Expression of E1AF, an ets-oncogene transcription factor, highly correlates with malignant phenotype of malignant melanoma through up-regulation of the membrane-type-1 matrix metalloproteinase gene

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Abstract. Matrix metalloproteinase (MMP) is closely involved in the degradation of extracellular matrix and confers invasive and metastatic potential to malignant tumors. MMP-2 is a type-IV collagenase secreted as a proenzyme that is activated on the surface of the tumor cell by membrane-type 1-MMP (MT1-MMP). MT1-MMP plays a critical role during tumor progression and metastasis. We investigated the expression levels of E1AF and MT1-MMP in malignant melanoma cell lines and specimens from patients in order to clarify the mechanisms responsible for the invasion and metastasis of malignant melanoma. High levels of E1AF and MT1-MMP mRNA expression were observed in melanoma cells by Northern blotting and real-time PCR. The expression level was highly correlated with an invasive potential determined by an *in vitro* invasion assay. The down-regulation of MT1-MMP was identified when E1AF was knocked down by RNA interference. These results suggest that E1AF plays a crucial role in the invasion and metastasis of malignant melanoma through up-regulating the MT1-MMP expression.

Introduction

Malignant melanoma is characterized by its high degree of malignancy with widespread metastatic ability. The histopathological evaluation of melanoma (1,2) has been utilized to predict the prognosis of malignant melanoma. Melanomas with a predominantly vertical phase that deeply invades tissues, were shown to have a poor prognosis, although the details of the metastatic factor of malignant melanoma remain obscure.

The mechanism of tumor invasion and metastasis has been described as a complex series of sequential processes that involve the proliferation of transformed cells, local invasion and destruction of extracellar matrix components, migration of tumor cells away from the primary tumor mass and penetration of cancer cells through the blood vessel walls (3-5). We focused on the degradation of type IV collagen by malignant melanoma cells, which is the main component of the basement membrane.

Matrix metalloproteinases (MMPs) play an important role in the invasive process of tumor cells by degrading various substrates within the extracelluar matrix (ECM) (6-9). MMP-2 is a type IV collagenase/gelatinase and it was shown to be produced as a latent form from fibroblasts surrounding tumor tissues by *in situ* hybridization (10). The conversion of the latent (inactive) form of MMP-2 into an active form is an important step for metastasis by degrading the basement membrane (11). MT1-MMP (MMP14) was shown to have an essential role in MMP-2 activation (12). MT1-MMP was expressed on tumor cell surfaces that induce the activation of pro-MMP-2 and enhance cellular invasion *in vitro*. However, the activation mechanism of MT1-MMP is still unclear at the transcription level.

E1AF is an ets-oncogene family transcription factor (13,14) and we reported that E1AF confers to the invasiveness of epithelial tumors by transcriptional activation from several MMPs (6-8). We investigated the correlation of E1AF in malignant melanoma with regards to metastasis promoting the MMP-2 activation by MT1-MMP.

Materials and methods

Cell line and culture conditions. The human malignant melanoma cell lines TXM18, UT, MEWO and 96E were used

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Table I. Primers and probe sequences for real-time PCR.

Primer	
E1AF	
Sense	5'-TCA GGTACC AGA CAG TGA TGA GCA GTTTGT-3'
Antisense	5'-AAG GGC GTG AGG GGC GAG TG-3'
MT1-MMP	
Sense	5'-GAG AAG CAG GCC GAC ATC A-3'
Antisense	5'-GCA CAG CCA CCA GGA AGA T-3'
GAPDH	
Sense	5'-GCC TCC TGC ACC ACC AAC TG-3'
Antisense	5'-CGA CGC CTG CTTCAC CAC CTTCT-3'
Hybridization probes	
E1AF	
Sense	5'-GCG AGC AGT GCC TTTACTCCA GT-3' FITC
Antisense	5'-LCRe d640 CCTATG ACC CCC CCA GAC AAA T-3' P[a]
MT1-MMP	
Sense	5'-AGG CCC CAA CAT TGG AGG AGA-3' FITC
Antisense	5'-LCRe d640 ACC CAC TTTGAG TCTGCC GAG C-3' P[a]
GAPDH	
Sense	5'-GGG AAG CTC ACTGGC ATG GC-3' FITC
Antisense	5'-LCRe d640 TTC CGTGTC CCC ACTGCC AA-3' P[a]

