism by which N-*myc* amplification leads to the acquisition of invasive phenotypes.

Matrix-degrading metalloproteinases (MMPs), a family of structurally related enzymes capable of degrading specific components of extracellular matrix (ECM),[11] play an important role in the invasive process.[11–13] In normal tissue, the enzyme activities of MMPs are tightly regulated to prevent them from damaging the tissue, but in metastatic tumor cells, deregulated expression of MMPs causes degradation of ECM that will result in invasion.[14–16] It has been proposed that several members of the *MMP* gene family are positively regulated at the transcriptional level through the action of AP-1 and *ets* family transcription factors.[17–21]

E1AF is a human member of the *ets* oncogene family that is located on 17q21, and was cloned based on its ability to bind to an enhancer element of the adenovirus *E1A* gene.[22, 23] Recently it was found that the *E1AF* gene is fused with the *EWS* gene in Ewing's sarcoma with a novel chromosome translocation t(17;22)(q12;q12), suggesting causal involvement in the neoplastic process of this tumor.[24] E1AF, as well as *ets* family members *ets*-1 and *ets*-2, activates promoters of different subclasses of the *MMP* genes, including interstitial collagenase (MMP-1), stromelysin (MMP-3), and 92 kD type IV collagenase (MMP-9).[25] We found that introduction of the *E1AF* gene causes a non-invasive human breast cancer cell line to acquire high invasiveness and MMP-9 production.[26]

It is possible that *ets* family members and N-myc protein may stimulate MMP expression and upregulate invasive activity of neuroblastoma cells. To understand better the molecular basis of neuroblastoma invasion, we are

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currently examining the roles of *ets* transcription factor E1AF and the expression of several matrix-degrading proteases and their inhibitor proteins. In the present work, we compared the invasive activity of five human neuroblastoma cell lines (TGW, GOTO, SK-N-BE, SK-N-SH and SK-N-AS) and their expression of *E1AF*, *MMP* and tissue inhibitor of metalloproteinases (*TIMP*) genes. Since these cell lines possess distinct copy numbers of the N-*myc* gene,[27–30] we also wished to assess the correlation between amplification of N-*myc* gene and expression of the invasion-associated genes.

MATERIALS AND METHODS

Cells and cell culture Neuroblastoma cell lines, TGW,[27] GOTO,[27, 30, 31] SK-N-BE,[29, 32] SK-N-SH,[27, 29] and SK-N-AS,[31] were used in the present investigation. Cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Filtron Pty Ltd., Brooklin, Australia) and penicillin/streptomycin (GIBCO, Grand Island, NY).

In vitro **invasion assay** Biocoat Matrigel invasion chambers (Becton Dickinson, Bedford, MA) were used for the invasion assay.[33] Cells (1×10^5) were suspended in serum-free DMEM, and added to the upper chamber. The lower chamber contained DMEM and human cellular fibronectin (12.5 μg/ml, as a chemoattractant). Cells were incubated for 6 h at 37°C in a CO_2 incubator. At the end of incubation, the cells were fixed in methanol and stained with Giemsa solution. Cells on the upper surface of the filter were completely removed by wiping with a cotton swab. Cells that had invaded through the Matrigel and reached the lower surface of the filter were counted under a light microscope at a magnification of ×200.

Northern blot assay Approximately 5×10^6–1×10^7 cells were washed with phosphate-buffered saline and suspended in hypotonic buffer.[34] Cells were lysed by adding NP-40 at a final concentration of 0.5%. Cytoplasmic RNA was extracted twice with a phenol/chloroform mixture and precipitated with two volumes of ethanol.[34] RNA (15 μg/lane) was applied to 1.0% agarose gels containing 2.2 M formaldehyde in MOPS-running buffer, transferred onto a nitrocellulose filter (S&S, BA85) and probed at high stringency with [^{32}P]DNA (specific activity, $1–3 \times 10^8$ cpm/μg) labeled by the random priming method. The following probes were used for the northern blot assay: a 0.6 kb *Xba-Bam*H I fragment of E1AF cDNA,[22] 1.7 kb *Eco*R I fragment of MMP-1 cDNA, 1.5 kb *Eco*R I–*Bam*H I fragment of MMP-2 cDNA, 1.4 kb *Sac* I–*Xho* I fragment of MMP-3 cDNA, 1.2 kb *Pst* I–*Eco*R I fragment of MMP-9 cDNA, 0.6 kb *Cla* I–*Bam*H I fragment of TIMP-1 cDNA, and a 0.7 kb *Eco*R I–*Bgl* II fragment of TIMP-2 cDNA.[14] Plasmid pEMBL8 carrying urokinase-type plasminogen activator (uPA)

