Cloning and Characterization of the Expression Pattern of a Novel Splice Product MIA (Splice) of Malignant Melanoma-derived Growth-inhibiting Activity (MIAY CD-RAP)

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Melanoma-inhibiting activity/cartilage-derived retinoic acid-sensitive protein, a 11 kDa protein, is mainly expressed in cartilage during embryogenesis, and is related to invasion, metastasis, and immunomodulation of melanoma and glioma cells *in vivo* and *in vitro*. Here, we describe an alternative splice product of this gene termed melanoma-inhibiting activity (splice), lacking exon 2 of the original protein. A predicted frameshift by alternate splicing results in a unique C-terminal portion of the protein. Consistent with this, a protein migrating at the predicted molecular weight of the splice form (3.5 kDa) was detected using an N-terminal specific antibody. This band was undetectable when using a C-terminal specific antibody. In addition, we describe the expression pattern of melanoma-inhibiting activity (splice) in different human tumors. Expression was shown in tissue samples of five of six primary melanomas, 11 of 12 primary sites of metastatic melanomas, 10 of 10 systemic metastases of melanomas, four of four central nervous system metastases of melanomas, six of eight primary melanoma cultures, and five of five melanoma cell lines. Only a faint signal was obtained in tissue samples of five of six naevi. Interestingly, seven of eight nonmelanocytic tissue samples and five of seven glioma cell lines showed weak expression of melanoma-inhibiting activity (splice). Approaching first functional aspects, reverse transcriptase–polymerase chain reaction showed weak expression of melanoma-inhibiting activity (splice) in relation to melanoma-inhibiting activity in nonmelanocytic and strong expression in melanocytic cells. Staining with a specific anti-serum raised against a synthetic peptide resembling the amino acid sequence of melanoma-inhibiting activity (splice) showed a more nuclear staining pattern in comparison with melanoma-inhibiting activity. Furthermore, incubation of melanoma and glioma cell cultures with transforming growth factor-β2 showed inverse regulation of the mRNA of melanoma-inhibiting activity and melanoma-inhibiting activity (splice), both suggesting also a different function within the physiologic role of this unique family of proteins. Melanoma-inhibiting activity (splice) has no homology to any other known protein so far. Whereas the biologic function of melanoma-inhibiting activity (splice) is not clear yet, it might provide a relevant diagnostic and therapeutic tool for malignant melanomas. ***Key words: alternative splicing/cartilage-derived retinoic acid-sensitive protein/melanoma-inhibiting activity/melanoma. J Invest Dermatol 119:562–569, 2002***

The 11 kDa protein melanoma-inhibiting activity (for review: Apfel *et al*, 1998; Bosserhoff *et al*, 1999a) has been previously identified within growth-inhibitory activities secreted by a primary culture from the central nervous system (CNS) metastasis of a patient with malignant melanoma and was purified from tissue culture supernatants (Apfel *et al*, 1992). Recent studies have shown that the expression of melanoma-inhibiting activity (MIA) in adult tissue is mainly restricted to malignant melanoma, cartilage, and to a lesser degree to breast carcinoma, carcinoma of the colon, and glioblastoma (Dietz and Sandell, 1996; Bosserhoff *et al*, 1999a; de Vries *et al*, 1999). MIA expression is correlated to some degree with embryonic development as well as with differentiation of cartilage cells *in vitro* (Dietz and Sandell, 1996). Summarizing both biologic functions, the gene/protein is now referred to as MIA/cartilage-derived retinoic acid-sensitive protein (MIA/CD-RAP). The MIA/CD-RAP-gene is located on chromosome 19q13.32–33 (Koehler *et al*, 1996). Recently, some other proteins with similar sequences were described, all of which reside in one family of genes (Cohen-Salmon *et al*, 2000; Pan *et al*, 2000; Robertson *et al*, 2000; Rendtorff *et al*, 2001).

Comparing expression levels in melanocytes, melanocytic nevi, and melanomas, MIA expression was shown to parallel the progressive malignancy of melanomas (Bosserhoff *et al*, 1999b). In

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Abbreviations: MIA, melanoma-inhibiting activity; cartilage-derived, retinoic acid-sensitive protein

clinical studies, MIA serum levels correlate with the clinical tumor stage in melanoma patients, providing an enzyme-linked immunosorbent assay (Roche Diagnostics, Mannheim, Germany) to monitor therapy and progression of this disease. Serum levels are enhanced in 13% of patients with stage I melanoma and in 100% of patients with stage IV melanoma as tested by enzyme-linked immunosorbent assay (Bosserhoff *et al*, 1999b; Dreau *et al*, 1999; Brochez and Naeyaert, 2000).

The biologic function of MIA in adult organisms is still unclear, but evidence is growing that it might be involved in cellular motility, metastasis, and modulation of immune responses. These effects may be explained by interaction with components of the extracellular matrix, such as laminin and fibronectin (Bosserhoff *et al*, 1997). Therefore, MIA may have a function in regulating detachment of melanoma and possibly other cells from the extracellular matrix, which is an important step in metastasis, and in cartilage development. Furthermore, MIA inhibits proliferation and activation of peripheral blood mononuclear cells in a concentration-dependent manner. As cytotoxicity of T cells is mediated by $\alpha 4/\beta 1$ and $\alpha 5/\beta 1$ integrins, an immune escape of malignant melanoma and possibly other tumors producing MIA could be facilitated by this mechanism (Jachimzak *et al*, in press).

