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Advances in Brief

Identification by Differential Display of Annexin-VI, a Gene Differentially Expressed during Melanoma Progression

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Abstract

To identify genes involved in the melanocyte to malignant melanoma conversion, we have applied differential display to the comparison of syngeneic murine B16F10 (metastatic melanoma) and Melan-a-immortalized melanocyte cell lines. Approximately 7000 bands were analyzed, revealing approximately 80 to be differentially displayed. Reverse Northern blotting and subsequent Northern blotting confirmed the reproducible differential expression of four transcripts. Three B16F10-specific bands encode novel genes or partially sequenced cDNAs of unknown function. One Melan-a-specific band was found to be identical to the 3′ end region of the mouse Annexin VI mRNA and shown to have a reduced message expression in B16F10 relative to Melan-a. Differential expression was confirmed at the protein level with Western blotting using a rabbit polyclonal antiserum. Immunohistochemistry of human melanoma specimens with this antiserum revealed a decrease or loss of Annexin VI expression as melanomas progressed from a benign to a more malignant phenotype. Our results provide further evidence for a potential role of Annexin VI in tumor suppression.

Introduction

Malignant melanoma is a disease thats incidence is increasing dramatically in various parts of the world, and there is a need to characterize the genetic basis of the progression of this cancer. The superficial location of cutaneous melanoma, and its often pigmented nature, has meant that this tumor has been well characterized in histological terms for its development and evolution (1, 2). The availability of a number of human and murine cell lines established from the various stages of tumor development has allowed some progress in understanding the molecular processes associated with the pathology of this disease (for an overview, see Refs. 3 and 4).

DD[2] is a rapid and effective technique (5) to use to identify differences in gene expression between related cell lines (6). This procedure already has been applied to melanoma. Recently, von Groningen *et al.* (7) identified selective expression of melanoma inhibitory activity, as well as other genes, with DD comparison. Their study compared human melanoma cell lines of different metastatic capacity and pooled dysplastic nevi *versus* metastatic melanoma lesions (7). However, their analysis of the identified genes was confined to mRNA expression and was not confirmed at the protein level.

In this report, we describe the application of DD to the comparison of murine B16F10 (metastatic melanoma; Ref. 8) and Melan-a (immortalized melanocyte) cell lines (9). Both lines are syngeneic, being derived from C57BL/6 mice, and therefore provide an excellent model to study changes in gene expression associated with melanoma progression (9).

We report the isolation of four differentially expressed transcripts, one of which corresponds to Annexin VI. This protein, recently reported to have tumor suppressive activity in an unrelated tumor type (10), is a member of a calcium-dependent phospholipid-binding protein family (known as the annexins) for which the biological function(s) remains unclear (11). Immunohistochemical analysis of Annexin VI expression in normal human melanocytes and melanoma specimens of varying stages of tumor evolution suggests a possible role in melanoma progression.

Materials and Methods

RNA Isolation and Northern Blotting. Total RNA was isolated using the RNA-Stat-60 kit (Biogenesis, Poole, United Kingdom) following the manufacturer's instructions. RNA was DNase treated (as described in Ref. 12) and quantitated by U.V. absorbance. Twenty μg of total RNA were size fractionated on a 1% agarose-formaldehyde gel and transferred onto Hybond N filters (Amersham, Aylesbury, United Kingdom). Probes were [α-^{32}P]dCTP labeled using an oligolabeling kit (Pharmacia Biotech, Uppsala, Sweden) and hybridized to filters overnight at 42°C. Filters were washed twice in 1× SSC/0.1% SDS at room temperature for 15 min followed by 0.25× SSC/0.1%SDS for 15 to 30 min at 60°C and autoradiographed for 1 to 7 days.

