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Identification of Melanoma Inhibitory Activity and Other Differentially Expressed Messenger RNAs in Human Melanoma Cell Lines with Different Metastatic Capacity by Messenger RNA Differential Display

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ABSTRACT

The differential display technique was used to identify mRNAs differentially expressed in human melanoma cell lines with different metastatic capacity. We report the isolation of nine different clones, of which four were uniquely expressed in the highly metastatic human melanoma cell line MV3, whereas the other five clones were uniquely expressed in the poorly metastatic human melanoma cell line 530. The differences in expression identified by differential mRNA display were confirmed by Northern blot analyses. DNA sequencing followed by computer search analyses indicated that of the nine differentially expressed clones, five represented novel gene products. The other four were histocompatibility antigen HLA-DR, laminin B_2, melanoma inhibitory activity (MIA), and tissue inhibitor of metalloproteinases 3. MIA was also identified in RNA from human melanoma metastasis lesions in a comparison by differential display with pooled human nevi. Northern blot analysis confirmed MIA mRNA expression in nonmetastasizing melanoma cell lines and in melanoma metastasis lesions, while expression was absent in highly metastasizing cell lines and pretumor stages. In the 11 metastasis lesions examined, MIA mRNA expression was apparently inversely correlated with pigmentation.

INTRODUCTION

The incidence of human cutaneous melanoma increases more rapidly than any other cancer in the last decade (1). All lesions are thought to develop from melanocytes and represent subsequent stages of progression accompanied by increasing atypia. An early primary melanoma, characterized by horizontal growth (radial growth phase), can develop into an advanced melanoma that is also capable of growth in the vertical direction (vertical growth phase) and, eventually, of metastasis. Extensive research efforts are invested to identify reliable markers that should facilitate diagnosis of the neoplastic progression stage and the design of efficient therapeutic strategies. The characterization of relevant progression markers inherently provides better insight in the tumor biology of melanoma.

In the last few years, we reported the isolation of several potential progression markers, *e.g.*, calcyclin (2), thymosin β10 (3), *nma* (4), and *nmb* (5) by applying differential and subtraction hybridization techniques. As it is the case for other markers as well, the expression profile of these genes is not unequivocal, but shows overlap between different stages of tumor progression.

We applied the differential mRNA display method to obtain additional informative progression markers. The differential mRNA display technique is based on RT[2]-PCR and allows rapid isolation of differentially expressed transcripts from two or more cell types. The advantage of this technique lies in its ease of operation and the minimal quantities of total RNA required for analysis. Furthermore, it gives access to transcripts of low abundance that are hardly or not detectable using subtractive hybridization techniques (6, 7). By using human melanoma cell lines with different metastatic capacities, the application of this method led to the isolation of nine clones, which were uniquely or differentially expressed either in the highly metastatic human melanoma cell lines MV3 (8) and BLM (9), or in the poorly metastatic human melanoma cell lines 530 and 1F6 (9). From these clones, five represented novel genes, whereas four clones were recorded genes. One of the recorded genes, known as MIA was also identified by using the differential mRNA display method to compare pooled human metastases *versus* dysplastic nevi.

MIA was identified as a melanoma growth inhibitory activity in the culture supernatant of a cell line derived from a human melanoma metastasis in the central nervous system (10, 11). MIA is secreted by a number of malignant melanoma cell lines and acts as a potent growth inhibitor for malignant melanoma and other cell lines (12).

Our results on expression in melanoma lesions characterize MIA as an ontogenic melanoma marker apparently correlated with the loss of pigmentation.

MATERIALS AND METHODS

Melanoma Cell Lines. Human melanoma cell lines MV3, BLM, 530, and 1F6 (8, 9) were grown as monolayers on DMEM (GIBCO Laboratories, Grand Island, New York) supplemented with 10% FCS (GIBCO Laboratories), glutamine (2 mM), penicillin G (100 units/ml), streptomycin (100 μg/ml), and pyruvate (1 mM). Within this panel of cell lines, 1F6 and 530 represent poorly metastasizing cell lines, with a metastasis frequency of < 10% three months after s.c. inoculation into nude mice. The cell lines BLM and MV3 represent the highly metastatic phenotype, with over 50% metastasis frequency.

Human Tissues. After excision, large parts of melanoma metastases were immediately frozen in liquid nitrogen and stored at −80°C. Dissected melanoma metastases were processed individually and were taken from patients other than those from whom the nevi were removed. For RNA isolations from dysplastic nevi, a representative slice was taken. Most of the skin surrounding these lesions was cut off before freezing the material in liquid nitrogen. The remainder was processed for conventional histopathology. Nevi were obtained from patients without any history of melanoma, whereas normal skin was obtained from patients who had also developed melanoma. When using nevi or normal skin tissue, 6–22 biopsies of 6–17 patients were pooled to obtain enough material.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated using the lithium-urea procedure as described by Auffray and Rougeon (13). Ten μg of total RNA were glyoxylated (14), size fractionated on 1% agarose gels, and blotted to Hybond N-plus (Amersham, Aylesbury, United Kingdom). To confirm that equal amounts were loaded in each lane, the blots were hybridized afterwards with an 18S rRNA probe.

mRNA Differential Display. For differential display of mRNA, we used the RNAmap protocol from GenHunter (Brookline, MA). Two hundred ng of total RNA (after DNase treatment with the Message-Clean kit; GenHunter) from the human melanoma cell lines MV3 and 530 were reverse transcribed in a total reaction volume of 20 μl containing 1× reverse transcriptase buffer [125 mM Tris-HCl (pH 8.3), 188 mM KCl, 7.5 mM $MgCl_2$, and 25 mM DTT],

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[2] The abbreviations used are: RT, reverse transcription; MIA, melanoma inhibitory activity; dNTP, deoxyribonucleoside triphosphate; TIMP, tissue inhibitor of metalloproteinases.