During the process of the characterization of MIA using northern blot analysis and reverse transcriptase–polymerase chain reaction (reverse transcriptase–PCR), we detected a second transcript, indicating possible alternate splicing. Here, we describe the characterization, cloning, sequencing, and expression pattern of this novel 3.5 kDa peptide MIA(splice) as well as its tissue distribution and first data indicating a different functional role in comparison with MIA. An alternative splice form could be critically involved in the biologic function of MIA, could broaden the diagnostic value of MIA, give insight into the physiologic role of this protein, and might even be a target for novel therapeutic strategies.

MATERIALS AND METHODS

Primary cultures and cell lines The melanoma cell lines CRL-1424 and HTB-69 were obtained from American type culture collection (ATCC). The other melanoma cell lines designated with ''HTZ'' were established from human melanoma central nervous system tumor metastasis. All melanoma cultures were grown in Ham's F-12/Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as monolayers under standard culture conditions as described elsewhere (Bogdahn *et al*, 1989). HTZ-19 cells were grown in serum-free medium (Bogdahn *et al*, 1989). All glioma cell lines (HTB-10, HTB-11, HTB-185, HTB-186) that were obtained from ATTC were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum as standard monolayer cultures. All other glioma cell lines designated ''HTZ'' were established from human tumor biopsies and grown under the same conditions.

To elucidate the role of transforming growth factor (TGF)-β in the regulation of MIA, cultures were treated with 10 ng TGF-$\beta 2$ per ml (Boehringer Mannheim, Germany), and expression of MIA and MIA(splice) mRNA was analyzed by northern blot after harvesting cells after 3, 6, 12, 24, and 48 h in comparison with an untreated control.

Cloning and sequencing of recombinant cDNA clones Total RNA was prepared according to the method of Chomczynski and Sacchi (1987)). The RNA was amplified by reverse transcriptase–PCR as described with specific primers specified below and was run on an agarose gel. A southern blot was performed and stained with a specific ^{32}P-labeled 459 bp probe derived from the MIA sequence. Two signals, one for MIA and a second one, later identified as MIA(splice), were found. The film was used as a mask to cut out the corresponding second signal from an identical reverse transcriptase–PCR reaction. The amplified reverse transcriptase–PCR product was electroeluted and cloned into the vector pQE40 (Qiagen, GMbH, Max-Vollmer-Strasse 4, 40724 Hilden, Germany) under standard conditions. Clones were selected and amplified, and plasmids were prepared by alkaline lysis (Birnboim and Doly, 1979). Sequencing of the plasmids was performed according to a standard protocol (Sanger *et al*, 1977).

Reverse transcriptase–PCR The primer pair MIA-forward (5′-CAT GCA TGC GGT CCT ATG CCC AAG CTG-3′) and MIA-reverse (5′-GAT AAG CTT TCA CTG GCA GTA GAA ATC-3′) was employed for reverse transcriptase–PCR except for the studies shown in **Fig 1** and **Table I**, there the following primers were used: for amplification of MIA, a forward primer hybridizing to the exon–exon boundary of exon 2/3 (5′-TGG GGA GGC AGC GTT CAG G-3′), and for MIA(splice), a forward primer binding to the exon–exon boundary of exon 1/3 (5′-GAC CAG GAG TGC AGC CTT CAG-3′) were designed. As reverse primers, MIA-reverse (5′-ACA TCG ACT TTG CCA GGT TTC AGG-3′) and MIA(splice)-reverse (5′-TCA CAT CGA CTT TGC CAG TTT CA-3′) were used. The latter primers result in fragments of 100 bp for MIA and 114 bp for MIA(splice).

Twenty-eight cycles of PCR for MIA or 32 cycles of PCR for MIA(splice) were done using the following profile: 45 s at 95°C, 30 s at 55°C, and 60 s at 72°C. PCR reaction products were separated on a 1.8% agarose gel and subjected to southern blot analysis.

In parallel, β-actin or GAPDH mRNA was amplified to confirm equal amounts and integrity of different RNA preparations. The same reverse transcriptase–PCR conditions were used to perform PCR in all primary cultures shown here. Results were confirmed in part by direct sequencing of reverse transcriptase–PCR products and by northern blotting.

Northern blots RNA was isolated as described above. Total cellular RNA (20 μg per lane) was loaded on a 1% formaldehyde-agarose gel and transferred to nylon membranes according to a standard protocol (Sambrook *et al*, 1989). The complete human MIA cDNA insert was used as a probe for both MIA and MIA(splice) RNA. Final washes were performed in standard sodium citrate/chloride buffer two times for 1 h each at 68°C.

