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Annexin VII as a Novel Marker for Invasive Phenotype of Malignant Melanoma

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Both F10 and BL6 sublines of B16 mouse melanoma cells are metastatic after intravenous injection, but only BL6 cells are metastatic after subcutaneous injection. While examining the genetic difference between the two sublines, we found a marked reduction of annexin VII expression in BL6 cells. In addition, fusion cell clones of both sublines were as poorly metastatic as F10 cells after subcutaneous injection, and contained the annexin VII message as abundantly as F10 cells. Hence, we examined whether the annexin VII expression was correlated with the less malignant phenotype of clinical cases by immunohistochemistry. Immunoreactivities to anti-annexin VII antibody in melanoma cells were evaluated quantitatively by using skin mast cells as an internal positive control. Eighteen patients with malignant melanoma were divided into two groups: lymph node metastasis-negative and positive groups. The ratio of numbers of patients positive versus negative to the antibody was significantly larger in the former than in the latter group. These results not only indicated that annexin VII serves as a marker for less invasive phenotype of malignant melanoma, but also suggested a possible role of annexin VII in tumor suppression.

Key words: B16 melanoma — Ca^{2+}-binding protein — Fusion cells — Immunohistochemistry

An increasing number of people are suffering from malignant melanoma in various areas of the world. The major cause of death in the patients with melanoma is tumor metastasis. Several approaches, including immunologic examination,[1–3] single strand conformation polymorphism (SSCP),[4, 5] and serological tumor markers,[6–8] have been applied to evaluate the metastatic potentials of melanoma cells. Immunohistochemistry is one of the simplest and most commonly used methods. However, few probes have been identified that serve as prognostic markers of malignant melanoma in immunohistochemistry. To seek a novel and useful prognostic marker, we have examined a variety of sublines of B16 mouse melanoma cells, which possess distinct metastatic potentials.[9] The BL6 subline is one of the most malignant and is metastatic after both intravenous and subcutaneous injection, whereas F10 cells are metastatic only after intravenous injection.[10, 11] The F1 subline is a parent of F10 cells and is poorly metastatic even after intravenous injection.[12]

Previously, we determined the genetic dominance in the metastatic phenotype between F10 and F1 cells. Fusion cell clones of both cells were as highly metastatic after intravenous injection as F10 cells. High metastatic phenotype appeared to dominate in crosses between F1 and F10 cells.[13] In the present study, we determined the genetic dominance in the metastatic phenotype between F10 and BL6 cells, and found that the low metastatic phenotype was dominant in their crosses. This indicated that F10-derived metastasis-suppressive activity dominated over the BL6-derived metastasis-promoting activity. It is logical that factors essential for metastasis-suppressive activity are expressed in F10 cells, but missing in BL6 cells. To isolate genes encoding such factors, we performed an mRNA differential display (DD) procedure and constructed a subtracted cDNA library using poly(A)$^+$ RNA from F10 and BL6 cells. Among a pool of clones rescued from subtraction, annexin VII was found to be expressed at a higher level in F10 cells, but at a lower level in BL6 cells. Immunohistochemical study of annexin VII in human melanoma tissues provided the first evidence that annexin VII serves as a marker of less invasive phenotype of malignant melanoma.

MATERIALS AND METHODS

Cell culture B16 melanoma sublines were kindly provided by Dr. I. J. Fidler (The University of Texas). Human lung fibroblast cell line TIG-1[14] was purchased from Japanese Cancer Research Resources Bank-Cell (Tokyo). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. To maintain the drug-resistant cells, the culture medium was supplemented with G-418 (2 mg/ml) and/or hygromycin B (0.2

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mg/ml). Mast cells were established from spleen cells of C57BL/6 mice as described previously.[6)]