cDNA was obtained from ATCC and a 1.5 kb *Pst* I fragment was used as a probe. MT1-MMP cDNA (0.4 kb) was cloned by the reverse transcriptase-polymerase chain reaction method using specific 5′ and 3′ primers (5′-AAGCGGATCCAGACACCATGAAGG-3′ and 5′-TATCTAGAACAGAAGGCCG-3′).[35] This was confirmed by nucleotide sequencing. As an internal standard, a cDNA probe encoding ribosomal large subunit protein L38 was used. Hybridization was performed according to the supplier's manual. Filters were washed twice with 2× SSC/0.1% SDS at room temperature, and twice with 0.2×SSC/0.1% SDS at 55°C, then exposed to Fuji RX X-ray films with intensifying screens at −70°C. For quantitive analysis, radioactivity of the specific mRNA band was measured with a BAS2000 Bio-Imaging Analyzer (FUJIX, Tokyo). Radioactivity was adjusted to that of L38 RNA and is shown as a relative value normalized to TGW.

Statistical analysis In the invasion assay, the differences in the numbers of invaded cells among cell lines were examined using Fisher's PLSD test, and $P<0.05$ was taken as the criterion of significance.

RESULTS

Highly increased invasive activities in neuroblastoma cell lines with amplified N-*myc* To investigate the invasive potential of neuroblastoma cell lines, we first examined the *in vitro* invasive activity in five cell lines with different degrees of N-*myc* amplification using a reconstituted basement membrane-coated invasion chamber. As shown in Fig. 1 and summarized in Table I, TGW, GOTO and SK-N-BE cells carrying more than 10 copies of the N-*myc* gene had relatively high invasive activities, while SK-N-SH and SK-N-AS cells without N-*myc* amplification were only weakly invasive. Numbers (mean values±SE) of invaded cells were TGW (67.5 ± 7.7), GOTO (67.7 ± 8.1), SK-N-BE (53.5 ± 4.3), SK-N-SH (36.5 ± 2.8), and SK-N-AS (23.1 ± 1.8) (Fig. 1). The correlation between N-*myc* amplification and invasive activity was statistically significant (Fisher's PLSD test, TGW and SK-N-SH, $P=0.0002$; TGW and SK-N-AS, $P<0.0001$; GOTO and SK-N-SH, $P=0.0002$; GOTO and SK-N-AS, $P<0.0001$; SK-N-BE and SK-N-SH, $P=0.0341$; SK-N-BE and SK-N-AS, $P=0.0002$). On the other hand, neither the invasive activity within the three cell lines (TGW, GOTO and SK-N-BE) with N-*myc* amplification, nor that in the two cell lines (SK-N-SH and SK-N-AS) without N-*myc* amplification showed any statistically significant difference (Fisher's PLSD test, TGW and GOTO, $P=0.9731$; TGW and SK-N-BE, $P=0.0809$; GOTO and SK-N-BE, $P=0.0753$; SK-N-SH and SK-N-AS, $P=0.0913$), though the data indicated that N-*myc* amplification was associated with the *in vitro* invasive activity of neuroblastoma cells.

Increased expression of the *E1AF* gene in cell lines with higher invasive activity   We previously reported that expression of the *E1AF* gene was high in several human tumor cells showing invasive phenotypes.[26] To examine the correlation with invasive potential of neuroblastoma cells, E1AF mRNA was analyzed by northern blot assay. Amounts of E1AF mRNA were relatively high in TGW, GOTO and SK-N-BE cells with N-*myc* amplification, compared with those of SK-N-SH and SK-N-AS cells without N-*myc* amplification (Fig. 2A). Levels of E1AF
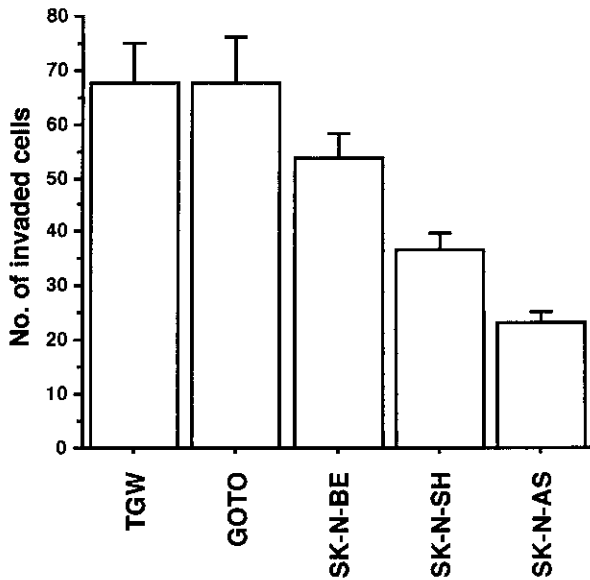