Western blots Supernatants from cell cultures for western blots were prepared with centricon-3 ultrafiltration vials using a kit of Amicon, Millipore GmbH, Am Kronberger Hang 5, 65824 Schwalbach, Germany. The samples were separated on a high-resolution tricine-polyacrylamide gel electrophoresis according to the method described by Schägger and Jagow (1987). Western transfers to nitrocellulose membranes were performed according to the method of Khyse-Anderson (1984). After blocking with 1% bovine serum albumin in phosphate-buffered saline, blots were incubated for 1 h with a biotin-conjugated C-terminal and a N-terminal monoclonal antibody against MIA (Boehringer Mannheim) in two different reactions to detect MIA and MIA(splice) separately. Washing the blots was followed by incubation with a second streptavidin–peroxidase conjugated antibody. For color development, diaminobenzoid was used as a substrate.

Anti-serum against MIA(splice) A synthetic peptide, resembling the putative amino acid sequence of MIA(splice), was synthesized (QCB, #3 Avenue D, Hopkinton, MA 01748), purified, and used as an antigen to immunize rabbits. The synthetic peptide contains all 36 amino acids of the original endogenous peptide MIA(splice). Serum of the rabbits was affinity purified and tested for specificity (not shown).

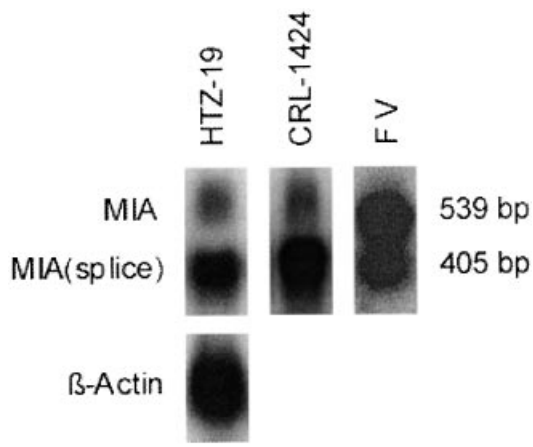

Figure 1. Expression of MIA(splice) in a northern blot analysis. The bands show MIA (runs at 539 bases), MIA(splice) (runs 134 bases lower at 405 bases), and β-actin (used as a control). In the lanes, two cell lines of cerebral metastases of human malignant melanoma, HTZ-19 and CRL-1424 (ATCC), and FV, a human fibroblast cell line, are shown.

Table I. Expression pattern list of MIA and MIA(splice) in melanoma and control tissue samples or cell cultures as detected by reverse transcriptase–PCR. Tumors are graded according to the pTNM system. Expression of MIA(splice) is strongly correlated to the expression of MIA in all primary cultures tested so far. The ratio of the PCR products of MIA to MIA(splice) in melanocytic cells is almost constant in all primary cultures tested, whereas in nonmelanocytic MIA-positive controls, the expression of MIA(splice) is relatively low compared with MIA.

	mRNA expression MIA(splice)	mRNA expression MIA
Melanocytic tumors		
Nevus		
A1	–	+++
A2	++++	+++++
A3	++++	++++
A4	++	+++++
A5	++++	++++
A6	+++	+++++
Melanoma (pTx Nx M0)		
B1	++	++++
B2	++	+++
B3	++	+++
B4	+++	++
B5	–	+
B5	++	+++
Primary site of metastatic melanoma (pTx Nx M1b)		
C1	–	+
C2	++++	++++
C3	+++++	+++
C4	+	+
C5	++	++
C6	+++	++
C7	+	++
C8	++	++
C9	++	++
C10	++	++
C11	++	++
C12	+	++
Systemic metastases of melanoma (pTx Nx M1b)		
D1	+	++++
D2	+	+++
D3	+++	++++
D4	+++++	+++
D5	++++	+++
D6	++++	+++++
D7	++	++++
D8	++	++++
D9	+	++++
D10	+++	++
CNS metastases of melanoma (pTx Nx M1b)		
E1	+	+
E2	+	++
E3	+	+
E4	++	+
Primary cultures of melanoma		
F1	+++	++++
F2	++++	+++
F3	++++	+++
F4	+++	++++
F5	++++	+++
F6	–	+
F7	++++	+++
F8	–	++
Nonmelanocytic tumors		
G1	++	+++
G2	–	+++
G3	+	+++
G4	+	++
G5	+	+++
G6	+	+++
G7	+	+++
G8	+	++

pTx Nx M0, no distant metastasis; pTx Nx M1b, visceral distant metastasis; A, melanocytic nevi; B, melanomas; C, primary sites of metastatic melanomas; D, systemic metastasis of melanomas; E, CNS metastasis of melanomas; F, primary cultures of melanomas; G, controls consisting of nonmelanocytic tumors: G1–4, basalioma; G5, endothelium; G6, healthy colon; G7, colitis; and G8, carcinoma of the colon.

Immunohistochemistry was done in a 1 : 200–500 dilution of the serum as described below.

Immunohistochemistry For immunohistochemistry, standard 5 μm sections of formalin-fixed and paraffin-embedded tissues were used. Immunohistochemical staining was performed using an indirect immunoperoxidase protocol according to the LSAB2 kit (Dako, Hamburg, Germany). Sections were incubated with the following antibody dilution: mouse monoclonal anti-MIA, 1 : 40, and rabbit polyclonal anti-MIA(splice), 1 : 200–500. Characterization of the 2F7 anti-MIA antibody included immunoprecipitation of MIA protein from tissue culture supernatant visualizing a single 11 kDa signal specifically for melanoma cells.