100 units of the Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD), 20 μM dNTPs and 20 pmol $T_{12}MG$, $T_{12}MC$, $T_{12}MA$, or $T_{12}MT$ (where M may be dG, dC, or dA), respectively, as a primer. Reactions were carried out in a Gene E thermocycler (Techne Limited, Cambridge, United Kingdom): 65°C for 5 min, 37°C for 1 h, and then 95°C for 5 min. Reverse transcriptase was added after 10 min at 37°C.

One tenth of the cDNA was then amplified by PCR in a total volume of 20 μl containing 1× PCR buffer [10 mM Tris-HCl (pH 9.0) and 1.5 mM $MgCl_2$], 2 μM dNTPs, 0.5 μl ^{35}S-dATP (Amersham), 1 unit Taq polymerase (1 unit/ml) using 20 pmol of the corresponding $T_{12}MN$ and 4 pmol of an arbitrary 10 mer (AP 6–15). The samples were overlaid with 25 μl mineral oil and subjected to 40 cycles of PCR in a Gene E thermocycler using the following parameters: 94°C for 30 s, 42°C for 2 min, and 72°C for 30 s; the last cycle was followed by a 5-min extension at 72°C. Two μl of loading dye were added to 3.5 μl of the amplification products, heated at 80°C for 2 min, and loaded on a 6% DNA sequencing gel. Gels were dried without fixation onto filter paper and subjected to autoradiography using Kodak XAR-5 films.

We also applied the mRNA differential display to isolate differentially expressed mRNAs in human dysplastic nevi and melanoma metastasis lesions. Melanoma metastasis RNA consisted of a pool of metastases from 8 patients, whereas dysplastic nevi RNA was a pool of 17 lesions from 16 patients. Differential mRNA display was performed using RNA map kit B (AP 6–10) as described above, except that [^{32}P]dATP was used instead of ^{35}S-dATP.

Recovery and Amplification. The cDNA bands representing differentially expressed mRNAs were excised from the gel, rehydrated in 100 μl distilled H_2O for 15 min, and boiled for 10 min. Solid debris was removed by centrifugation, and the cDNA in the supernatant was precipitated by the addition of 10 μl 3 M sodium acetate (pH 5.3), 5 μl glycogen (10 mg/ml; Sigma Chemical, St. Louis, MO), and 450 μl 100% ethanol. The pellet was redissolved in 10 μl H_2O. For each band, 4 μl extracted cDNA were reamplified for 30 cycles in a total volume of 40 μl with the same primer set and PCR conditions used in the initial RT-PCR, except that no radioactive dNTP was included. After PCR 30 μl of the samples were run on a 1.5% agarose gel and stained with ethidium bromide. PCR bands of the expected size were cut from the gel, purified (15), and used as probes for Northern blot analyses.

Cloning and Dot-Blot Selection. Unique or differential expression of the reamplified PCR products in the human melanoma cell lines was checked by Northern blot analyses, and the positive fragments were cloned into the pCRII vector by the TA cloning system (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Selection of positive clones by the dot-blot procedure was carried out as described by Callard *et al.* (16) using the recovered PCR fragments as probes.

DNA Sequencing and Computer Analyses. Cloned reamplified PCR fragments were sequenced according to the dideoxy method as described by Sanger *et al.* (17) using a Sequenase sequencing kit (United States Biochemical, Cleveland, OH). Large fragments were sequenced by constructing a set of deletion clones with exonuclease III (Erase-a-base kit; Promega, Madison, WI). Identification of known sequences was performed using the Genbank and EMBL data bases (18, 19).

Construction of cDNA Libraries. A cDNA library was constructed from 5 μg oligodeoxythymidine-selected RNA using a λZap cDNA synthesis kit (Stratagene, La Jolla, CA) according to the procedure recommended by the manufacturer. Oligodeoxythymidine-selected RNA from the poorly metastatic human melanoma cell line 530 was used for the construction of the 530 λZAP cDNA library.

DNA Probes and Hybridization. DNA probes were radiolabeled by the random prime labeling method as described by Feinberg and Vogelstein (20). Hybridization of cDNA libraries was performed according to standard protocols (15).

DNA Isolation and Southern Blot Analysis. Chromosomal DNA was isolated according to the method of Blin and Stafford (21). DNA was digested with *Eco*RI, and 10 μg were size fractionated on 0.6% agarose gels, transferred to Hybond N-plus, and hybridized as described previously for Northern blot analysis. To check for equal loading of DNA, the blot was hybridized afterward to a chromosome 18-specific centromeric probe. No abnormalities involving chromosome 18 are known in relation to melanoma.