DD Reverse Transcription-PCR. Primers HAP 1–8 were purchased from GenHunter (Brookline, MA). Primers HAP9A-55A, 13-mers designed with a 5′ *Hin*dIII site and an arbitrary 3′ end, were obtained from the Oligonucleotide Synthesis Laboratory (Imperial Cancer Research Fund Clare Hall, South Mimms, United Kingdom). A 0.4-μg aliquot of total RNA was reverse transcribed in 1× reverse transcriptase buffer [25 mM Tris-Cl (pH 8.3), 37.6 mM KCl, 1.5 mM $MgCl_2$, and 5 mM DTT] with 0.2 μM $HT_{11}M$ (where M is C, G, or A) and 20 μM deoxynucleotide triphosphates. Conditions were 65°C for 5 min and then cooling to 37°C for 10 min. This was followed by the addition of 1.0 μl of 100 mM DTT and 2 μl of murine Moloney leukemia virus reverse transcriptase (50 units/μl; Stratagene, Cambridge, United Kingdom) in a total volume of 20 μl, which was incubated for 50 min at 37°C. Reverse transcriptase was inactivated by 75°C for 5 min. For each PCR reaction, 2 μl of cDNA were made to 20 μl in 1× PCR buffer [10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$], 2.0 μM deoxynucleotide triphosphates, 0.2 μM upstream 13-mer (HAP1-55A), 0.2 μM downstream $HT_{11}M$, 0.4 μCi [α-^{33}P]dATP (Amersham), and 1 unit *Pic Taq* polymerase (Imperial Cancer Research Fund). Conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 40°C for 2 min, and 72°C for 30 s. A final extension was carried out at 72°C for 5 min. Eight μl of each PCR reaction were made to 5% glycerol and run on a nondenaturing 6% polyacrylamide gel at 55 W constant power for 2 to 3 h. Gels were dried and exposed overnight. Identified bands were isolated (as described in Ref.12), reamplified, and cloned into pCRII (Invitrogen BV, NV Leek, the Netherlands).

Reverse Northern Blotting. Reamplified bands were resolved on a 1.5% agarose gel, purified (using QUIAEX gel extraction kit II; Qiagen, Dorking Surrey, United Kingdom), made to 50 μl in distilled water, and applied to Hybond N filters using a slot blot manifold (13). Ten μg of total RNA (in 10 μl dH_2O) from B16F10 or Melan-a cells was reverse transcribed as described above, except 5 μg *Not*I-dT_{18} was used as primer and 5 μl [α-^{32}P]dCTP (3000 mCi/mmol) were included. The labeled cDNA was RNase A treated (2 μl of 10 mg/ml; 37°C for 30 min), purified on a G-50 Sephadex column, and counted using a beta counter. Probes (2 × 10^6cpm) were added to each filter and hybridized (in 10% dextran sulfate/1 M NaCl/1% SDS and 50 μg/ml of

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[2] The abbreviation used is: DD, differential display.