Fig. 1.   *In vitro* invasive activity. Invasive activity of five neuroblastoma cell lines through reconstituted basement membrane was assayed with Matrigel invasion chambers. TGW, GOTO and SK-N-BE cells with N-*myc* amplification showed relatively high invasive activities that were significantly higher than those of SK-N-SH and SK-N-AS cells without N-*myc* amplification.

mRNA normalized to TGW were as follows: TGW, 100%; GOTO, 95.7%; SK-N-BE, 67.4%; SK-N-SH, 48.0%; and SK-N-AS, 5.0% (Table I). Thus, the E1AF expression level was correlated with the invasive activity and N-*myc* amplification.

Correlation of *MMP* gene and *E1AF* gene expression with more invasive phenotype   Transient expression assays showed that E1AF activated promoters of three different subclasses of *MMP* genes.[26] It is also known that constitutive expression of exogenously added *E1AF* gene induces 92 kD type IV collagenase (MMP-9) expression in human breast cancer cells.[26] To investigate the expression of *MMP* genes in neuroblastoma cell lines, northern blot assay was performed using cDNAs for MMP-1, MMP-2, MMP-3, MMP-9 and MT1-MMP as probes (Fig. 2B). MMP-2 (72 kD type IV collagenase) mRNA was detected not only in the three cell lines with N-*myc* amplification, but also in the two cell lines without N-*myc* amplification (Fig. 2B) (RNA levels: TGW, 100%; GOTO, 27.3%; SK-N-BE, 130.9%; SK-N-SH, 102.4%; SK-N-AS, 66.3%). MMP-2 activity in the conditioned media of these cells was also compared by gelatin-zymography (data not shown). There was no obvious relation between expression of the *MMP-2* gene and invasive activity (Fig. 1 and Table I). Membrane-bound type 1 MMP (MT1-MMP) mRNA was detected in all three cell lines with N-*myc* amplification (Fig. 2B) (RNA levels: TGW, 100%; GOTO, 124.8%; SK-N-BE, 23.5%) (Table I). By contrast, the two cell lines without N-*myc* amplification (SK-N-SH and SK-N-AS) did not express MT1-MMP mRNA (Fig. 2B). Transcripts of MMP-1 (interstitial collagenase), MMP-3 (stromelysin), and MMP-9 were undetectable in all cell lines (Fig. 2B). Therefore, only MT1-MMP expression showed a good correlation with invasive phenotype.

Expression of uPA and TIMPs in neuroblastoma cell lines   In addition to MMPs, uPA[36–39] and TIMPs[40, 41] have been shown to be involved in tumor invasion.

Table I.   Invasive Characteristics of Neuroblastoma Cell Lines

Cell line	N-*myc* amplification[a]	Invasive activity[b]	E1AF[c]	MT-MMP[c]	TIMP-1[c]	MMP-2[c]	TIMP-2[c]	MMP-1, 3, 9 and uPA[c]
TGW	>10	+++	++++	++++	++++	++++	++++	−
GOTO	>10	+++	++++	+++++	++++	++	++	−
SK-N-BE	>10	+++	+++	+	+++++	+++++	++++	−
SK-N-SH	1	++	++	−	++	+++++	+++++	−
SK-N-AS	1	+	+	−	++	+++	+	−