RESULTS

Differentially Expressed mRNAs in Human Melanoma Cell Lines. Human melanoma cell lines MV3 and 530 were used to isolate potential melanoma progression markers. MV3 is a highly metastatic human melanoma cell line, while 530 has a very low potential to metastasize. To analyze the expression pattern of both cell lines by RT-PCR, 40 different combinations of primer sets were used composed of four degenerate anchored oligo(dT) primers ($T_{12}MG$, $T_{12}MA$, $T_{12}MC$, or $T_{12}MT$) and 10 short arbitrary 10 mers (AP 6–15). A total of 19 fragments was found to be uniquely expressed by either of two human melanoma cell lines MV3 or 530. These cDNAs fragments were reamplified by PCR and used as probes for hybridization of Northern blots containing total RNA of the human melanoma cell lines MV3 and 530. Of 19 MV3/530 differential display products, 9 fragments were confirmed to be differentially or uniquely expressed. A similar comparison of RNA from dysplastic nevi and melanoma metastasis lesions yielded 12 differential display products, of which only one was confirmed to be uniquely expressed by Northern blot analysis of the human melanoma cell lines MV3 and 530. The differential display of the 9 + 1 PCR-amplified cDNAs (clones 1–10), which detect uniquely or differentially expressed mRNAs on a Northern blot, is shown in Fig. 1. Some fragments appeared to be doublets or even sets of three or four bands with nearly the same intensity. These bands represent the two strands of one fragment and probably result from an additional A known to be added by Taq polymerase. The 9 + 1 PCR fragments that detected differentially expressed mRNAs between the human melanoma cell lines MV3 and 530 were cloned into the TA cloning vector, and recombinants were selected by the dot-blot procedure. The cloned PCR fragments were used as probes to hybridize a Northern blot containing total RNA of two highly metastatic (MV3 and BLM) and two poorly metastatic (1F6 and 530) human melanoma cell lines. As shown in Fig. 2, clone 1 detected a 1.7-kb transcript expressed only in 530 cells (Fig. 2*A*). Clone 2 detected a 1.8-kb transcript highly expressed in 530, weakly in 1F6, and not detectable in MV3 and BLM cells (Fig. 2*B*). A 4.6-kb transcript was detected by clone 3, showing high expression in BLM and modest expression in MV3 while expression was absent in 530 and 1F6 cells (Fig. 2*C*). Clone 4 detected a 0.55-kb transcript present only in 530 and 1F6 cells (Fig. 2*D*). Clone 5 displayed a weak expression in MV3 and even weaker expression in BLM cells of a 0.45-kb transcript (Fig. 2*E*). Clone 6 detected a 2.0-kb transcript only present in 530 and 1F6 cells (Fig. 2*F*). A 5.0-kb transcript was detected by clone 7, with a high expression in BLM and weakly detectable in MV3 and 1F6, whereas expression was absent in 530 cells (Fig. 2*G*). Clone 8 detected a 4.4-kb transcript highly expressed in 1F6, moderately in 530, and very weakly in MV3 and BLM cells (Fig. 2*H*). A 4.7-kb transcript was detected only in the MV3 and BLM cells by clone 9 (Fig. 2*I*). Clone 10 detected a 0.55-kb transcript only in the 530 and 1F6 cells (Fig. 2*J*).

DNA Sequencing and Computer Analysis. All 10 differentially expressed cDNA fragments were analyzed by DNA sequencing (data are shown in Fig. 3). DNA sequencing revealed that clones 4 and 10 are identical. A computer search against Genbank and EMBL DNA data bases revealed that three clones (clones 2, 5, and 6) had no significant homology to any DNA sequences. Clone 1 showed 99% homology to human mRNA for the histocompatibility antigen HLA-DR (α chain; Ref. 22). Clone 3 showed 96.5% homology (11 mismatches; 3 at the upstream AP-9 primer) to a 518-bp human cDNA clone (EMBL accession no. T57750). Both clones 4 and 10