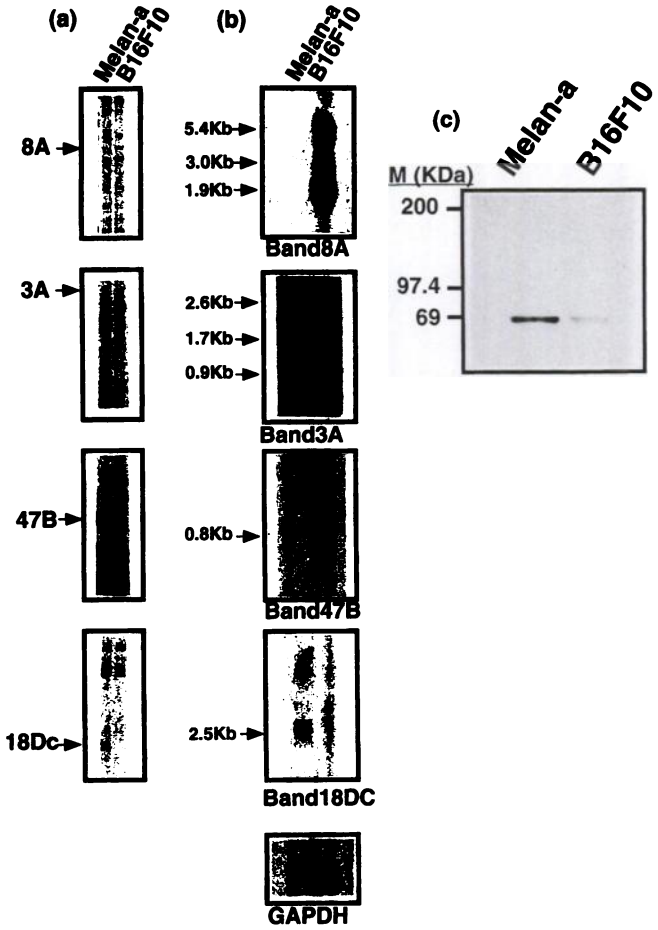

Fig. 1. Analysis of identified bands for DD. *a*, total RNA was reverse transcribed followed by PCR using [^{33}P]dATP as a label and was resolved on a 6% nondenaturing sequencing gel. *b*, Northern blotting using 20 μg of total RNA/lane. Probes were labeled using [^{32}P]dCTP. Size of bands was approximated by relative migration with respect to 18S and 28S bands. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe was used as loading control. *c*, Western blot of 5 μg of crude cell lysate/lane blotted using MC2 (anti-Annexin VI) polyclonal antibody and detected using ECL. *KDa*, kilodaltons.

sheared salmon sperm DNA) overnight at 57°C. Filters were washed in 2× SSC/1% SDS at 57°C for 30 min and exposed for 3 to 4 days.

Cell Culture. Growth conditions for B16F10 cells were in Eagle's MEM supplemented with 10% FCS and 4 mM L-glutamine. The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (200 nM) and cholera toxin (100 pg/ml) were also included for growth of Melan-a cells (9).

Immunohistochemical Staining. Tissue sections from human benign nevus and melanoma were microwaved in 0.1% sodium citrate for 10 min and then incubated with rabbit anti-Annexin VI polyclonal antibody, MC2 (14), at 1:100 dilution. For detection, the standard streptavidin-biotin complex and diaminobenzidine were used. Normal infiltrating lymphocytes and fibroblasts acted as internal positive controls.

Western Blotting. Whole-cell lysates were prepared by adding 10 times the volume of the lysis buffer (2% SDS/100 mM DTT/60 mM Tris-Cl, pH 6.8) to a 10^6 cell pellet. After a brief vortex, lysates were passed several times through a 26-gauge needle to shear genomic DNA. Five μg of protein lysate (measured by U.V. absorbance at 260 nm) were boiled for 5 min. and loaded onto an 8% polyacrylamide-SDS gel and run at a constant current of 35 mA for 2 h. Transfer was performed with electroblotting using Hybond C filters. Filters were blotted using MC2 antibody at a 1:1000 dilution followed by peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) at a 1:1000 dilution. For detection, ECL was used (Amersham).

Results

DD and Reverse Northern Blotting. Total RNA was isolated from Melan-a and B16F10 cells grown to approximately 70% confluency. Reverse transcription was carried out using three $HT_{11}M$ and combined in PCR reactions with fifty-five 13-mers (HAPs1-55A) producing approximately 7000 bands (corresponding to roughly one third of the estimated total message population). Approximately 80 bands were observed to be differentially displayed on a repeat experiment. These bands were excised from the gels, purified, and tested using reverse Northern blotting (which included a positive control,

(a) Band 47B.

1 AAGCTTGCTC AGAACATGGG CAAAAAGCCC ATTCATTTTA GACTGTGAAG
51 AAACTCGCTA CCAACTCTAC TGTATTCTGA CTATAAATAT TTATTAATAA
101 AACAGATCCC ACGGTAGAAC CTGAGGACCT GCGAGGACCT GAGGTGTATG
151 ACACGAGGTG TCATTGACAA CAATAGTGAT GATCTGATGA CATGTAATAA
201 ATGATACACT GCCTGCAAAA AAAAAAAGCTT

(b) Band 8A.