[a]Phosphorylation.

in this study. The human fibrosarcoma cell line, HT1080 was used as a positive control for the E1AF and MT1-MMP expression. MCF7, a human breast carcinoma cell line, was used as a negative control for the E1AF and MT1-MMP expression. The human fibroblast cell line MRC5 was cultured, and its conditioned medium was used as a conditioned medium for the *in vitro* invasion assay. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Northern blot assay. For Northern blot, analysis probes were labeled with [^{32}P]dCTP, using a BucaBest labeling kit (Takara, Tokyo, Japan). The following probes were used for Northern blot analysis: a 0.6 kb *Xba*I-*Bam*HI fragment of E1AF cDNA and a 1.2 kb of *Hin*dIII and *Eco*RI fragment of MT1-MMP cDNA. A 531 bp human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal control for the amount of RNA on each RNA blot. Labeling probes were purified through a Sigma spin post reaction purification column (Sigma, Tokyo, Japan) before hybridization. Total-cell RNA (15 mg/lane) was applied to 1.0% agarose gel containing 2.2 M formaldehyde in MOPS running buffer, transferred onto a nitrocellulose membrane (Schleichner and Schuell, Dassel, Germany) and cross-linked by UV irradiation using an Ultraviolet cross linker. Membranes were pre-hybridized at 42˚C for 4 h in pre-hybridization solution containing 50% formamide, 5X SSC (0.75 M NaCl, 75 mM sodium citrate and pH 7.0), 5X Denhardt's solution, 0.1% SDS and 100 mg/ml of denatured salmon sperm DNA. A labeled probe was then added to the same pre-hybridization solution with 2.5% dextran sulfate and hybridization was allowed to proceed for 48 h at 42˚C. After hybridization, the membrane was washed twice under highly stringent conditions and exposed to Kodak X-Omat X-ray film at -80˚C for 48 h.

The intensities of these results were measured with a BAS-2000 Bio-Imaging analyzer (Fuji-Film Co., Tokyo, Japan). Radioactivity was adjusted to that of GAPDH RNA.

Real-time RT-PCR. Total RNA (1 μg each) was reverse transcribed into cDNA with ReverTra Ace reverse transcriptase (Toyobo, Tokyo, Japan), then diluted 200-fold with distilled water as templates of PCR. Real-time PCR was performed on a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) using a Fast Start DNA Master Hybridization probe kit (Roche Molecular Biochemicals). The primer design and hybridization probes used are shown in Table I. After adding the primers (final concentration: 0.25 pM), 3 mM $MgCl_2$ and template DNA to the master mix, 37 cycles of denaturation (95˚C for 1 min), annealing (55˚C for 5 sec) and extension (72˚C for 20 sec) were performed. All templates were heated and cooled at 20˚C per second.

Quantification was performed by online monitoring for identification of the exact time point at which the logarithmic linear phase could be distinguished from the background (crossing point). Serially diluted samples of the plasmid (10-10^5 copies) were used as the external standard in each run. The cycle numbers of the logarithmic linear phase were plotted against the logarithm of the concentration of template DNA. The concentrations of PCR products were calculated by comparing the cycle number of the logarithmic linear phase of the sample with the cycle number of the external standard.

In vitro invasion assay. Bio-coat matrigel invasion chambers (Becton-Dickinson, San Jose, CA, USA) were used for the invasion assay. Cells (1x10^5) were suspended in serum-free DMEM and added to the upper chamber. The lower chamber contained a condition medium from the human fibroblast MRC5. Cells were incubated for 7 h at 37˚C in a CO_2 incubator. At the end of incubation, the cells on the upper surface of the filter were completely removed and cells were fixed in 100% ethanol and stained with Giemsa solution. Cells that invaded the lower surface of the filter were counted under a light microscope at a magnification of x200. Each assay was performed in triplicate.