mRNA differential display Total RNA was extracted by the guanidine thiocyanate/CsTFA method from F10 and BL6 cells, then poly(A)$^+$ RNA was purified using oligo(dT) cellulose. One microgram of poly(A)$^+$ RNA was mixed with 50 pmol of 3′-downstream primer labeled with Rhodamine (Takara, Ohtsu) in 6 μl of diethylpyrocarbonate-treated water and heated at 70°C for 10 min. This solution was added with 2 μl of 10× RNA PCR buffer (Takara), 2 μl of dNTPs (10 m*M* each), 0.5 μl of RNase inhibitor (40 U/ml) (Toyobo, Osaka), 8.5 μl of water and 1 μl of Superscript II (5 U/ml) (Gibco BRL, Rockville, MD). Reverse transcription (RT) reaction mixture was incubated for 1 h at 45°C then for 10 min at 70°C. Nine tubes of RT reaction mixture were obtained with nine different downstream primers. The mixture was diluted 5-fold by addition of TE buffer (10 m*M* Tris HCl [pH 7.5], 1 m*M* EDTA). The resulting cDNA solution was subjected to polymerase chain reaction (PCR). The PCR reaction mixture was composed of 2 μl of the RT reaction solution, 2 μl of 10× RNA PCR buffer, 0.8 μl of 25 m*M* $MgCl_2$, 0.4 μl of 2.5 m*M* dNTPs, 0.1 μl of *Taq* DNA polymerase (Takara), 20 pmol each of 3′-downstream primer labeled with Rhodamine and 5′-upstream primer (Differential Display Kit, Display Systems Biotech, Vista, CA). Reactions were performed under the following conditions: one cycle of 2 min at 94°C, 5 min at 40°C, and 5 min at 72°C, then 34 cycles of 30 s at 94°C, 2 min at 40°C, and 1 min at 72°C, followed by 5 min at 72°C. Samples were electrophoresed on a 4% acrylamide 7 *M* urea sequencing gel. The PCR products were visualized by using FMVIO II Multi-View (Takara). Bands of interest were selected by slicing the gel and DNA was eluted through a QIA quick column (Qiagen, Hilden, Germany) into a 30 μl Tris solution. For reamplification (second PCR), a 5 μl aliquot was used for PCR under the same conditions and with the same primers as used for the first PCR. The second PCR products were electrophoresed on a 3% gel containing HA yellow (Takara) to separate PCR products in an AT/GC content-dependent fashion. Amplified products were recovered and subcloned into the pT7-Blue vector (Novagen, Madison, WI).

Subtraction of cDNA libraries The method for the preparation of the original and subtracted cDNA libraries was described previously.[15)] To prepare single-stranded plasmid DNA, the plasmid DNA prepared from the F10 and BL6 cell cDNA libraries was introduced into *Escherichia coli* DH5F′ IQ cells by electroporation. After culture for 1 h in rich medium (2× YT), transformed cells were infected with R408 helper phages. Then, single-stranded DNA was purified from the supernatant of the 8-h culture. Poly(A)$^+$ RNA was labeled with photobiotin (Vector Lab., Burlingame, CA). Single-stranded DNA (1 μg) was hybridized with 10 μg of biotinylated RNA at 42°C in 25 μl of hybridization buffer containing 40% formamide, 50 m*M* *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES) (pH 7.5), 1 m*M* ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 200 m*M* NaCl and 1 μg of oligo-poly(A). After hybridization for 42 h, the mixture was transferred to 400 μl of SB (50 m*M* HEPES [pH 7.5], 2 m*M* EDTA, 500 m*M* NaCl), and 10 μg of streptoavidin (Gibco BRL) was subsequently added. The mixture was incubated at room temperature for 5 min and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The organic phase was back-extracted with 100 μl of TE (10 m*M* Tris-HCl [pH 8.0], 1 m*M* EDTA [pH 8.0]). The aqueous phases were pooled. Streptoavidin binding and phenol treatment were repeated once more. The recovered single-stranded plasmid DNA was subtracted with biotinylated RNA several times. After repeating the subtraction process, the recovered single-stranded DNA was converted to double-stranded plasmid DNA by the BcaBEST DNA polymerase (Takara) reaction at 65°C for 30 min. After phenol extraction and ethanol precipitation, the DNA was dissolved in 20 μl of TE buffer and 3 μl aliquots were introduced into *E. coli* MC1061A cells by electroporation. A single clone plasmid was purified from each colony.

Northern blot analysis Five microgram aliquots of total RNA prepared from F10 and BL6 cells and their fusion cells were loaded in lanes, fractionated on 1% agarose-formaldehyde gels, and transferred to nylon membranes by capillary action in 20× salt sodium citrate (SSC). Baked membranes were prehybridized for 3 h at 42°C in a buffer containing 50% formamide, 5× SSC, 5× Denhardt's solution, and 0.1% SDS. The membranes were hybridized with the [γ-^{32}P]dCTP-labeled DNA probes at 42°C for 15 h in the same buffer. DNA probes were prepared by the random hexamer labeling method. After hybridization, the membranes were washed to a final stringency of 0.1× SSC and 0.1% SDS at 50°C, and autoradiographed at −80°C.

DNA sequencing Dideoxy-chain termination sequencing reactions were performed with T7 or T3 dye-labeled primers and thermal cycle sequencing kits (Li-cor, Lincoln, NE). The reaction products were analyzed by a model 4000L automated DNA sequencer (Li-cor). The DNA sequences were used to search the htgs database with the BLASTN algorithm.