1 AAGCTTTTAC CGCAACTCAA AGACCCATTG GAACAAGACA ATACACGGCT
51 TGATATACAA CGCCCTGAAA CTCTTCATGG AGATGAACCA AAAACTCTTC
101 GATGACTGCA CTCAGCAGTT CAAAGCAGAG AAACTCAAAG AGAAGCTAAA
151 AATCGAAGAG CGAGAAGAAG CATGGGTTAA AATAGAAATC TAGCCAAAGC
201 GAATCCCCAG GTACTAAAAA AGAGAGTAAC TCGGGAGTGT TGAGGCTTTG
251 CGTGAATGTC TGAGATAGGG CCTGGCTCCA CCCCAGGAAG GGAGGCCAAC
301 GTCACTAACA CTGTATGTGC AAATGTCCGA ATA AAACACT TTCCAACTTT
351 GTAAAAAAAA AAAGCTT

(c) Band 3A.

1 AAGCTTTGGT CAGGGTATTC GGTCATCCGA CAATGCCTTG CAGGTAGATC
51 TCTGCTGTGG GAAAGGAATG GGGTGAGGAA GGCCGGAAGA CAGGGGGCAG
101 GGAGGTAGGC TCATCACTCT GAGCCTGCAG GAGAGAGGAA AGAATGTGAG
151 ATTGGATTGT GTGTGTCTGA GGAATACCCA TTCTGTCCTA TTTTGGTATT
201 GTCATGAAGT CACCAGGATG ACTCTATCTT TCTAGTTGCC CACCACCTGT
251 GGCTTTAGCT CCGTCCCCAT ATTTCCCTAC CCTTATACCT CCTATCCCGC
301 CATCTCTACC CCCCTGGGCA TTTTCTGGCT AGCCTGGATA GATTTAGAGA
351 CCTGCTTGTT GGCTATAATG TCTTTTCATT CTTTCTCTTC CTTTTTTAAA
401 AAATCGGAGC AAAACCAAAA AGTGTCCAAA AAAAAAGCTT

(d) Band 18DC.

1 AAGCTTCATG GTCTAAGGAA GACGCAAGGG GTGGGGTTGG ACTGTCCATC
51 CAGCTGGGGC ACTCTCTAGC CATTGAGGTC CAGGCGGTCC CTTCTGTTAT
101 CCCTCCCGCT CATGGCTAAG GGGCTGGTGA ACCACTGCCA TATACTGCAT
151 TCAACTCTGA TCCCTTCTAC CTACTCCTCC GCCCGCCCTG GTTTCCATAA
201 CTCCACATAA CCCTGCCTGA CTTGAGCTTT GTCACATCTC AAGACATACC
251 AACTCTGTCT ATGAAAAAA AAAGCTT

Fig. 2. Sequence of identified bands 47B (Genbank accession no. U59417), 8A (Genbank accession no. U59418), 3A (Genbank accession no. U59419), and 18DC. *Underlined*, primers used in the PCR.

Table 1 *Summary of immunohistochemical analysis of malignant and benign human melanocytic lesions using anti-Annexin VI antibody, MC2*

	Weakest area of lesion					Staining	
Lesion	3+	2+	1+	+/−	Negative	Uniform	Patchy
Malignant	1	0	2	2	5	1	8
Benign	8	2	1	0	0	10	1