RNA interference. The E1AF sequence (5'-AAA TCG CCC GGA AAT GGG AGC TT-3') was targeted by RNAi. siRNAs (sense: 5'-AUCGCCCGGAAAUGGGAGCdTdT and antisense: 5'-GCUCCCAUUUCCGGGCGAUdTdT) were synthesized and applied to knock down the E1AF expression into a TXM18 melanoma cell line. E1AF and MT1-MMP mRNA levels were monitored by real-time PCR as described above.

Results

Expression of E1AF and MT1-MMP in malignant melanoma cell lines. The expression of E1AF mRNA in human melanoma

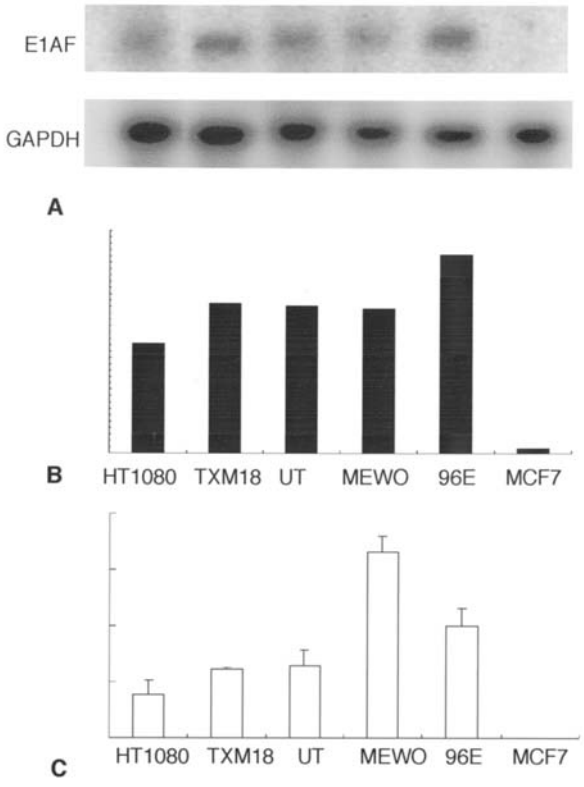

Figure 1. Expression of E1AF in melanoma cell lines. (A) Northern blot analysis of E1AF. A 2.5 kb E1AF mRNA band was detected in TXM18, UT, MEWO and 96E melanoma cell lines. HT1080 is a positive control of a fibrosarcoma cell line and MCF7 is a negative control of a breast carcinoma cell line. (B) Quantitative results of Northern blotting standardized with GAPDH by BAS-2000 Imaging analyzer and a high-level expression of E1AF in melanoma cell lines. (C) Results of real-time PCR and a corresponding expression with Northern blotting of high levels of E1AF was observed in melanoma cell lines.