RESULTS

Detection, molecular cloning, and sequencing of MIA(splice) Using northern blot to analyze MIA expression pattern, we detected a second signal when using complete MIA cDNA as a probe. As the known MIA mRNA runs at 539 bases, the new signal ran at approximately 405 bases (**Fig 1**).

Several approaches were used to assess the biologic relevance of the signal and to analyze the peptide. The band for the putative MIA(splice) was cut out of the agarose gel, using the autoradiogram as a template, and cloned into the vector pCR-Script Cam SK(+) (Stratagene, 11011 N. Torrey Pines Road, La Jolla, CA 92037) by reverse transcriptase–PCR amplification of eluted RNA. Subsequent sequencing revealed a size reduction in the mRNA of 134 bp, indicating an alternative splice product of the MIA gene. Owing to alternative splicing, exon 2 is missing and, in addition, splicing causes a frameshift in the open reading frame, resulting in a distinct and unique C-terminal portion of the protein. The loss of exon 2 combined with the frameshift results in a protein consisting of 36 amino acids with a molecular weight of 3.5 kDa. This theoretical weight could be verified by recombinant expression of a fusion protein (with dihydrofolate reductase) and by western blot analysis of supernatants from cell cultures (**Fig 2**), showing immunoreactivity to the originally described full length MIA, running at 11 kDa, when using a N-terminal specific antibody, as

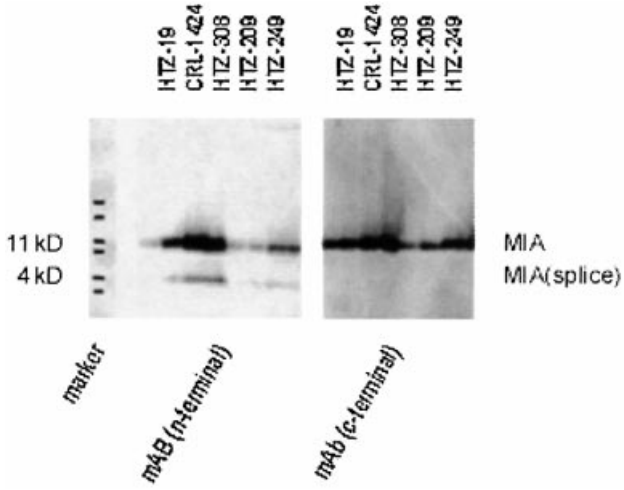

Figure 2. Western blot analysis of supernatants from cell cultures, showing immunoreactivity to the originally described full length MIA, using a N-terminal specific antibody, as well as to a protein band migrating at the predicted molecular weight of the splice form of about 3.5 kDa. Staining of this lower band is not detectable when using a C-terminal-specific antibody against MIA. In the bands, MIA and MIA(splice) are displayed. In the lanes, a marker, which was enhanced in this figure, and the reaction with a N-terminal antibody in the left panel and a C-terminal antibody in the right panel are shown. In each panel, the lanes show 1 melanoma cell line obtained from ATCC (CRL-1424), two cell lines of cerebral metastasis of human malignant melanoma and two cell lines of human malignant glioma (HTZ-cell lines, established in our laboratory).