a) Number of N-*myc* copies.
b) Graded as numbers of cells invaded through Matrigel. +, 0–25; ++, 25–50; +++, 50–75.
c) Radioactivity of specific mRNA bands (adjusted to that of L38 mRNA) was graded as a relative value normalized to TGW (++++ 100%). +, 0–25%; ++, 25–50%; +++, 50–75%; ++++, 75–100%; +++++, >100%.
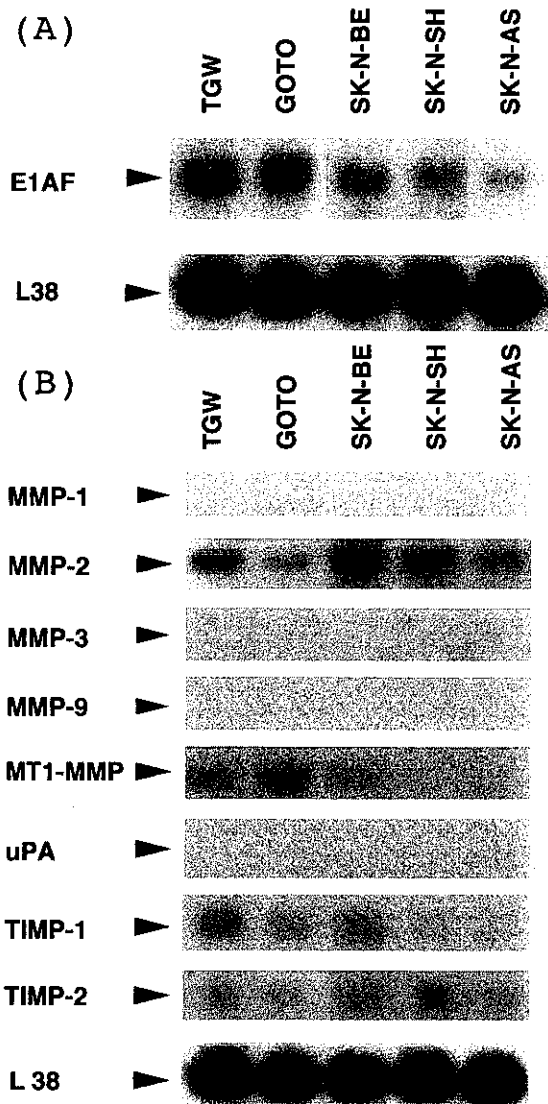
Fig. 2. Amounts of E1AF, MMP, uPA and TIMP mRNAs in neuroblastoma cells. Cytoplasmic RNA was extracted from five cell lines, transferred onto a nitrocellulose membrane and hybridized with ^{32}P-labeled cDNA probes of E1AF, MMPs, uPA, TIMPs and L38 as a control. A, E1AF mRNA was detected in all five neuroblastoma cell lines. L38 mRNA is an internal marker of applied RNA amount. B, MT1-MMP mRNA was detected only in the three cell lines with N-myc amplification (TGW, GOTO and SK-N-BE), while MMP-2 and TIMP mRNAs were detected in all five neuroblastoma cell lines. All the cell lines expressed TIMP-2 at lower levels. Expression of MMP-2 and TIMP-2 had no simple correlation with N-myc amplification levels of the cell lines. MMP-1, MMP-3, MMP-9 and uPA mRNA were undetectable in all the cell lines examined. Sizes of mRNA were as follows: E1AF, 2.5 kb; L38, 0.7 kb; MMP-1, 2.5 kb; MMP-2, 3.2 kb; MMP-3, 2.3 kb; MMP-9, 2.9 kb; MT1-MMP, 4.2 kb; uPA, 2.6 kb; TIMP-1, 0.9 kb; TIMP-2, 3.5 and 1.0 kb (1.0 kb TIMP-2 mRNA not shown). L38 mRNA is an internal marker of applied RNA amount.

Expression of uPA, TIMP-1 and TIMP-2 genes was monitored by northern blot assay (Fig. 2B). Transcripts of uPA were not detected in any cell line (Fig. 2B). TIMP-1 and TIMP-2 inhibit MMP activities by forming complexes with the proenzymes of MMP-9 and MMP-2, respectively.[42, 43] The mRNAs for TIMP-1 and TIMP-2 were detected in all cell lines (Fig. 2B). The cell lines with N-myc amplification (TGW, GOTO and SK-N-BE) expressed TIMP-1 mRNA at high levels compared with the cell lines without N-myc amplification (SK-N-SH and SK-N-AS) (Fig. 2A). Levels of TIMP-1 mRNA in cell lines were as follows: TGW (100%), GOTO (81.5%), SK-N-BE (125.8%), SK-N-SH (47.5%), and SK-N-AS (48.6%) (Table I). Relative levels of TIMP-2 mRNA were TGW (100%), GOTO (31.0%), SK-N-BE (79.3%), SK-N-SH (158.6%), and SK-N-AS (23.6%) (Table I). No simple correlation was found between the expression of TIMP-2 and invasive activity of individual cell lines (Table I).