Somatic cell fusion F10 and BL6 cells were transfected with pKOneo vector[13)] carrying the gene for G-418 resistance or pSV2hph vector[13)] carrying the gene for hygromycin B resistance by calcium phosphate co-precipitation or electroporation. Resistant clones were selected in medium containing 2.0 mg/ml of G-418 or 0.2 mg/ml of hygromycin B, and were grown to confluence. Cells were fused by the polyethyleneglycol-mediated cell fusion procedure as described previously.[13)] Then, 1×10^6 cells were plated on a

60-mm culture dish and incubated for 18–24 h at 37°C in 5% CO_2/95% air. After removal of the culture medium, 3 ml of 50% (w/v) polyethyleneglycol (*Mr.* av. 3350; Sigma, St Louis, MO) was added and the cells were exposed for 1 min at room temperature. After the removal of polyethyleneglycol, the cells were washed three times with Tris-saline buffer, refed with culture medium for 24 h and selected in medium supplemented with G-418 (2.0 mg/ml) and hygromycin B (0.2 mg/ml). The dual selective medium was changed every 4 days and growing colonies were cloned 2 to 3 weeks after the polyethyleneglycol treatment. The characteristics of the hybrid cells were confirmed by measuring chromosome numbers. To do this, confluent cells were harvested with trypsin-EDTA solution and one-third of the cell suspension was plated on a new culture dish. Eighteen hours later, colcemid was added to a final concentration of 20–40 ng/ml and the culture was incubated for 3 h. The cells were harvested with trypsin-EDTA solution and the chromosomes were banded using Giemsa staining. The chromosome numbers in ten G-banded cell spreads of each hybrid cell clone were counted.

Spontaneous metastasis assay Spontaneous metastasis assay was performed according to the method of Fidler.[10] Either F10, BL6, plasmid-transfected or fusion cells (2.5×10^5) were injected subcutaneously into the footpad of 4-week-old, male C57BL/6 mice (7 mice per cell line). Three weeks later, when tumors reached a diameter of 8–10 mm, the legs bearing tumors were amputated. The mice were allowed to survive a further 4 weeks. During this period, grayish or black metastatic nodules grew to about 1–2 mm in diameter in the lungs. Four weeks after the amputation, autopsies were performed. We counted the number of macroscopic metastatic nodules on the surface of the bilateral lungs. In the preliminary experiments, this number was confirmed to be parallel with the number of metastatic nodules countable in the coronal section of the lungs. Lung tissues were also weighed.

Western blotting Whole cell lysates were prepared by adding 10 times the volume of the lysis buffer (50 m*M* Tris-Cl [pH 8.0], 150 m*M* NaCl, 1% Triton X-100, 1 m*M* PMSF) to a 10^6 cell pellet. The lysate (50 μg of protein) was boiled for 5 min, separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred onto Immobilon membrane (Millipore, Eschborn, Germany). The membrane was incubated with goat anti-annexin VII antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h, then incubated with horseradish peroxidase-conjugated anti-goat IgG antibody (1:1000 dilution; MBL, Nagoya). Blots were washed with phosphate-buffered saline (PBS) and reacted with Renaissance chemiluminescence reagents (Dupont/NEN, Boston, MA) before exposure. After stripping, the blot was reacted with mouse monoclonal anti-actin antibody (clone C4; Boehringer Mannheim, Mannheim, Germany) plus horseradish peroxidase-conjugated anti-mouse IgG antibody (Pharmingen, San Diego, CA), using a similar procedure.

Human melanoma and nevus samples Eighteen patients with melanoma received surgical operation at the time of diagnosis and were followed-up for more than five years in the Osaka University Hospital. Metastatic or recurrent lesions were also excised. Three lesions with intradermal nevus were excised in the same hospital. Surgical samples were fixed with formalin and used for histologic examination.

Histologic examination Formalin-fixed, paraffin-embedded malignant melanoma or intradermal nevus tissues were cut serially. Of three adjacent sections, the first was stained with hematoxylin and eosin (H&E), the second was used for immunohistochemistry, and the third was stained with alcian blue to detect mast cells. The second sections were boiled in 0.1% sodium citrate for 5 min and incubated with goat IgG anti-annexinVII polyclonal antibody at 1:100 dilution for 4 h. After incubation, the sections were washed with PBS, incubated with fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG antibody (1:1000) and observed with a darkfield fluorescence microscope.

RESULTS

Cloning of genes transcriptionally downregulated in BL6 cells We performed a DD procedure to isolate genes whose expression was upregulated in F10 cells. Poly(A)$^+$ RNA was reverse-transcribed with 9 downstream anchor primers and combined in PCR reactions with 24 upstream arbitrary primers. Approximately 9000 bands were produced on acrylamide gel. Fifteen bands were differentially displayed in a repeat experiment. Such bands as DD 8-20U, DD 8-20M and DD 8-20D (Fig. 1A) were excised and reamplified in the second PCR. The PCR products were separated on 3% agarose gels containing HA yellow (Fig. 1B). Major bands, denoted by arrowheads in Fig. 1B, were excised and subcloned into pT7-Blue vector. Cloned inserts were used as a probe for the northern analysis. Three clones derived from DD 8-20U, DD 8-20M and DD 8-20D gave a stronger hybridization signal in F10-derived RNA than in BL6-derived RNA (Fig. 1C).