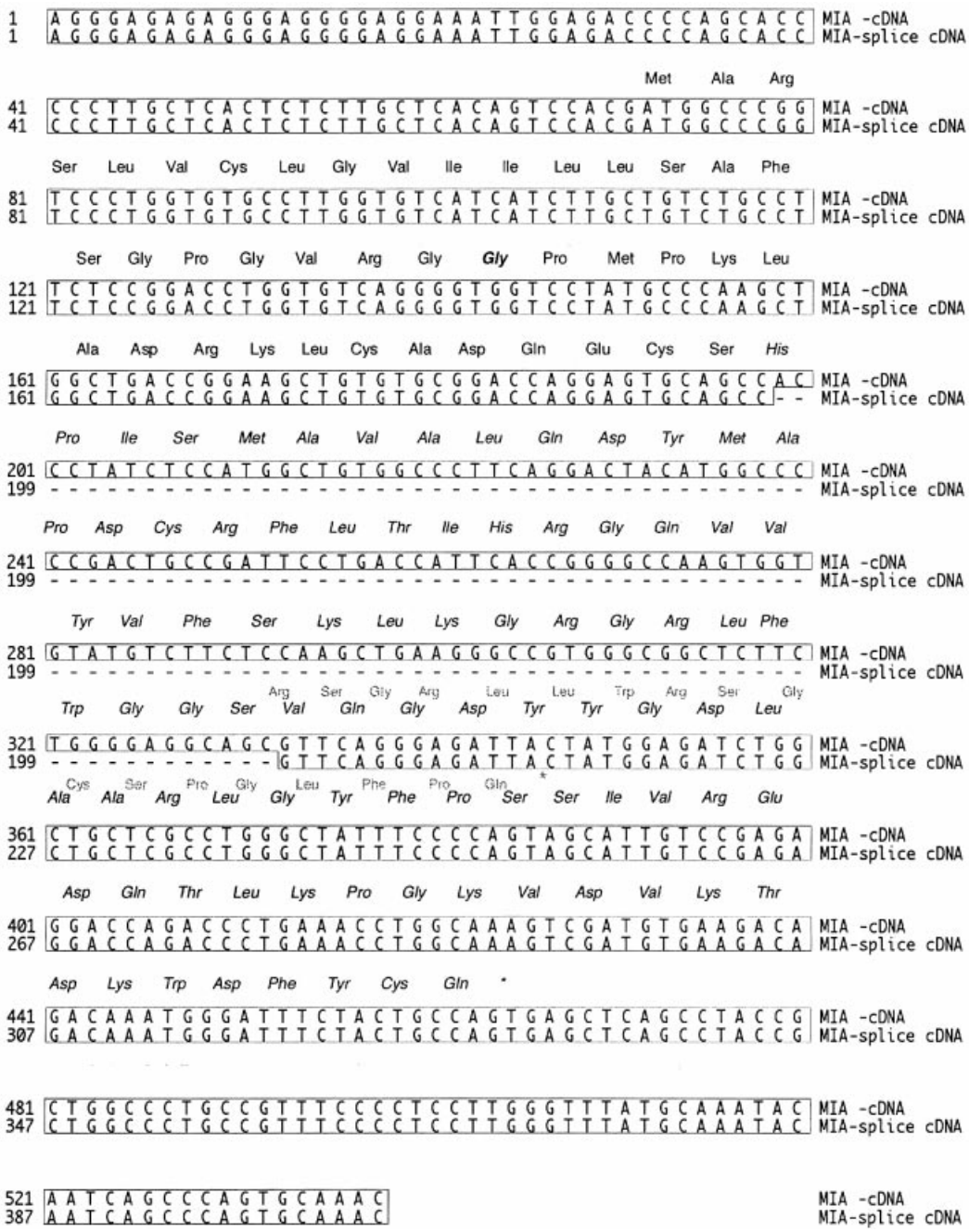

Figure 3. Using a PCR approach, sequencing of MIA(splice) was performed revealing a sequence identical to that of MIA, however, lacking 134 bp of exon 2 of the original sequence and thus resulting in a frameshift. Regular typed amino acids indicate the identical sequence of the proteins MIA and MIA(splice). Italic typed amino acids indicate the unique sequence of MIA and regular and lighter typed amino acids indicate the unique C-terminal protein sequence of MIA(splice).

well as to a second protein band migrating at the predicted molecular weight of the spliced form of about 3.5 kDa. This lower band, however, was not detectable when using a C-terminal specific antibody against MIA.

An extensive database search showed no homology to known sequences. The consensus sequences for the splicing donor and acceptor sites are preserved. All splicing sites start with GT at the 5′-end and end with AG at the 3′-end of the exons (Breathnach *et al*, 1978). Exon 1 contains the secretion signal (72 bp coding for 24 amino acids) and the codons for the first 18 amino acids of the mature peptide as described for MIA. After complete excision of exon 2, a frameshift occurs, causing a different amino-acid sequence in the C-terminal part of the peptide. After another 18 amino acids of exon 3, a stop codon (TAG) interrupts the sequence of the spliced peptide (**Figs 3 and 4**).

Three of the four cysteine residues of MIA are preserved, allowing intramolecular and intermolecular disulfide bonds. As MIA(splice) could be detected by an N-terminal specific antibody, three-dimensional confirmation seems to be preserved.

Expression pattern of MIA(splice) Cell lines of melanocytic origin and of gliomas and tissue of melanocytic and other origin were screened for expression of the full length protein and the alternative spliced form. MIA(splice) is coexpressed with MIA in almost all tissues and cell lines tested. Expression was shown in tissue of five of six primary melanomas (stage pTx Nx M0), 11 of

Figure 4. The genomic DNA consists of four exons and two noncoding introns. Exon 1 consists of the bases for the first 18 amino acids of the peptide as described for MIA above. After complete lack of exon 2, a frameshift causes a different amino-acid sequence in the second part of the peptide. An additional Histidine residue (amino acid 43) is generated by regular splicing of exon 1 to exon 2 and is not indicated here.

12 primary sites of metastatic melanomas (stage pTx Nx M1b), 10 of 10 systemic metastasis of melanoma (stage pTx Nx M1b), four of four CNS metastases of melanoma (stage pTx Nx M1b), and six of eight primary melanoma cell cultures. Only a faint signal was obtained in tissue samples of five of six naevi. Interestingly, seven of eight controls representing nonmelanocytic tissue positive for MIA (basalioma, endothelium, healthy colon, colitis, and carcinoma of the colon) showed weak expression of MIA(splice) (**Fig 5, Table I**).

The ratio of the PCR products of MIA and MIA(splice) in melanocytic cells is almost constant in all tissues and cultures tested, whereas in nonmelanocytic MIA-positive tissues or cultures, the expression of MIA(splice) is relatively low compared with the nonspliced cDNA (**Tables I and II**).

In addition, five of seven malignant glioma cell cultures tested were found positive as well (**Table II**). Translation of MIA and MIA(splice) mRNA to protein was confirmed in a selected number of cell lines by western blot analysis. Protein expression was confirmed in three of three cell lines of malignant melanomas and in two of two glioma cell lines as specified above and correlated strongly to the expression of mRNA (**Fig 2**).