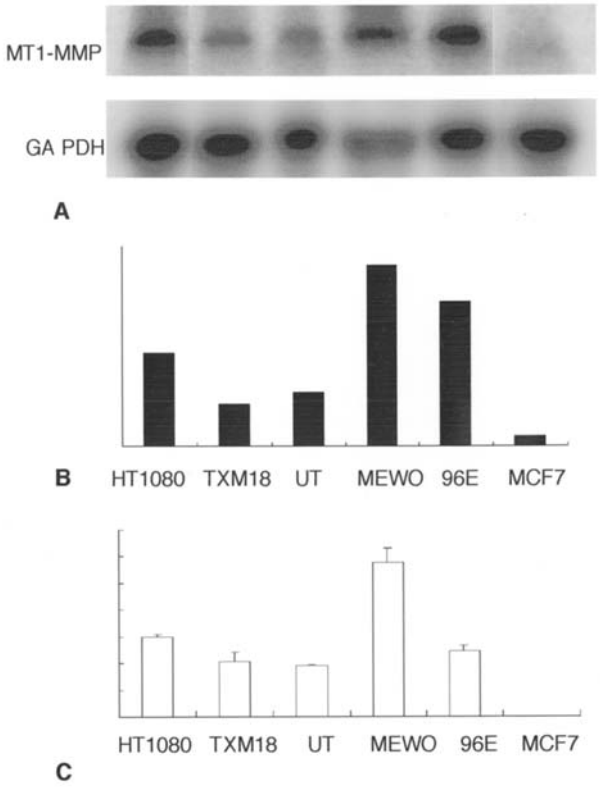

Figure 2. MT1-MMP expression in melanoma cell lines. (A) A 4.2 Kb MT1-MMP mRNA was detected in all melanoma cell lines. (B) The expression level was analyzed by an image analyzer. Melanoma cell lines showed a high level of MT1-MMP. (C) A correlated expression was observed by real-time PCR.

cell lines was analyzed by Northern blot hybridization and the real-time PCR. A single 2.5 kb band for E1AF was detected in all melanoma cell lines by Northern blotting. Expression levels were quantified by the BAS-2000 Imaging analyzer (Fuji Photo Film) standardized with the amount of GAPDH as an internal control. A high-level expression of E1AF was observed in melanoma cell lines (TXM18, 137.4%; UT 135.2%; MEWO, 133% and 96E, 182.6%) compared to the expression levels of HT1080, a positive control of the fibrosarcoma cell line as 100%, and MCF7, a negative control of breast carcinoma cell line showed 3.9% of expression compared to HT1080. The real-time PCR data showed that all human melanoma cells constitutively produced E1AF mRNA which was in agreement with the results of Northern blotting (Fig. 1). The expression level of MT1-MMP mRNA in human melanoma cell lines was analyzed by the same methods as E1AF. Single bands (4.2 kb) were detected by Northern blotting and the correlated high level of MT1-MMP expression was observed by real-time PCR in all melanoma cell lines (Fig. 2).

The invasive activity of malignant melanoma cell lines. To investigate the invasive potential of malignant melanoma cell lines, we examined the *in vitro* invasion assay in 4 malignant melanoma cell lines using a reconstituted basement membrane-coated invasion chamber (Matrigel, Becton-Dickinson). The cells that invaded the lower surface of the filter were counted. The invasive activity of these cells was examined three times and standardized. HT1080 and all melanoma cell lines had high invasive activities. The numbers (mean values ± SE) of invading cells were HT1080 (124±17), TXM18 (41.83±5.8), MEWO (82.17±7.2), UT (54.83±9.5) and 96E (49.5±4.7). On the other hand, MCF7 showed no invasive activity (Fig. 3).

Statistical analysis. To compare these data, we used Spearman's regression analysis. The expression levels of E1AF mRNA and MT1-MMP mRNA markedly correlated with each other (P=0.0001). The expression levels of E1AF and MT1-MMP were significantly correlated to the invasive activity, respectively (E1AF, P=0006 and MT1-MMP, P=0.0014). These data indicated that E1AF positively regulated the transcription of MT1-MMP and led to the mechanism of human melanoma cell invasion (Fig. 4).

RNAi interference. The direct correlation between E1AF and MT1-MMP was investigated using RNA interference. The dsRNA molecule of E1AF was induced in a TXM18 melanoma cell line. Real-time PCR analysis was performed and the expression level of E1AF was reduced to ~60% compared to the mock cells after treatment with dsRNA. A corresponding reduction of MT1-MMP was observed in dsRNA-treated TXM18 cells (Fig. 5).