We also constructed a subtracted cDNA library using F10- and BL6-derived mRNA. Plasmid clones carrying cDNA inserts complementary to BL6-derived mRNA were removed from a F10 cDNA library. After three rounds of subtraction, 600 clones were rescued and examined one by one for mRNA expression in F10 and BL6 cells. One clone, FB #3-19, was found to show a marked induction of expression in F10 cells (Fig. 1C).

We sequenced four clones isolated above. Three cDNAs obtained by DD were found to have the expected primer

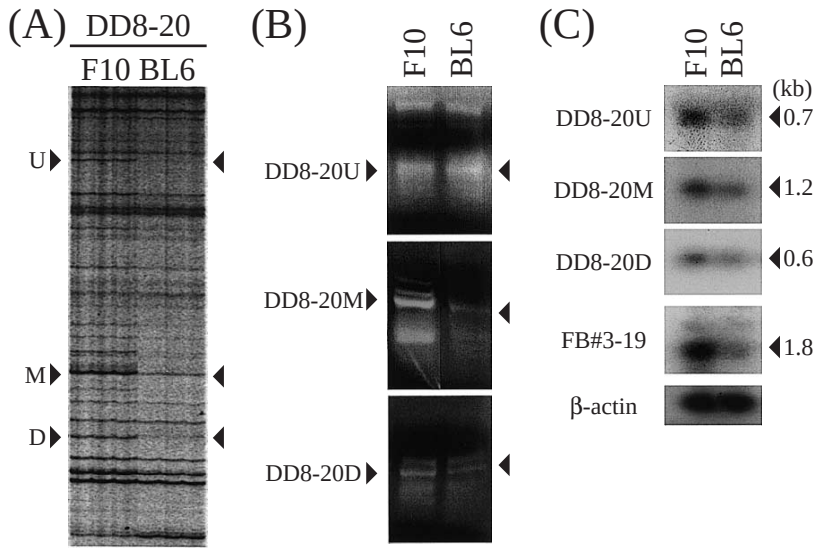

Fig. 1. Isolation of genes transcriptionally downregulated in BL6 cells by DD and subtraction. (A) Representative result of the first PCR products. The PCR was done with the downstream primer TTTTTTTTTTTGC and the upstream primer GATCAAGTCC. The product was separated on acrylamide gel and visualized with FMVIO II. Three bands of interest, denoted by arrowheads and letters (U, M and D), were excised. (B) The second PCR of the three bands in (A). The PCR products were separated on 3.0% agarose gel containing HA yellow. Major bands, denoted by arrowheads, were excised and subcloned. (C) Northern blot analysis of total RNA from F10 and BL6 cells. Cloned inserts derived from the second PCR products were used as a probe. FB #3-19 was obtained while screening the subtracted cDNA library.

sequence (downstream primer; TTTTTTTTTTTGC and upstream primer; GATCAAGTCC) at the 5′ and 3′ ends. DD 8-20M, throughout 282 nucleotides, was 97% identical with the deposited sequence (GenBank accession no. AU041176), having neither a functional nor a structural motif. DD 8-20U, throughout 331 nucleotides, was 83% identical with rat transferrin mRNA.[16] DD 8-20D contained 245 nucleotides and matched (97% identical) the 5′ end region of mouse ribosomal protein L26.[17] FB #3-19 contained an approximately 0.8-kb insert corresponding to the coding sequence of mouse annexin VII.[18]