Tissue distribution In contrast to the localization of MIA, which is a secreted protein and consequently is detected mainly in the cytoplasm of MIA-positive cells, MIA(splice) shows a more nuclear localization (**Fig 6**).

The scattered appearance within a given cell culture or tissue specimen, respectively, leads to the speculation that the expression

Table II. Expression pattern list for mRNA and protein of MIA and MIA(splice) in tissue samples and cell lines of melanomas, low- and high-grade gliomas and other nonmelanocytic tissue as specified in Table I. Melanoma cell lines are CRL-1424 and HTB-69 from ATCC, cell lines from CNS-metastases of melanomas are HTZ cell lines established in our laboratory. Glioma cell lines were in part from ATCC as specified in *Materials and Methods*, and in part established in our laboratory. mRNA was tested by reverse transcriptase–PCR and confirmed by northern blot in some cases. Western blot was not done (ND) from tissue samples due to lack of material and was performed in a representative selection of MIA and MIA(splice)-positive cell lines of malignant melanomas and of high-grade gliomas. MIA(splice) is expressed in a wide variety of tumors and expression is correlated to MIA as shown in **Table I**. Numbers indicate cell lines positive in comparison with cell lines tested (n/n)

	MIA(splice) mRNA	MIA mRNA mRNA	MIA(splice) protein	MIA protein
Melanoma				
Melanocytic nevus tissue	5/6	6/6	ND	ND
Melanoma tissue	5/6	6/6	ND	ND
Primary site of metastatic melanoma tissue	11/12	12/12	ND	ND
Systemic metastasis of melanoma tissue	10/10	10/10	ND	ND
CNS metastasis of melanoma tissue	4/4	4/4	ND	ND
Melanoma primary cultures	6/8	8/8	ND	ND
Melanoma cell lines	2/2	2/2	2/2	2/2
CNS metastasis of melanoma cell lines	3/3	3/3	3/3	3/3
Malignant glioma				
Low-grade (WHO I/II) cell lines	1/3	1/3	ND	ND
High-grade (WHO III/IV) cell lines	6/6	4/4	2/2	
Nonmelanocytic tumors				
Tissue	7/8	8/8	ND	ND

of MIA(splice) may be associated with the regulation of the cell cycle. Interestingly, in a series of stainings of cutaneous nevi, melanoma *in situ*, and melanoma metastasis, there is a clear relation of the expression of MIA(splice) to the stage of tumorigenesis (**Fig 7**).

Regulation of MIA and MIA(splice) MIA and MIA(splice) are inversely regulated if incubation TGF-β2 is performed in cell culture (**Fig 8**). The mRNA of MIA is, antagonistic to TGF-β2, almost completely downregulated after 3 h of incubation, whereas the signal for MIA(splice) is, agonistic to TGF-β2, upregulated. Both signals are back to untreated levels 48 h after incubation.

DISCUSSION

MIA is mainly expressed in cartilage during embryogenesis, and, in adulthood, is related to invasion, metastasis, and immunomodulation of melanoma and glioma cells *in vivo* and *in vitro* (Jachimczak *et al*, manuscript in preparation). Here, we describe an alternative splice product termed MIA(splice), lacking exon 2 of the original protein. A predicted frameshift results in a unique C-terminal portion of the protein. A protein migrating at the predicted molecular weight of the splice form (3.5 kDa) was detected using a N-terminal specific antibody. Expression was shown in most primary melanomas, primary sites of metastatic melanomas, systemic metastasis of melanoma, CNS metastases of melanomas, primary melanoma cultures, and malignant melanoma cell lines. Only a faint signal was obtained in tissue samples of naevi. Interestingly, some nonmelanocytic cell lines and glioma cell lines showed weak expression of MIA(splice) as well. An extensive search in commonly used databases showed that MIA(splice) has no homology to any other protein known so far.

Alternative splicing is a widely used mechanism involved in physiologic regulatory processes. In most cases, alternative splicing leads to functional proteins as in the case of the NK-TR-gene (''natural killer-cell tumor-recognition protein''; Rinfret and Anderson, 1993). During activation of natural killer cells by interleukin-2, a change in the splicing pattern with enhanced production of unspliced mRNA is induced. Consecutively, the full length form of the protein is translated, and an enhanced response of the natural killer cells against tumor cells is possible. In contrast, alternative splicing can also lead to insufficient gene products and