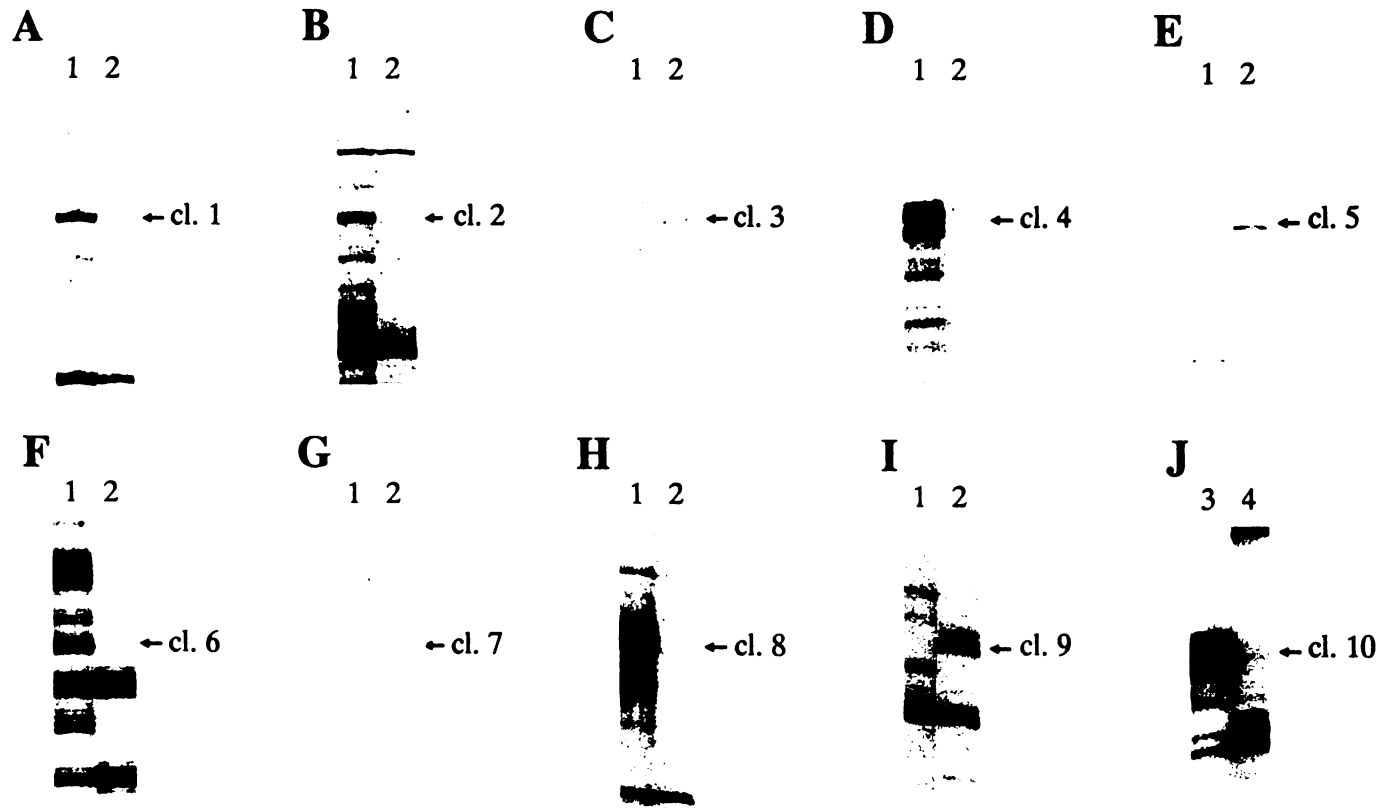

Fig. 1. Differential mRNA display using total RNA isolated from human melanoma cell lines 530 (*Lanes 1*), MV3 (*Lanes 2*), pooled human melanoma metastases (*Lanes 3*), and dysplastic nevi (*Lanes 4*). Total RNA was reverse transcribed, followed by PCR amplification in the presence of [^{35}S]-dATP (*A-I*) or [^{32}P]dATP (*J*). The PCR fragments were fractionated on 6% DNA sequencing gel and autoradiographed as detailed in "Materials and Methods." Primers used are: *A*, clone 1 (*cl. 1*) AP-9/T_{12}MC; *B*, clone 2 (*cl. 2*) AP-7/T_{12}MC; *C*, clone 3 (*cl. 3*) AP-9/T_{12}MT; *D*, clone 4 (*cl. 4*) AP-6/T_{12}MC; *E*, clone 5 (*cl. 5*) AP-8/T_{12}MT; *F*, clone 6 (*cl. 6*) AP-10/T_{12}MG; *G*, clone 7 (*cl. 7*) AP-12/T_{12}MT; *H*, clone 8 (*cl. 8*) AP-12/T_{12}MT; *I*, clone 9 (*cl. 9*) AP-15/T_{12}MT; and *J*, clone 10 (*cl. 10*) AP-6/T_{12}MC. *Arrows*, cDNA fragments.