Metastatic properties of hybrid cells between F10 and BL6 cells To examine the genetic dominance of the gene expression and the metastatic phenotype between F10 and BL6 cells, we constructed hybrid cells by somatic cell fusion of both cells. To obtain clones carrying different drug-resistant markers, F10 and BL6 cells were transfected with plasmids conferring resistance to G-418 or hygromycin B. Four clones were isolated: F10-G (G-418-resistant F10 cells), F10-H (hygromycin B-resistant F10 cells), BL6-G (G-418-resistant BL6 cells), and BL6-H (hygromycin B-resistant BL6 cells). The drug-resistant cells did not exhibit any alteration in metastatic potential (data not shown). We also checked if genetic complementation in the hybrid cells of two low-metastatic clones resulted in the acquisition of metastatic ability.[19] Homofusion hybrid clones were isolated on the basis of a hexomic chromosome composition, because both F10 and BL6 cells displayed a trisomic chromosome composition of, on average, 61 chromosomes. Two F10 homofusion clones (FF-1 and FF-2) derived from F10-G and F10-H cells, and two BL6 homofusion clones (BB-1 and BB-2) derived from BL6-G and BL6-H cells were examined for the metastatic phenotype. FF and BB clones were poorly and highly metastatic, respectively (Table I). These control experiments indicated that genetic complementation did not occur in these clones. Three hexomic heterofusion clones (FB-1, FB-2, and FB-3) were obtained by fusing F10-G cells with BL6-H cells, and 3 clones (BF-1, BF-2, and BF-3) were obtained by fusing BL6-G cells with F10-H cells. In the spontaneous metastasis assay, three clones (FB-3, BF-2, and BF-3) were as poorly metastatic as F10 cells (Table I). The other clones (FB-1, FB-2 and BF-1) were a little more metastatic than the former three clones, but much less metastatic than BL6 cells. When the chromosome number of cells in lung nodules was examined, the original chromosome composition was conserved during *in vivo* proliferation and metastasis (data not shown). These results indicated that the low-metastatic phenotype of F10 cells was dominant over the high-metastatic phenotype of BL6 cells.

mRNA expression of the four genes isolated by DD and subtraction was examined in FB-3 and BF-2 clones, both of which were as poorly metastatic as F10 cells (Fig. 2).

Table I. Metastatic Properties of Hybrid Cells between F10 and BL6 Cells

Clone	Number of lung nodules		Lung weight (mg) mean±SD
	mean±SD	(range)	
F10	2.9±6.2	(0–20)	167.1±49.7
BL6	34.7±26.5	(5–70)	311.8±155.7
FF-1	3.7±3.1	(1– 7)	175.3±41.1
FF-2	3.0±1.7	(2– 5)	199.7±8.1
BB-1	21.0±11.2*	(10–33)	247.5±46.5
BB-2	19.8±16.0**	(6–36)	281.8±38.1
FB-1	6.3±7.6	(1–15)	150.1±11.0
FB-2	9.5±9.5	(1–20)	181.8±22.2
FB-3	6.6±5.9***	(0–15)	188.2±72.0
BF-1	8.2±12.9	(0–30)	156.8±39.4
BF-2	0.3±0.6***	(0– 1)	132.7±10.1
BF-3	4.0±5.1***	(0–15)	157.6±21.8

Homofusion and heterofusion clones were examined with a spontaneous metastasis assay.
∗ $P<0.05$ by t-test when compared to the values of FF-1 and FF-2 clones. ∗∗ $P=0.15$ and 0.14 by t-test when compared to the values of FF-1 and FF-2 clones. ∗∗∗ $P<0.05$ by t-test when compared to the value of BL6 cells.

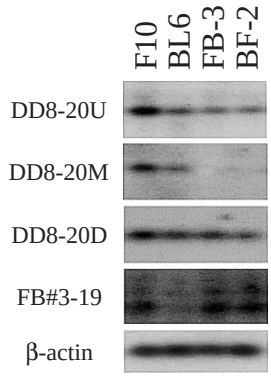

Fig. 2. Genetic dominance of gene expression between F10 and BL6 cells. Northern blot analysis of RNA from F10, BL6 and two heterofusion clones, FB-3 and BF-2. The blots were probed with the four cloned inserts used in Fig. 1C. Reprobing with β-actin probe verified equal loading.

The two fusion clones expressed the annexin VII gene (FB #3-19) as abundantly as F10 cells. The expression of other genes than annexin VII in the fusion cells was similar to or below that in BL6 cells. Since both F10 cells and the two fusion clones were poorly metastatic, annexin VII was the most probable candidate for a metastasis-suppressor, as judged from the mRNA expression profiles. Subsequently, we focused our attention on annexin VII.

Annexin VII expression in human melanoma cells We examined the annexin VII expression in clinical cases of malignant melanoma. While examining the expression in tissue sections, we found a prominent expression of the annexin VII protein in skin mast cells (denoted by arrowheads in Fig. 3). The expression was confirmed by western blot analysis (Fig. 4). Human lung fibroblast cell line TIG-1 was used as a positive control, because the annexin VII mRNA has been detected in lung tissues and a fibroblast cDNA library.[18, 20–22] Cultured mast cells (CMC) established from spleen cells expressed the annexin VII protein as abundantly as TIG-1 cells. The expression in F10 cells was detectable, but weaker than that in mast cells. In contrast, the expression in BL6 cells was below the limit of detection, consistent with the difference in the annexin VII message level between F10 and BL6 cells (Fig. 4). The immunoreactivity of melanoma cells to anti-annexin VII antibody was evaluated in comparison with the positivity of mast cells resident in the same section. When more than 90% of melanoma cells were immunoreactive to anti-annexin VII antibody, the annexin VII staining was expressed as "positive." In some cases, the immunoreactivity was expressed as "strong" because it was as strong as that of mast cells. When more than 90% of melanoma cells were not immunoreactive, the annexin VII staining was expressed as "negative." In some cases, melanoma cells showed a heterologous immunoreactivity: approximately 20% of melanoma cells were positive to the antibody. The annexin VII staining for these cases was expressed as "patchy."