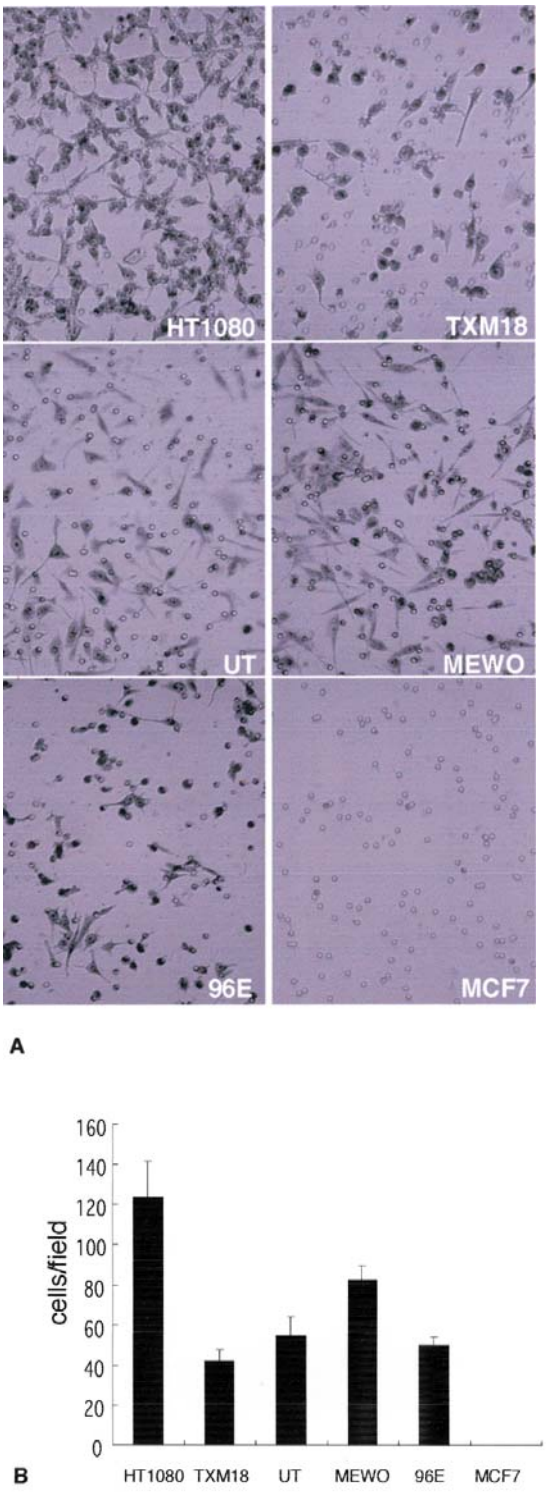

Figure 3. Results of the invasion assay. (A) A high-power field view of invading cells from matrigel. HT1080, a fibrosarcoma cell line that was shown to express a high-level of E1AF and MT1-MMP and showed a large number of invading cells which were also seen in the melanoma cell lines (TXM18, UT, NEWO and 96E). In contrast, MCF7, a breast carcinoma cell line with a low E1AF and MT1-MMP expression, had no invasive ability. (B) Values represent the mean of three measurements; bars show standard deviation (SD).