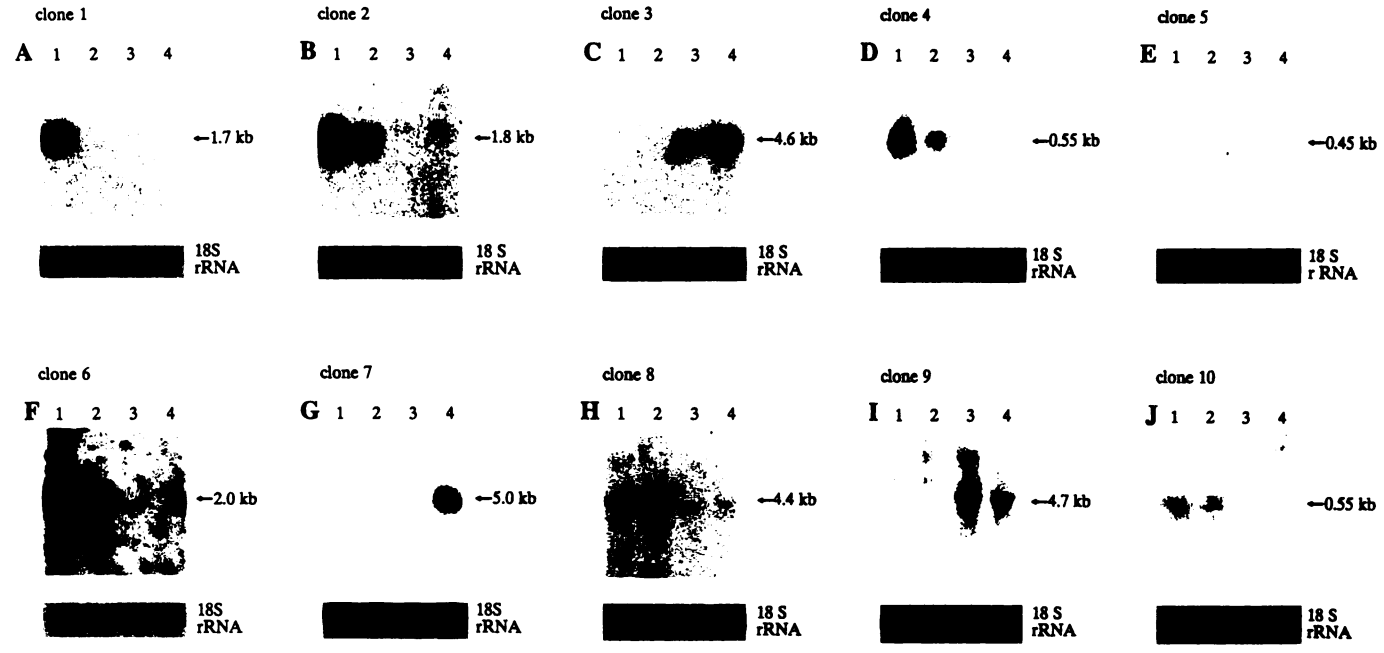

Fig. 2. Northern blot analysis of a panel of four human melanoma cell lines with differential display products as probes. Ten μg of total RNA were loaded in each lane. *Lanes 1*, 530; *Lanes 2*, 1F6; *Lanes 3*, BLM; *Lanes 4*, MV3. *Blots A-J* were hybridized to cloned PCR fragments 1–10, respectively. The molecular weight marker was λDNA digested with restriction endonuclease *Hin*dIII. As a control for the amount of RNA loaded in each lane, an 18S rRNA hybridization is shown.

```
clone 1
  1 cgtggcaata tgacctcagt gaaagcagtc atcttcagcg
 51 ttttccagcc ctatagccac ctcaagtgtg gttatgcctc ctcgattgct
101 ccgtactcta acatctagct ggcttccctg tctattgcct tttcctgtat
151 ctatttcct ctatttccta tcatttatt atcaccatgc aatgcctctg
201 gaataaaaca tacaggagtc tgtctctgct atggaatgcc ccatgactct
251 cttgtgtact tattgtttaa ggtttcctca aactgtgatt ttctgaacac
301 aataaactat ttgatgatct tgggtggaaa aaaaaaaaa

clone 2
  1 ccgaagaatg aaaagagagc tctaaccaga tggaacactg gaacattcca
 51 gtggaccctg gaccattcca ggaaaactgg gacataggat cgtcccgcta
101 tgatggaagt gttcagacag tttataatag taagcccctg tgaccctctc
151 acttacccg agacctcact ttattacaag atctttccaa atacccaaat
201 gtccctgcaa gcccgttaaa taattcccta tgctaccctt aataacatac
251 aatgaccaca tagtgtgaga acttccaaca agcctcaaag tcccttgaga
301 ctccccaata cctaataagg catgcgaaat gttctcatga actaccccac
351 aacacgccta aaactcaaaa cacccaaaaa tacctcctcc aatgtcctga
401 aacatgaacc caaaaagaga cccacaataa actcgtgact tgtccccaaa
451 aaaaaaaaa

clone 3
  1 ggtggcaata gagagagtta tgctacaatt atttcttggt ttccacttgc
 51 aatggttaat taagtccaaa aacagctgtc agaacctcga gagcagaaca
101 tgagaaactc agagctctgg accgaaagca gaaagtttgc cgggaaaaaa
151 aaagacaaca ttattaccat cgattcagtg cctggataaa gaggaaagct
201 tacttgttta atggcagcca catgcacgaa gatgctaaga agaaaaagaa
251 ttccaaatcc tcaacttttg aggtttcggc tctccaattt aactctttgg
301 caacaggaaa caggttttgc aagttcaagg ttcactccct atatgtgatt
351 ataggaattg ttgtggaaat ggattaacat acccgtctat gcctaaaaga
401 taataaaact gaaatatgtc ttcacaaaaa aaaaaaa

clone 4
  1 gcaatcgatg tgaagacaga caaatgggat ttctactgcc agtgagctca
 51 gcctaccgct ggccctgccg tttcccctcc ttgggtttat gcaaatacaa
101 tcagcccagt gcaaaaaaaa aaaa

clone 5
  1 ggattgtgcg atgttatgtt catgttaatc ctatttgtaa aatgaagtgt
 51 tcccaacctt atgttaaaag agagaagtaa ataacagact gtattcagtt
101 attttgccct ttattgagga accagatttg tcttcttttt gtttgtaatc
151 tcatttttgaa ataatcagca agttgaggta cttcttcaaa tgctttgtac
201 aatataaact gttatgcctt cagtgcatta ctatgggagg agcaagaaaa
251 ataaagactt acaaaaagga gtattttccc acaaaaaaaa aaaa

clone 6
  1 tagcaagtgc agttaagtat gatgggaaag ctaaaatggg tatgtacata
 51 agatcggcaa aggaaaccaa gttctgtaaa atgagttctc cctccctcca
101 gggtagctga ttatgaggaa aataagaaag agctttgctt ttctccttag
151 tagtaatggt ctacaataag ctgcacacac acatccctca tcacacctct
201 ctccaaaaa aaaaaaa

clone 7
  1 tgctgacctg gagaatgaag ccaagaagca ggtaggctgc cattatggac
 51 tataaccgag atatcgagga gatcatgaag gacattcgca atctggagga
101 catcaggaag accttaccat ctggctgctt caacacccg tccattgaaa
151 agccctagtg tctttagggc tggaaggcag catccctctg acaggggggc
201 agttgtgagg ccacagagtg ccttgacaca aagattacat ttttcagacc
251 cccactcctc tgctgctgtc catccactgt ccttttgaac caggaaaagt
301 cacagagttt aaagagaagc aatttaaaca tcctgaatcg ggaacaaagg
351 gttttatcta ataaaagtgt ctcttccata aaaaaaaaaa a

clone 8
  1 tgctgacctg aaacccagaa gtgatggaga gaaaccaaca agagatctcg
 51 aaccctgtct agaaggaatg tatttgttgc taaatttcgt agcactgttt
101 acagttttcc tccattttat ttatgaattt tatattccgt gaatgtatat
151 tgtcttgtaa tgttgcataa tgttcacttt ttatagtgtg tcctttattc
201 taaacagtaa agtggtttta tttctatcac aaaaaaaaaa aa

clone 9
  1 agggcctgtt ggggaagtgg gagatagagg agatattggt taaaggatag
 51 aaaatttttag ttagacaaga ggaataagtt caagaatatt gtctaacatg
101 gtgactatac ttacaacaac aactgcatta atgatatatt tgtacttgaa
151 atttgcttag agtagattta gtgtttaccc acaaaaaaaa aaaa

clone 10
  1 gcaatcgatg tgaagacaga caaatgggat ttctactgcc agtgagctca
 51 gcctaccgct ggccctgccg tttcccctcc ttgggtttat gcaaatacaa
101 tcagcccagt gcaaaaaaaa aaaa
```

Fig. 3. Partial cDNA nucleotide sequence of 10 mRNAs differentially expressed in human melanoma cell lines with different metastatic potential. Flanking sequences representing primer sets used for PCR amplification are *underlined*. The GenBank accession numbers for the novel clones are: clone 2, U31214; clone 3, U30999; clone 5, U31000; clone 6, U30998; and clone 9, U31001.

showed 97.5% homology (three mismatches at the upstream AP-6 primer) to human mRNA for MIA (12). Clone 7 showed 98% homology (four mismatches; two at the upstream AP-12 primer) to human mRNA fragment for laminin B_2 (23). Clone 8 showed 99.6% homology (one mismatch at the upstream AP-12 primer) to human TIMP-3 (24, 25). Clone 9 showed 69% homology to the human cytotoxic serine protease B (*CSP-B*) gene flanking sequence (data base accession no. M62716). All clones were, as expected, flanked by the sequences of the primer set used for PCR amplification. The summarized data on the isolation of these 10 clones, their expression pattern, and computer search results are listed in Table 1.

Characterization of MIA. Clones 4 and 10 (MIA) were identified by the differential display technique when using melanoma cell lines with different metastatic capacities (clone 4) and also by using pooled melanoma metastases *versus* pooled dysplastic nevi (clone 10). Clone 4 was analyzed in more detail. A cDNA library was constructed from the poorly metastatic human melanoma cell line 530. The library had a complexity of 4×10^6 recombinant clones, with an average insert length of 1.7 (range, 0.9–3.4) kb. The cDNA insert of clone 4 was used as a probe to screen a 530 λZap cDNA library, resulting in the isolation of a 0.55-kb cDNA clone, designated pJG460. DNA sequencing (Fig. 4) and computer data base search revealed, as expected, that this cDNA was identical to mRNA for MIA (12), while our MIA cDNA clone is extended at the 5′ end with 14 nucleotides (positions 1–14) and at the 3′ end with 43 nucleotides (positions 474–516, poly(A) tail excluded).