Eighteen patients with malignant melanoma were retrospectively divided into two groups; less (cases 1–11) and more (cases 12–18) malignant phenotype groups (Table II). All the patients had received operations, wide excision or amputation, at the time of diagnosis. Patients of the less malignant phenotype group survived without lymph node metastasis or recurrence for more than 5 years after the first operations. Patients of the more malignant phenotype group died of malignant melanoma or suffered from lymph node metastasis or recurrence within 5 years after the first operations. According to the clinicopathologic staging by the International Union Against Cancer (UICC),[23] all patients of the less malignant phenotype group were classified into stage I or II. In the more malignant phenotype group, four were at stage I or II, two (cases 13 and 18) were at stage III due to lymph node metastasis at diagnosis, and one (case 14) was at stage VI due to lung metastasis at diagnosis. The primary lesions varied in depth from Clark's level II to level V.[24] The two groups were compatible in this respect. In the less malignant phenotype group, nine out of eleven cases showed an annexin VII-positive staining in melanoma cells of the primary lesions. Of the nine, three cases (cases 1, 8 and 10) showed a strong staining (Fig. 3, A and B). In contrast, only one (case 12) out of seven cases belonging to the more malignant phenotype group was positive in the pri-

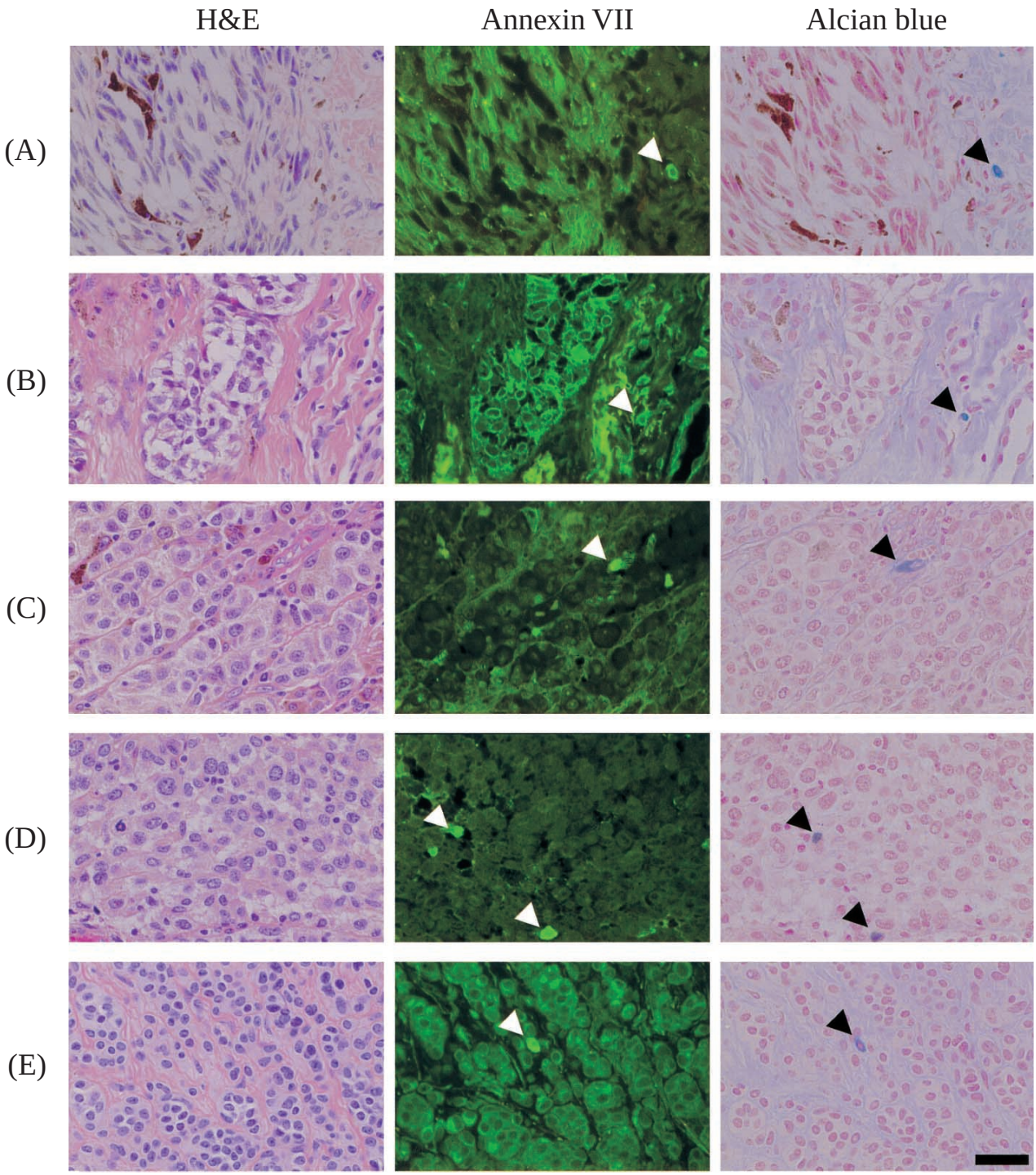

Fig. 3. Examples of immunohistochemistry using anti-annexinVII antibody. Of three serial sections, the first was stained with H&E (left column), the second was reacted with anti-annexin VII antibody (middle column), and the third was stained with alcian blue (right column). Arrowheads indicate the same mast cells recognized in two adjacent sections. All the tissues were observed at the same magnification. Bar=50 μm. The immunoreactivity is summarized in Table I. (A) Primary lesion of case 10 of malignant melanoma. (B) Primary lesion of case 1 of malignant melanoma. (C) Primary lesion of case 17 of malignant melanoma. (D) Lymph node metastasis of case 16 of malignant melanoma. (E) Sections of case 2 of intradermal nevus.