DISCUSSION

Tumor invasion, the first step of metastasis, requires complex interactions, including recognition and attachment of tumor cells to the ECM-binding sites, proteolytic dissolution of ECM and tumor cell migration within the surrounding tissue.[44] In particular, the degradation of ECM is significant, and therefore enzymes that have a proteolytic effect on ECM, such as MMPs and uPA, have been investigated.[11-13] However, to our knowledge, a correlation between N-myc amplification and expression of these proteolytic enzymes has not hitherto been demonstrated in neuroblastoma. Here we compared five human neuroblastoma cell lines showing distinct amplification of the N-myc gene and found that N-myc amplification in these cell lines was highly correlated with the invasive activity and the expression of E1AF, MT1-MMP and TIMP-1.

The normal single-copy locus of N-myc has been mapped to the short arm of chromosome band 2p-23-24,[45] and the N-myc gene encodes a transcription factor that regulates gene expression during cell differentiation and growth.[3, 4] Clinical studies of neuroblastoma have shown that the genomic amplification of N-myc is correlated with malignant phenotype, invasion and metastasis.[6, 7] Our in vitro invasion study confirmed the increased invasiveness of N-myc-amplified neuroblastoma cells.

The ets transcription factor E1AF stimulates MMP transcription, cell motility and invasive activity, and is thus implicated in tumor cell invasion.[25, 26, 36] Expression of the E1AF gene is observed in a variety of cell lines, especially those with large numbers of motile and invasive cells.[35] In this investigation, we found that E1AF and N-myc were associated with increased invasiveness

of neuroblastoma cells. Although the possibility of cooperative action between the two proteins remains to be examined, enzymes with a proteolytic effect on ECM might be among the target genes modulated by these transcription factors. In this regard, it is of interest that MT1-MMP is highly expressed in N-*myc*-amplified neuroblastoma cells.

Our results indicated that the *MT1-MMP* gene, recently identified in human placenta,[46] was correlated with E1AF expression and N-*myc* amplification level. Our preliminary transfection experiments using human osteosarcoma cells revealed that E1AF had the ability to activate the MT1-MMP promoter in an E1AF dose-dependent manner (data not shown). However, it is unknown whether the promoter region of the *MT1-MMP* gene contains PEA3 sites recognized by *ets* family members or an E-box sequence motif recognized by *myc* family members. MT1-MMP has been proposed to be a protease processing inactive proMMP-2 to the active form on the cell surface.[46, 47] Our analysis of the conditioned media by gelatin zymography did not show any significant difference in amounts of the active MMP-2 form between MT1-MMP-expressing cells and those that did not express it (data not shown). Further experiments will be required to elucidate the role of MT1-MMP in the invasiveness of neuroblastoma cells.

TIMP-1 expression was also high in N-*myc*-amplified cells. TIMP-1 not only inhibits MMP-9 by forming a complex with the activated enzyme,[42, 43, 48] but also has limited effects on other MMPs.[49, 50] It should be noted that the balance between TIMP-1 and MMPs regulates the activity of tumor cells to invade the ECM.[40, 41] TIMP-1 expression has been observed in a variety of human tumor cells.[14] Although the TIMP-1 promoter possesses PEA3 sites, it remains unknown which *ets* family member is responsible for modulating TIMP-1 transcription.[51]

In summary, we have shown that neuroblastoma cells with N-*myc* amplification highly express the *ets* transcription factor E1AF and MT1-MMP, and their levels are well correlated with the invasive activity. These findings raise the possibility that the increased expression of the two transcription factors E1AF and N-*myc* is related to malignant phenotypes of neuroblastoma. We are currently investigating the effects of E1AF and N-*myc* transcription factors on the MT1-MMP promoter and expression of these proteins in samples of neuroblastoma at various stages of progression.

ACKNOWLEDGMENTS

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