To exclude the possibility that gene amplifications or rearrangements could be responsible for the differences in MIA mRNA expression between poorly and highly metastatic human melanoma cell lines, we hybridized a Southern blot containing *Eco*RI-digested genomic DNA from a panel of human melanoma cell lines with the 0.55-kb MIA cDNA insert of pJG460. A 17-kb *Eco*RI fragment was detected by the 0.55-kb MIA cDNA insert (Fig. 5). The hybridization pattern of MIA is comparable for the different human melanoma cell lines (with only a slight elevation of MIA signal in BLM cells).

The expression of MIA in human melanocytic lesions was examined by a Northern blot analysis of several human melanocytic metastases and nevi. From 11 melanoma metastases, 8 (Fig. 6, *Lanes G, I, J, K, L, N, O,* and *P*) were used as a pool for mRNA differential display. Total RNA was hybridized with the 0.55-kb MIA cDNA insert of pJG460 as a probe (Fig. 6). MIA mRNA is not detectable in melanocytes (Fig. 6, *Lane A*), normal skin (Fig. 6, *Lane B*), dysplastic nevi (Fig. 6, *Lane C*), and 2 (Fig. 6, *Lanes*

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                                        26                                  53
GG AAA TTG GAG ACC CCA GCA CCC CCT TGC TCA CTC TCT TGC TCA CAG TCC ACG

                                        80                                 107
ATG GCC CGG TCC CTG GTG TGC CTT GGT GTC ATC ATC TTG CTG TCT GCC TTC TCC
Met Ala Arg Ser Leu Val Cys Leu Gly Val Ile Ile Leu Leu Ser Ala Phe Ser

                                       134                                 161
GGA CCT GGT GTC AGG GGT GGT CCT ATG CCC AAG CTG GCT GAC CGG AAG CTG TGT
Gly Pro Gly Val Arg Gly Gly Pro Met Pro Lys Leu Ala Asp Arg Lys Leu Cys

                                       188                                 215
GCG GAC CAG GAG TGC AGC CAC CCT ATC TCC ATG GCT GTG GCC CTT CAG GAC TAC
Ala Asp Gln Glu Cys Ser His Pro Ile Ser Met Ala Val Ala Leu Gln Asp Tyr

                                       242                                 269
ATG GCC CCC GAC TGC CGA TTC CTG ACC ATT CAC CGG GGC CAA GTG GTG TAT GTC
Met Ala Pro Asp Cys Arg Phe Leu Thr Ile His Arg Gly Gln Val Val Tyr Val

                                       296                                 323
TTC TCC AAG CTG AAG GGC CGT GGG CGG CTC TTC TGG GGA GGC AGC GTT CAG GGA
Phe Ser Lys Leu Lys Gly Arg Gly Arg Leu Phe Trp Gly Gly Ser Val Gln Gly

                                       350                                 377
GAT TAC TAT GGA GAT CTG GCT GCT CGC CTG GGC TAT TTC CCC AGT AGC ATT GTC
Asp Tyr Tyr Gly Asp Leu Ala Ala Arg Leu Gly Tyr Phe Pro Ser Ser Ile Val

                                       404                                 431
CGA GAG GAC CAG ACC CTG AAA CCT GGC AAA GTC GAT GTG AAG ACA GAC AAA TGG
Arg Glu Asp Gln Thr Leu Lys Pro Gly Lys Val Asp Val Lys Thr Asp Lys Trp

                                       458                                 485
GAT TTC TAC TGC CAG TGA GCT CAG CCT ACC GCT GGC CCT GCC GTT TCC CCT CCT
Asp Phe Tyr Cys Gln

                                       512
TGG GTT TAT GCA AAT ACA ATC AGC CCA GTG C(A)n
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Fig. 4. cDNA sequence of pJG460 (human MIA). 5′ and 3′ extensions to the published sequence by Blesch *et al.* (12) are *underlined*.

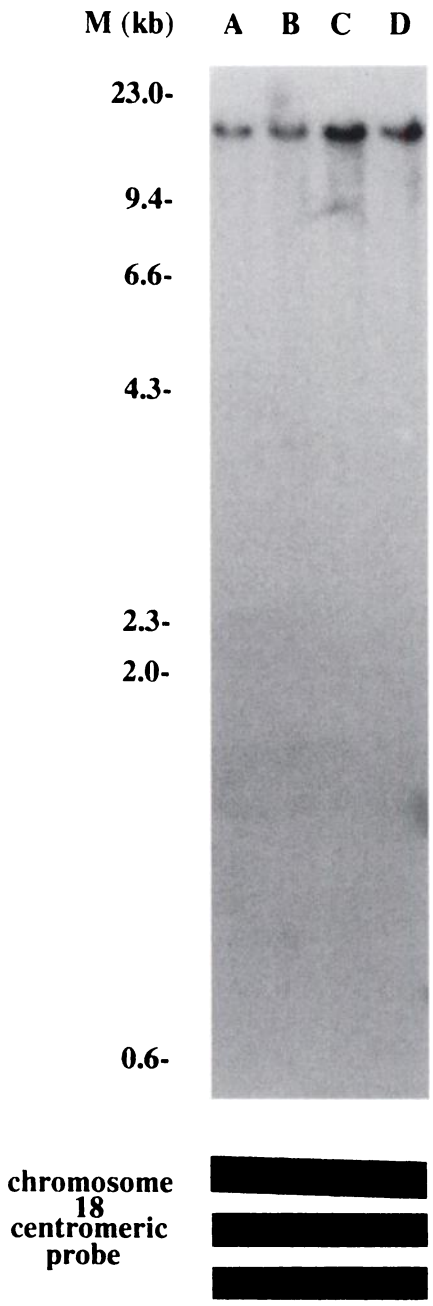

Fig. 5. Southern blot analysis of several human melanoma cell lines. Ten μg *Eco*RI-digested chromosomal DNA were loaded in each lane. *Lane A*, 530; *Lane B*, 1F6; *Lane C*, BLM; *Lane D*, MV3. The blot was hybridized to a radiolabeled 0.55-kb MIA cDNA insert of pJG460. The molecular weight marker was λDNA digested with restriction endonuclease *Hin*dIII. As a control to the amount of DNA loaded in each lane, a chromosome 18-specific centromeric hybridization is shown (three bands of approximately 20, 1.3, and 0.6 kb are visible). Abnormalities involving chromosome 18 were not described in relation to melanoma.