mary lesions. Two cases (cases 16 and 18) showed a heterologous, patchy staining. The ratios of numbers of patients positive versus negative and patchy to annexin VII were 9/11 and 1/7 in the less and more xmalignant phenotype groups, respectively. The difference in the ratios between the two groups was significant by the χ^2-test ($P<0.05$). In addition, the metastatic and recurrent lesions of all the cases were negative (Fig. 3, C and D). We also examined three cases with intradermal nevus, a benign melanocytic lesion, for annexin VII expression (Table III). In all cases, the nevus cells were positive (Fig. 3E). These results indicated that annexin VII can serve as a clinical marker for less malignant phenotype of malignant melanoma.

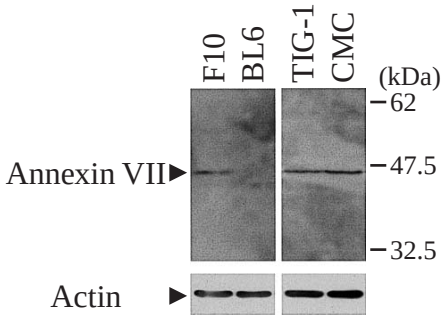

Fig. 4. Western blot analysis to detect the annexin VII protein expression. Cultured mast cells (CMC) contained annexin VII as abundantly as TIG-1 cells. The expression level of the annexin VII protein corresponded to that of the message in F10 and BL6 cells. Reprobing with anti-actin antibody verified equal loading.

DISCUSSION

In our previous report, the method for constructing a subtracted cDNA library was applied to isolate genes that were transcriptionally upregulated in BL6 cells.[9] Plasmid clones carrying cDNA inserts complementary to F10-derived mRNA were removed from a BL6 cDNA library. More than ten clones were isolated. On the other hand, only one clone carrying annexin VII cDNA was obtained

Table III. Clinical and Immunohistochemical Data of Intradermal Nevus Patients

	Sex/Age at diagnosis	Primary site /diameter (cm)	Annexin VII staining
Case 1	M/14	head/1.5	positive
Case 2	F/38	head/1.2	positive
Case 3	F/16	arm/1.0	positive

Nevus cells showed a uniform positive staining to the anti-annexin VII antibody.