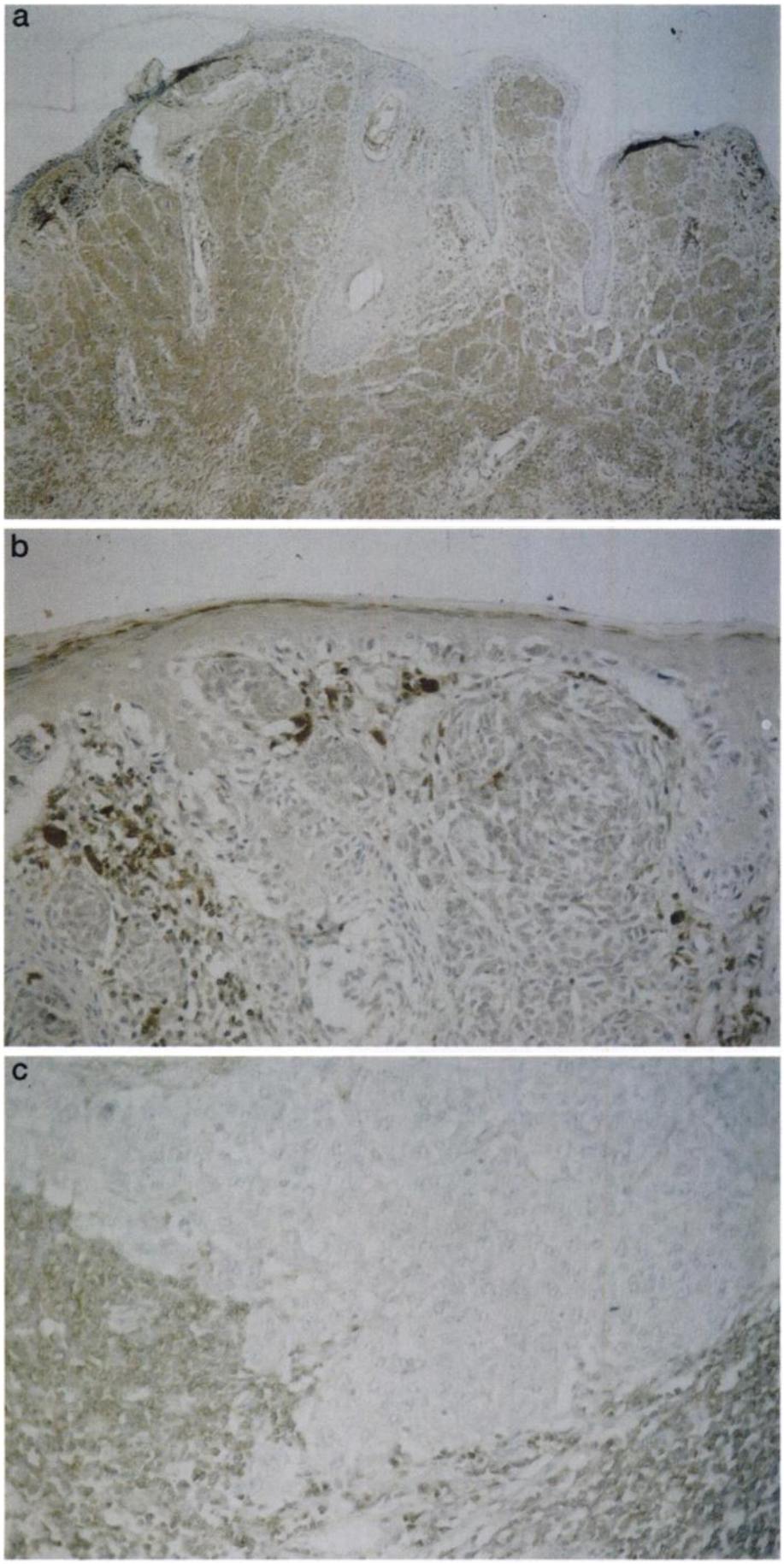

Fig. 3. Examples of immunohistochemistry using anti-Annexin VI polyclonal antibody (MC2). Sections of benign nevus (*a*, uniform positive brown staining), melanoma (*b*, patchy staining), and lymph node metastasis with lymph node staining positive (*c*); the metastatic lesion is negative. Adjacent highly positive lymphocytes acted as positive controls.

band 27A, as well as a negative control, the bacterial plasmid ptZ18). Four bands (8A, 3A, 47B, and 18DC; Fig. 1*a*) were observed to be differentially expressed using the reverse Northern blotting. However, the majority of bands (~90%) were revealed as false positives by this technique. This result was confirmed with Northern blotting using six arbitrarily selected bands which failed to show a difference of expression, suggesting that the value volume for false positives was true rather than a consequence of some deficiency in the reverse Northern blotting procedure (data not shown).