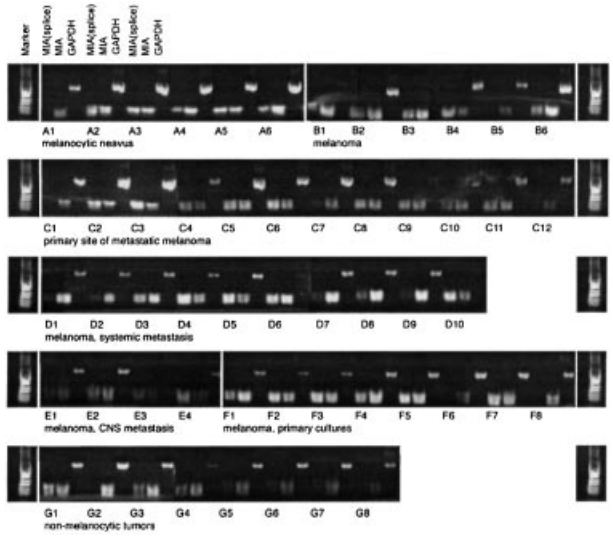

Figure 5. Reverse transcriptase–PCR from tissue of six naevi, six melanomas, 12 primary sites of metastatic melanomas, 10 systemic metastasis of melanomas, four CNS metastasis of melanomas, eight primary melanoma cultures, and eight controls (basalioma, n = 4; endothelium, n = 1; healthy colon, n = 1; colitis, n = 1; and carcinoma of the colon, n = 1). In the lanes, MIA(splice), MIA, and GAPDH (control) are shown for each specimen; marker 8 (Roche Pharma, Roche Diagnostics AG, Industriestrasse 7, 6343 Rotkreuz, Switzerland) was used as a weight marker. Expression levels of melanocytic tumors correlate with the malignancy of tumors. MIA(splice) can be detected in nonmelanocytic controls as well, but to a much weaker extent.

therefore can be correlated with pathologic conditions. Alternative splicing of the MEN1 tumor suppressor gene, for example, leads to an enhanced range of multiple endocrine neoplasias (Mutch *et al*, 1999). Splicing of the transcription factor PAX-2 leads to the development of inborn colobomas (Tavassoli *et al*, 1997), mutation of the receptor for interferon-α to the development of myeloma (Abramovich *et al*, 1994). The splicing of the NF-2-gene leads to

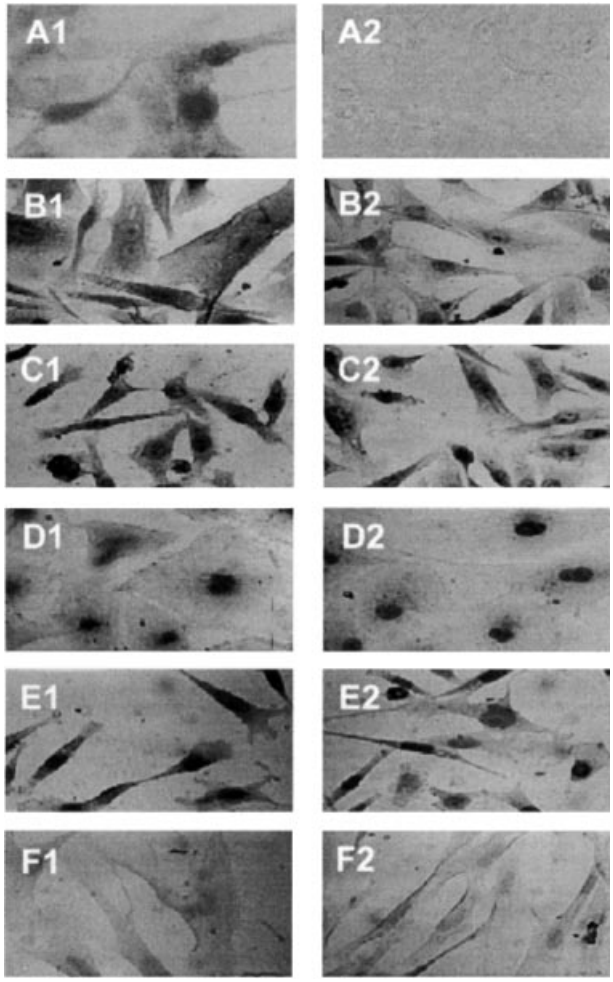

Figure 6. PAP stain of different tumors and fibroblasts treated with serum of a rabbit immunized against MIA(splice) (A1, first row, left) and MIA (B1, C1, D1, E1, F1, next rows, left), and controls (A2, B2, C2, D2, E2, F2, right). (*A,B*) HTZ-19, malignant melanoma; (*C*) HTZ-318, malignant melanoma; (*D*) HTZ-17, glioblastoma; E: HTZ-146, glioblastoma; and (*F*) H-36, embryonal fibroblasts. With the antibody generated against MIA(splice), a more nuclear staining pattern is detected in comparison with the antibody against MIA, where a more cytoplasmatic staining pattern was detected.