Table II. Clinical and Immunohistochemical Data of Malignant Melanoma Patients

	Sex/Age at diagnosis	Primary site/ diameter (cm)	Operation at diagnosis	Post-operative chemotherapy	Years of survival	Staging at diagnosis (UICC, 1987)	Clinical phenotype of malignancy				Annexin VII staining			
							Primary lesion (Clark's level)	Lymph node metastasis		Recurrence	Primary lesion	Lymph node metastasis		Recurrence
								at diagnosis (1st operation)	post-operative follow-up			at diagnosis (1st operation)	post-operative follow-up	
Case 1	M/66	sole/0.5	wide excision	DAV-Feron	>5	I	II	no	no	no	strong			
Case 2	F/52	sole/1.0	wide excision	DAV-Feron	>5	I	II	no	no	no	positive			
Case 3	F/43	toe/1.0	amputation	DAV-Feron	>5	I	II	no	no	no	positive			
Case 4	M/56	arm/0.4	amputation	DAV-Feron	>5	I	II	no	no	no	positive			
Case 5	M/49	abdomen/2.1	wide excision	DAV-Feron	>5	I	III	no	no	no	positive			
Case 6	F/54	sole/0.8	amputation	decarbazine	>5	I	III	no	no	no	positive			
Case 7	M/54	sole/0.5	wide excision	DAV-Feron	>5	I	III	no	no	no	negative			
Case 8	F/49	sole/0.4	wide excision	DAV-Feron	>5	II	IV	no	no	no	strong			
Case 9	M/10	back/3.0	wide excision	none	>5	II	IV	no	no	no	positive			
Case10	F/45	toe/1.5	amputation	DAV-Feron	>5	II	IV	no	no	no	strong			
Case11	M/57	head/0.8	wide excision	DAV-Feron	>5	II	IV	no	no	no	negative			
Case12	M/61	sole/0.6	amputation	DAV-Feron	>5	I	II	no	yes	yes	positive		negative	negative
Case13	F/59	finger/1.0	amputation	DAV-Feron	>5	III	II	yes	no	no	negative	negative		
Case14	M/86	sole/1.7	wide excision	Feron	1	IV	III	yes	yes	yes	negative	negative	negative	negative
Case15	F/60	sole/0.8	wide excision	DAV-Feron	1	I	III	no	no	yes	negative			negative
Case16	F/58	leg/2.0	wide excision	DAV-Feron	>5	II	IV	no	yes	no	patchy		negative	
Case17	F/54	sole/0.9	wide excision	DAV-Feron	>5	II	IV	no	no	yes	negative			negative
Case18	M/41	sole/0.8	amputation	DAV-Feron	2	III	V	no	yes	yes	patchy		negative	negative

Eighteen patients with malignant melanoma received surgical operations at diagnosis. The operations included wide excision or amputation. Wide excision excised the melanoma lesions with surrounding skin to a width of 5 cm. Most amputation operations were done at finger or ankle joints. After operation, all cases but case 9 received adjuvant chemotherapy. Abbreviations: DAV, decarbazine, nimustine and vincristine; Feron, interferon-β.

as a gene transcriptionally upregulated in F10 cells, although the same subtraction method was applied. Ishiguro *et al*. isolated genes expressed differently between F10 and BL6 cells by differential hybridization.[25] Consistent with our results, the number of clones transcriptionally upregulated in F10 cells was smaller than that of clones transcriptionally upregulated in BL6 cells. In addition to examination of genetic difference, we determined the genetic dominance of the metastatic phenotype between F10 and BL6 cells. The fusion cells of both cells were poorly metastatic, indicating that the high-metastatic phenotype is a genetically recessive trait in hybrids between F10 and BL6 cells. This also suggested that F10 cells may possess a metastasis-suppressor, which dominates over BL6-derived high metastatic potential in the fusion cells. Among genes isolated by DD and subtraction, annexin VII was found to be expressed at a higher level not only in F10 but also in the fusion cells. This expression profile was consistent with that of a putative metastasis-suppressor.

We examined the annexin VII expression in melanoma cells of clinical cases. We found that the annexin VII expression was inversely correlated with malignant phenotype, but independent of Clark's level staging (Table II).[24] This suggested that annexin VII could be a clinical marker that directly reflects the metastatic properties of melanoma cells. Consistent with this suggestion, two cases of the more malignant phenotype group showed a patchy staining in the primary lesions, but were negative in the metastatic lesions. Only annexin VII-negative melanoma cells, but not annexin VII-positive cells, could metastasize into lymph nodes. Skin mast cells were also annexin VII-positive and always resident around melanoma lesions. Thus, mast cells served as a positive control to evaluate the immunoreactivity in melanoma cells.

Annexins are a large group of calcium-dependent cytoskeleton- and membrane-associated proteins whose characters largely depend upon their phospholipid-binding activity.[26] They have been implicated in multiple aspects of cell biology including regulation of membrane trafficking, transmembrane channel activity, transduction of mitogenic signal, and inhibition of phospholipase A_2.[27–32] Balch and Dedman reported that annexins II and V inhibit Lewis lung carcinoma cell migration.[33] They supposed that annexins II and V binding to phosphatidylserine might restrict phospholipid movement in the plasma membrane, resulting in increased membrane rigidity and blocking of membrane flow necessary for tumor cell migration. Recently, annexin VI has been shown to be downregulated during melanoma progression from a benign to a more malignant phenotype.[34, 35] The annexin VII expression profile demonstrated here was similar to that of annexin VI. Since all annexins have phospholipid-binding activity, annexins VI and VII might suppress melanoma progression via the same mechanism as was proposed for annexins II and V. Even though the exact role of these annexins in malignant progression remains elusive, further characterization of annexins as a malignant cell marker should provide deeper insights into the molecular mechanisms of metastasis.

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