Northern Blotting. Band 8A hybridized to three transcripts of 1.9, 3.0, and 5.4 kb, which all were B16F10 specific; band 3A identified three transcripts of 0.9, 1.7, and 2.6 kb, all specific or preferentially expressed in B16F10; band 47B recognized a 0.9-kb transcript as B16F10 specific. Band 18DC was preferentially expressed in Melan-a, recognizing a 2.5-kb transcript (Fig. 1*b*).

Sequence Analysis. All four bands were found to have the expected primers at the 5′ and 3′ ends (Fig. 2). A search of the European Molecular Biology Laboratory data base revealed band 47B and band 3A to be novel. Band 8A was found to be >90% homologous to a partially sequenced human cDNA from the myeloblast cell line KG1 (KIAA0044; EMBL accession no. D26445). Band 18DC matched perfectly (98% identical), throughout 276 bp, the 3′ end region of the mouse Annexin VI mRNA.

Western Blotting and Immunohistochemistry. Using the MC2 antibody, a M_r 68,000 band was detected in the tested lines. This was markedly reduced in B16F10 relative to Melan-a cells, as expected from the level of mRNA transcripts (Fig. 1*c*). Immunohistochemical staining of 11 benign and 9 malignant human melanocytic lesions indicated a clear inverse relationship between Annexin VI staining and tumor progression (Table 1 and Fig. 3).

Discussion

Previously identified genes associated with melanoma progression have been revealed by DD using cell lines or tissues of differing origin (*i.e.*, isolated from various donors; Ref. 7). To overcome the potential problem of detecting origin-specific differences [*e.g.*, MHC allotype (7)], we chose to use a syngeneic mouse melanoma model composed of two cell lines: B16F10, which is highly metastatic in syngeneic mice, and Melan-a, which is a nontumorigenic melanocyte line (9). To determine the relevance, if any, of observed differences, we evaluated their presence (or absence) in material derived from human tumor material. We report that 4 out of approximately 7000 transcripts were shown by DD, reverse Northern blotting, and Northern blotting to be differentially expressed between the two cell types. Three transcripts were expressed preferentially by melanoma as compared to melanocyte cells. It may be interesting to pursue further investigation into their role in melanoma tumor progression.

The fourth transcript, expressed preferentially by nontumorigenic melanocytes, was identified as encoding for mouse Annexin VI. Expression of this protein in A431 human squamous epithelial carcinoma cells (which lack Annexin VI) led to a reduction in their *in vitro* growth (15) and tumorigenicity in nude mice (10). To investigate further the possible involvement of Annexin VI in melanoma development and progression, we looked at expression of this molecule in human melanoma tissue specimens. Fig. 3 and Table 1 show a clear difference between benign (uniform positive staining of the cytoplasm) and malignant (patchy or negative staining) tissue with regard to expression of this protein.

Intracellular expression of Annexin VI has been suggested to have tumor-suppressive effects by contributing to cell-cell contact-dependent growth regulation (10). Our results are compatible with this hypothesis. As far as we know, ours is the first study to document a correlation (*i.e.*, down-regulation) of Annexin VI expression and tumor development using naturally occurring human tumors as distinct from rodent model systems. However, there are reports of extracellular expression in human and rodent cell lines (16, 17), where it is proposed that Annexin VI could participate in malignant spread by contributing to tumor cell attachment to the endothelium. It may be that the subcellular location of Annexin VI (as distinct from absolute levels) plays an important role in modulating the effect this protein has on cell behavior. Transfection of Annexin VI into A431 cells using a plasmid construct led to intracellular, but not extracellular, expression with concomitant abrogation of tumorigenic capacity.[3] Interestingly, in view of this finding, B16F10 cells have been reported not to express Annexin VI on the cell surface (17), although we detected expression in total cell lysates (Fig. 1*c*), although, relative to melanocytes, at low levels. We are currently producing B16F10 transfectants overexpressing Annexin VI to further address the role of this protein in melanoma behavior.

This article describes how the application of DD to an appropriate cell line model, coupled with an effective screening of identified bands (reverse Northern blotting and analysis of clinical samples), can allow the rapid identification of genes potentially associated with tumor progression.

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[3] S. Moss, unpublished observation.

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