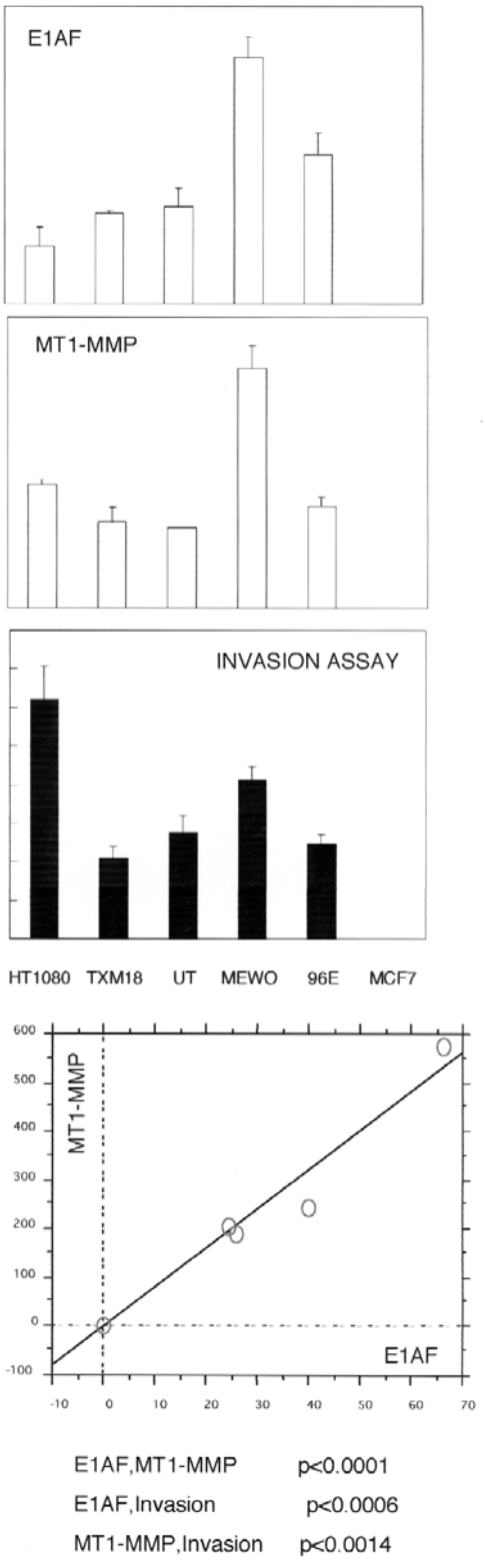

Figure 4. Statistical analysis between E1AF, MT1-MMP expression and tumor cell invasion. The expression levels of E1AF mRNA and MT1-MMP mRNA markedly correlated with each other (P=0.0001). The expression levels of E1AF and MT1-MMP were significantly correlated to the invasive activity, respectively (E1AF, P=0006 and MT1-MMP, P=0.0014).

Discussion

Malignant melanoma is shown to have a poor prognosis with its high metastatic potential. The expression of several proteinases and their activation are important factors in malignant melanoma by degrading the basement membrane and the extracellular matrix that leads to metastasis. It was reported that murine malignant melanoma cells with a high

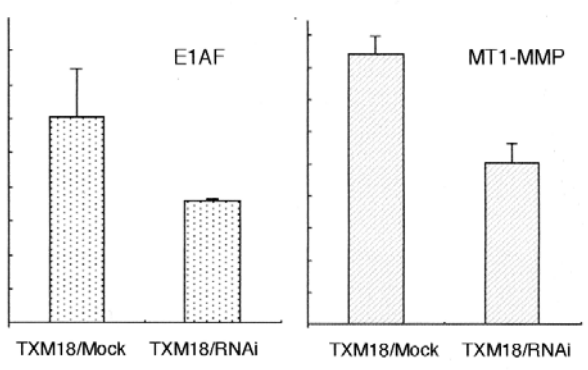

Figure 5. Effect of RNA interference for E1AF. The expression levels of E1AF and MT1-MMP were analyzed by real-time RT-PCR 48 h after dsRNA transfection into TXM18 melanoma cells. E1AF expression was reduced in TXM18/RNAi and a corresponding reduction of MT1-MMP was observed.

metastatic potential expressed a larger amount of type IV collagenase than low-metastasis types (15).

The matrix metalloproteinase (MMP) is a family of proteinases that has structural features of an N-terminal propeptide domain, a zinc-coordinating active site and a C-terminal hemopexin-like domain. MMP is comprised of >20 types and divided into certain subgroups as archetypal MMPs (collagenases, MMP-1, -8, -13; stromelycins, MMP-3, -10; other MMPs; MMP-12, -19, -20 and -27), matrilysins (MMP-7 and -26); gelatinases (MMP-2 and -9) and convertase-activatable MMPs (secreted, MMP-11, -21 and -28; membrane-types, MT1, MT2, MT3, MT4, MT5, MT6 and MMP-23) (16). Generally, MMPs are synthesized as a proform and enzyme activation is achieved by the removal of the N-terminal propeptide domain through exogenous or autocatalytic cleavage (16,17).