J and *L*) of 11 melanoma metastases. MIA mRNA expression is either absent or weak in melanoma metastases with strong pigmentation (Fig. 6, *Lanes I*, *J*, *L*, and *M*), whereas expression was moderate in melanoma metastases with weak pigmentation (Fig. 6, *Lanes F* and *N*) and high in melanoma metastases with no pigmentation (Fig. 6, *Lanes G*, *H*, *K*, *O*, and *P*). This could suggest a correlation between pigmentation and MIA mRNA expression in melanoma metastases. The results of the Northern blot analysis of the human melanocytic lesions confirm the differential display of melanoma metastases and dysplastic nevi, showing unique expression of clone 10 (MIA) human metastases. We were unable to detect MIA mRNA expression on a Northern blot containing total RNA from human kidney, lung, liver, placenta, spleen, prostate, and colon (results not shown).

DISCUSSION

This article describes the use of the differential mRNA display technique to identify potential progression markers for human melanoma. The analysis comparing the poorly metastatic cell line 530 *versus* the highly metastatic cell line MV3 yielded five cDNA fragments with unique expression in the poorly metastatic cell line 530 and four cDNA fragments uniquely expressed in the highly metastatic cell line MV3 (Fig. 1). Northern blots containing total RNA from a panel of human melanoma cell lines characterized for metastatic behavior confirmed the correlation between expression of these cDNA fragments and metastatic potential. Sequence analysis of the cDNA fragments revealed five clones to represent novel genes, whereas four clones represented reported genes.

Clone 4, detecting a 0.55-kb transcript in the poorly metastatic human melanoma cell lines 530 and 1F6 (Fig. 2*D*) is identical to the *MIA* gene (Table 1), a potent growth inhibitor for melanoma and other cells (10–12). The MIA cDNA fragment did not only emerge from the differential display analysis of mRNA present in the poorly metastasizing cell line 530 *versus* the highly metastatic cell line MV3, but also from a second analysis comparing pooled human nevi and pooled metastases. Surprisingly, in this analysis MIA mRNA expression was exclusively detected in pooled human metastases. Additional Northern blot analysis confirmed this paradoxical expression pattern: MIA is expressed in nonmetastasizing melanoma cell lines in culture (representative of the radial growth phase) and in melanoma metastasis lesions, while it is virtually absent in frequently metastasizing cell lines (representative of the vertical growth phase) and pretumor stages. The differences in MIA mRNA expression between poorly and highly metastasizing cell lines cannot be ascribed to major chromosomal rearrangements. MIA is apparently not expressed in normal somatic cells of adult organisms (see "Results" and Ref. 12). While Blesch *et al.* (12) reported MIA mRNA to be expressed in every melanoma cell line tested, in our panel of human melanoma cell lines MIA mRNA expression is restricted to poorly metastasizing cell lines. This would suggest it to be a potential marker for early stages of melanoma progression. Its presence in a considerable number of human melanoma metastasis lesions would be in contrast with this suggestion. Other potential early progression markers, notably *nm23* (26, 27), *nma* (4), and *nmb* (5) display a similar expression distribution. One could speculate that these genes and MIA may be involved in attenuating metastatic properties of melanoma cells, possibly as a consequence of tumor-host interactions. Tumor progression may require that genes are (temporarily) switched off in the course of metastasis, but turned on again later in a secondary, distantly growing tumor (4). In the limited number of lesions analyzed, it seems that MIA mRNA expression is inversely correlated with pigmentation of melanoma metastases, and because loss of pigmentation can be considered as a consequence of dedifferentiation, MIA may be regarded as a dedifferentiation marker. Immunohistochemical analysis of melanoma lesions may clarify this apparent paradox.

mRNA expression of clone 1 was only detected in the poorly metastatic human melanoma cell line 530 (Fig. 2*A*) and appeared to be identical to human histocompatibility antigen HLA-DR (Table 1). The cloning of this cDNA fragment is likely explained by the fact that

Table 1 *Isolation of differential expressed mRNAs in human melanoma cells*

Clone	PCR primers (5′ → 3′)	Fragment size (bp)	Northern blot expression				mRNA size (kb)	Homologous by computer search
			530	1F6	MV3	BLM		
cl.1	AP9:CGTGGCAATA T_{12}MC	344	++++	–	–	–	1.5	Human mRNA for histocompatibility antigen HLA-DR (99%)
cl.2	AP7:CCGAAGGAAT T_{12}MG	459	++++	++	–	–	1.8	Novel
cl.3	AP9:CGTGGCAATA T_{12}MT	437	–	–	++	++++	4.6	Human cDNA clone 79197 3′ end (97.5%)
cl.4	AP6:GCAATCGATG T_{12}MC	124	++	+	–	–	0.55	Human mRNA for melanoma growth regulatory protein MIA (97.5%)
cl.5	AP8:GGATTGTGCG T_{12}MT	294	–	–	+/–	+/–	0.45	Novel
cl.6	AP10:TAGCAAGTGC T_{12}MG	217	++++	++++	–	–	2.0	Novel
cl.7	AP12:TGCTGACCTG T_{12}MT	389	–	+	+	+++	5.0	Human mRNA fragment for laminin B_2 (98%)
cl.8	AP12:TGCTGACCTG T_{12}MT	242	+	+++	–	–	4.4	Human mRNA for TIMP-3 (99.6%)
cl.9	AP15:AGGGCCTGTT T_{12}MT	194	–	–	++	++	4.7	Human cytotoxic serine protease B (*CSP-B*) gene (69%)
cl.10	AP6:GCAATCGATG T_{12}MC	124	+	+	–	–	0.55	Human mRNA for melanoma growth regulatory protein MIA (97.5%)