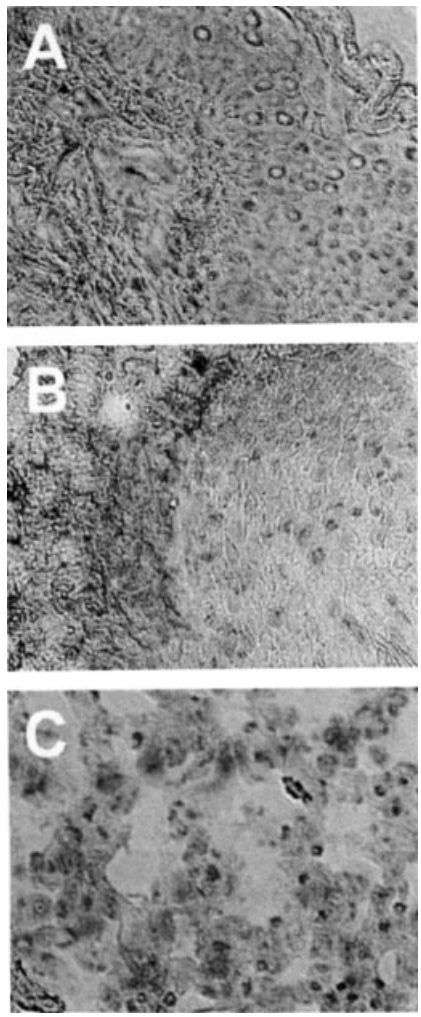

Figure 7. PAP stain of different stages of melanocytic tumors treated with the serum of a rabbit immunized against MIA(splice). (*A*) Naevus; (*B*) primary site of malignant melanoma; (*C*) metastasis of malignant melanoma. A very faint nuclear staining pattern is detected in the nevus, whereas in the primary site melanoma, staining is enhanced. In the metastasis, the most prominent nuclear staining pattern was detected.

the development of multiple schwannomas (Jacoby *et al*, 1994). Alternative splicing of the PTEN gene, which is important in a broad range of human primary brain tumors, is detected in about 20% of Burkitt's lymphomas and in about 3% of all non-Hodgkin lymphoma (Butler *et al*, 1999). Splicing of the BRCA1 gene with consecutive translation of a protein incapable of performing the hypothesized tumor suppressor function, was described in a family with cases of breast cancer (Hoffman *et al*, 1998).

Relating to that, elucidation of first functional aspects was a major concern after detection of MIA(splice). MIA(splice) can be detected with an N-terminal antibody, which is evidence for a three-dimensional configuration of the peptide and makes a functional role *in vivo* likely. MIA(splice) is frequently expressed in neuroectodermal tissue samples and cell cultures of different histologic origin, mainly in malignant melanoma and glioma. Expression of MIA(splice) is strongly correlated with the expression of MIA in all tumors tested so far. Interestingly, the ratio of the PCR products of MIA to MIA(splice) in melanocytic cells is almost constant in all specimens tested, whereas in nonmelanocytic MIA-positive specimens, the expression of MIA(splice) is relatively low compared with the nonspliced cDNA. The observation that an alternative splice product is coexpressed in all melanocytic cells tested here, but shows a significantly lower degree of expression in nonmelanocytic cells, may suggest that this new peptide is especially involved in the development of melanocytic tumors; this may define a role for MIA(splice) in the discrimination of different tumors with methods, such as enzyme-linked immunosorbent assay for MIA and MIA(splice) and eventually for therapeutic targeting of tumors expressing significant amounts of MIA(splice). In addition, MIA(splice) shows a more nuclear localization, whereas MIA, which is a secreted protein, is preferentially located in the cytoplasm, which leads to the speculation that MIA(splice) may be associated with the regulation of the cell cycle. Furthermore, as in MIA, there is a clear relation of the expression of MIA(splice) to the stage of tumorigenesis, which may indicate a role as a marker of progression. Finally, MIA and MIA(splice) are inversely regulated after incubation with TGF-β2. The mRNA of MIA is regulated in an antagonistic way in comparison with TGF-β2, whereas the signal for MIA(splice) is, agonistic to TGF-β2, upregulated. As TGF-β2 and MIA are known to be involved in the regulation of, for example, tumor invasion in malignant

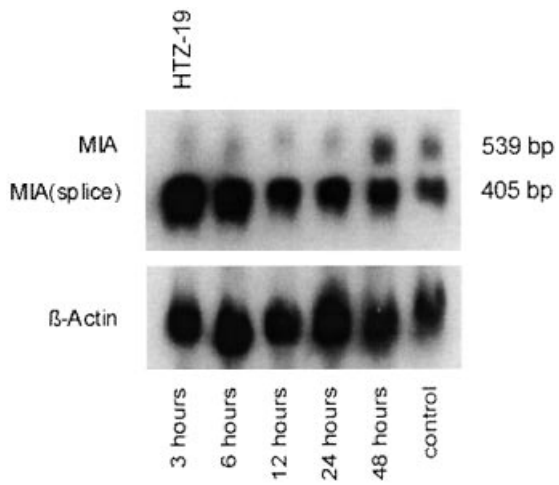

Figure 8. Northern blot of the melanoma cell line HTZ-19 treated with TGF-β2 10 ng per ml. Expression of MIA and MIA(splice) was analyzed by harvesting cells after 3, 6, 12, 24, and 48 h and isolating mRNA. The bands show MIA and MIA(splice), the lanes show labeled mRNA at the given time points and a untreated control. After incubation with TGF-β2, MIA is downregulated, whereas MIA(splice) is upregulated. At 48 h, both proteins are back to normal levels.

melanoma, MIA(splice) may have a different function, for example as an antagonist to MIA or as a transcription factor expressed if MIA is upregulated.

Taken together, MIA(splice) appears to be an important protein with a distinct function in comparison with MIA. It may play a major part in the development of malignant melanoma and glioma and could be a useful tool for the differential diagnosis of different stages of these tumors.

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