Gelatinases are shown to be strongly involved in cancer metastasis by degrading the basement membrane of capillary vessels and MMP-2 was shown to be the potent proteinase in this mechanism (11). The MMP expression and activity are tightly regulated by gene transcription and post-translational extracellular activation. However, the transactivation mechanism of MMP-2 had been obscure for a long time. Sato *et al* isolated a complementary DNA encoding 63 kDa matrix metalloproteinase with a transmembrane domain termed membrane-type MMP (MT-MMP) (12). The expression of the MT1-MMP gene product on tumor cell surfaces induced the activation of progelatinase A (MMP-2) and enhanced cellular invasion *in vitro*. It was recently shown that the activation of proMMP-2 by MT1-MMP depends on the presence of low amounts of tissue inhibitor of matrix metalloproteinase 2 (TIMP-2), a specific inhibitor of MMP-2 (18) which is required for the formation of a membrane-bound ternary complex consisting of MT1-MMP, TIMP-2 and latent MMP-2. High concentrations of TIMP-2 inhibit MMP-2 activation, whereas an excess amount of MT1-MMP located in proximity to the ternary complex cleaves proMMP-2 bound to the MT1-MMP/TIMP-2 complex.

We have previously identified E1AF, an ets-oncogene family transcription factor. E1AF was isolated from the HeLa cell λgt11 expression library that had a binding ability to the adenovirus E1A enhancer element (13). The ets family is characterized by the functional ETS domain composed of ~85 amino acids with a binding ability to the central GGAA/T core nucleotide motif (19), and we have reported that E1AF stimulates the transcription of MMP-1, -3 and -9 genes in transient expression assays (6). HSC3, a highly-invasive oral squamous cell carcinoma-derived cell line, showed the correlative E1AF and MMP-1 and -9 expression (8) and the transfection of the E1AF expression vector into MCF7, a weakly invasive human MCF7 breast cancer cell line, resulting in the induction of invasive and motile activity accompanied by an increase of 92 kDa type IV collagenase (MMP-9) gene expression (7). Moreover, the invasive activity was inhibited by the down-regulation of MMPs when HSC3 cells were transfected with an antisense E1AF expression plasmid (9). Thus, E1AF positively participates in epithelial cancer cell invasion by up-regulating MMP genes. However, MMP-2, another potent type IV collagense did not respond to E1AF by the reporter assay (unpublished data), although MMP-2 has the ets binding sites in the promoter sequences (16).

We examined the 5'-flanking region sequence of MT1-MMP and identified 3 putative binding sites for E1AF/PEA3 (20). Taguchi *et al* have reported that neuroblastoma cells with N-myc amplification highly express E1AF and MT1-MMP and that their levels are well correlated with the invasive activity (21). Habelhah *et al* have reported that the overexpression of E1AF in the mouse fibrosarcoma cell (QR-32) contributes to invasive phenotypes and enhanced cell migration with synergistical MT1-MMP up-regulation (22). Our results show that a correlated expression of E1AF and MT1-MMP were observed in malignant melanoma cell lines and *in vivo* tumors. The expression level of MT1-MMP was in agreement with E1AF expression estimated by Northern blotting and real-time PCR in melanoma cell lines as well as *in vivo* tumors. These findings were confirmed by the RNAi experiment. RNAi targeted for E1AF in TXM18 cells showed ~40% reduction of E1AF expression determined by real-time PCR which resulted in a correlated reduction of MT1-MMP. Thus, it is indicated that E1AF positively regulates the invasion activity of malignant melanoma by up-regulating MT1-MMP.

In this study, the synergistical up-regulation of E1AF and MT1-MMP was observed in melanoma cell lines. This indicates that E1AF possibly induces MT1-MMP expression that causes MMP-2 activation in malignant melanoma, which is related to the malignant phenotypes of malignant melanoma. There have been reports concerning genes responsible for tumorigenesis and the malignant phenotype expression of malignant melanoma. RAS, p53 and p16 as well as cell cycle regulating gene disorders were shown to be genes responsible for tumorigenesis (23-25). The high incidence of the E1AF expression followed by an MT1-MMP activation is associated with these gene disorders to induce a malignant phenotype of melanoma cells, especially the high metastatic potential.

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