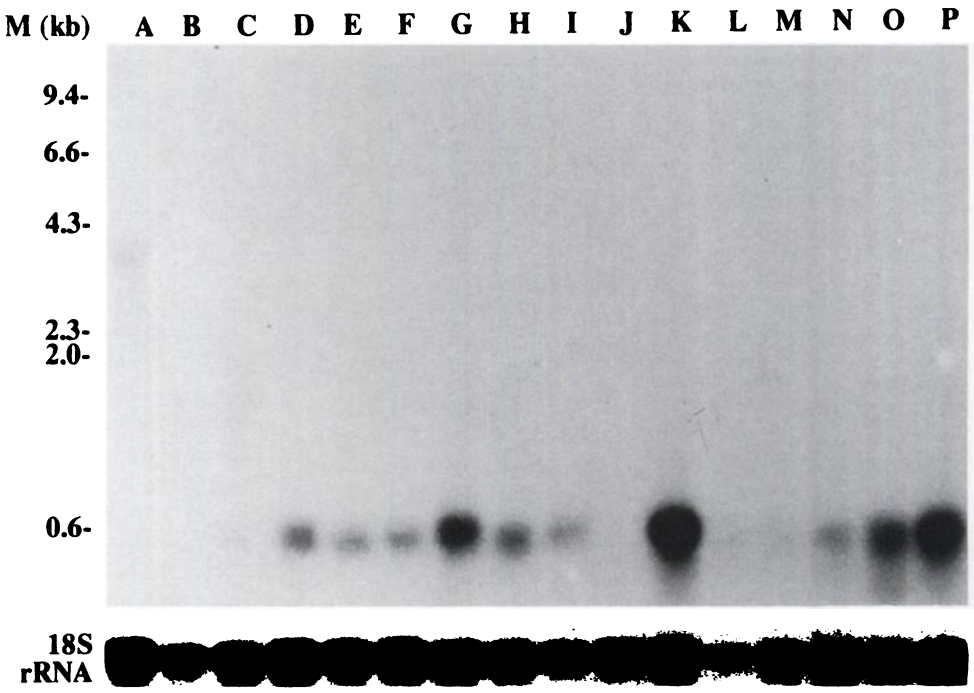

Fig. 6. Northern blot analysis of human cutaneous melanocytic lesions. Ten μg of total RNA were loaded in each lane. *Lane A*, melanocytes; *Lane B*, normal skin (13 biopsies from 12 patients); *Lane C*, dysplastic nevi (6 lesions from 6 patients); *Lane D*, xenograft derived from radial growth phase; *Lane E*, xenograft derived from vertical growth phase; *Lanes F-P*, melanoma metastases. Pigmentation was strong in the metastases in *Lanes I*, *J*, *L*, and *M*; weak in *Lanes F* and *N*; and absent in *Lanes G*, *H*, *K*, *O*, and *P*. The blot was hybridized to a radiolabeled 0.55-kb MIA cDNA insert from pJG460. The molecular weight marker was λDNA digested with restriction endonuclease *Hin*dIII. As a control to the amount of RNA loaded in each lane, an 18S rRNA hybridization is shown. Densitometric scanning showed that the maximum variation in 18S rRNA loading (between *Lanes L* and *D*) was not more than 3-fold, whereas this was 100-fold for MIA mRNA (between *Lanes M* and *K*, *Lanes A*, *B*, *C*, *J*, and *L* being negative). Pigmentation was scored by eyeball estimation of the pigment melanin intensity in the RNA samples. Pigmentation comparable to melanocyte samples was identified as strong; less pigmentation is scored as weak.

the human melanoma cell lines MV3 and 530 originated from different patients.

Clone 8, detecting a 4.4-kb transcript in the poorly metastatic human melanoma cell lines 530 and 1F6, appeared to be homologous to human TIMP-3 (Table 1). For TIMP-3 three transcripts of 2.2, 2.5, and 4.4 kb have been reported, probably as a result of alternative splicing or the presence of extended 3′ or 5′ untranslated regions in the TIMP-3 mRNA (24, 25). Clone 8 detected only the 4.4-kb transcript, and this indicates that the isolated 3′ end of the cDNA is absent in the two shorter mRNAs. TIMP-3, along with TIMP-1 and TIMP-2, belongs to a family of related but distinct genes functioning as naturally occurring inhibitors of matrix metalloproteinases, a group of enzymes implicated in degradation of the extracellular matrix (28). An imbalance between proteinases and their activators or inhibitors has been implicated in tumor invasion (29). Manipulation of the balance between matrix metalloproteinases and their inhibitors can induce or suppress abnormal cellular functions. Overproduction of TIMP-1 and TIMP-2 suppressed metastatic ability *in vivo* and markedly reduced tumor growth rate while completely suppressing local invasion (30, 31). We found only a strong TIMP-3 mRNA expression in the poorly metastatic human melanoma cell lines 530 and 1F6 and no expression in MV3 and BLM cells. These results suggests TIMP-3 to be a potential progression marker for early stages of melanoma development.

Our data substantiate that differential display is a powerful and fast technique to analyze gene expression and to identify both activated and repressed gene fragments from the same reaction. The low amounts of RNA required would even allow a direct comparative analysis of human lesions with a probability to detect early changes crucial for tumor progression. Further characterization of the novel genes and the two recorded genes MIA and TIMP-3 in regard to their biological functions should lead to a better understanding of the tumor biology of human cutaneous melanoma.

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