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Anne Gütgemann · Michaela Golob · Sandra Müller
Reinhard Buettner · Anja-Katrin Bosserhoff

Isolation of invasion-associated cDNAs in melanoma

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Abstract **Metastasis and invasion are key steps in the systemic spread of tumor cells. To identify the genes involved in this process we recently selected highly invasive and weakly invasive cell clones from a melanoma cell line. Both cell clones showed a stable phenotype over more than 40 passages and previous analyses revealed a fivefold difference in their invasive potential in vitro and in tumorigenesis in vivo. To compare gene expression of the two cell clones a cDNA array system (Clontech human cancer cDNA array) was used. Exact quantification of differentially expressed genes in each cell clone was performed by real-time RT-PCR. An evaluation of the array data revealed a total of 36 genes that were more than 1.5-fold differentially expressed, and 26 (72%) of these showed a differential expression pattern by quantitative RT-PCR. Previously known differences in expression patterns, including loss of p16 and HLA I, or equal expression of p73, and RAR α, β and γ were confirmed by the array data. In addition, reduced expression levels of several cytoskeletal proteins, such as vimentin, γ-actin, desmin and cytokeratins, in the highly invasive cell clone were reproducibly identified. Other genes strongly upregulated in the highly invasive cell clone included jagged 2, STAT1, tPA and c-myc, whereas MDA-6 (p21), caspase 2 and semaphorin were found to be downregulated. In conclusion, comparative hybridization of cDNA arrays identified a series of novel invasion-associated changes in gene expression and confirmed previously known expression patterns.**

The first two authors contributed equally to this work.

A. Gütgemann · S. Müller · R. Buettner · A. K. Bosserhoff (✉)
University Hospital RWTH Aachen, Institute of Pathology,
Pauwelsstr. 30, 52074 Aachen, Germany
e-mail: bosserhoff@pat.rwth-aachen.de,
Tel.: +49-241-8088080, Fax: +49-241-8888439

M. Golob
University Hospital Regensburg, Institute of Pathology,
93059 Regensburg, Germany

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Abbreviations *DVL* dishevelled · *FCS* fetal calf serum · *IGF* insulin-like growth factor · *ILK* integrin-linked kinase · *MDA* melanoma differentiation associated protein · *MMP* matrix metalloproteinase · *PTN* pleiotrophin · *PUF* nucleoside diphosphatekinase b · *RAR* retinoic acid receptor · *RBBP* retinoblastoma binding protein · *RT-PCR* reverse transcription-polymerase chain reaction · *STAT* signal transducer and transactivator · *tPA* tissue-type plasminogen activator

Introduction

Only a few genes have consistently been associated with the molecular changes leading to development and progression of malignant melanomas. Defects in the normal control of proliferation and apoptosis as well as changes in factors controlling migration, attachment and invasion have been shown, but none of the changes in gene expression identified has in itself been sufficient to initiate the development of melanoma in experimental models in vivo.

Invasion of melanoma cells through the basement membrane into the underlying dermis is a critical step in the progression of a primary melanoma to a systemic disease. Compared to other neoplasias, melanomas acquire the capacity to metastasize very early as small lesions. Therefore, identifying the mechanisms involved in invasion of melanoma cells is of great clinical significance. For the process of invasion a three-step hypothesis was proposed by Liotta (1986). The first step involves specific interactions of tumor cells with components of the extracellular matrix and the basement membrane via cell-surface receptors, including the family of integrins. Then tumor cell-associated or tumor cell-induced proteases such as MMPs are secreted or activated and start to degrade the surrounding matrix. Finally, the tumor cells migrate through the proteolytically degraded matrix to invade the stromal

compartment and, by the same stepwise mechanism, the lymphatic system and the blood vessels.

To analyze all three proposed steps of invasion, we and others have employed the Boyden chamber as an in vitro model (Albini et al. 1987; Jacob et al. 1995; Wach et al. 1996). Such in vitro studies using Boyden chambers have provided evidence that the invasive capacity and metastatic potential of melanoma cells comprise complex biological processes regulated by multiple genetic events. Among the features that have been found to be associated with the invasiveness of cell lines in vitro are altered expression of cell-cell and cell-matrix adhesion molecules, enhanced secretion of proteases or protease-activating proteins, reduced levels of protease inhibitors and deregulation of matrix protein production. Studies in vivo have confirmed the importance of these molecules and have demonstrated coregulation of the respective proteins with a highly metastatic phenotype of poorly differentiated, aggressive cancer cells.

We have recently selected highly invasive and weakly invasive cell clones from the melanoma cell line Mel Im (Jacob et al. 1995; Jacob et al. 1998). Characterization of the invasive phenotype of the two cell clones using the Boyden chamber model has revealed a fivefold difference in their invasive potential. The respective phenotypes are stable for more than 40 passages in cell culture. These cell clones were then used as a model system to clone genes which are expressed differentially in invasive melanomas. Previous experiments using subtractive cDNA hybridization identified γ-actin as being fivefold overexpressed in the weakly invasive cell clone and a series of further unknown differentially expressed genes.

In this study, we extended our analysis and used cDNA microarrays to compare the expression rate of 588 genes between the highly invasive and weakly invasive cell clones. Differential expression of the respective mRNAs was then confirmed and precisely quantitated by real-time PCR using β-actin as an internal standard.

Materials and methods

Cell culture conditions and selection of strongly and weakly invasive cell clones

The original cell line Mel Im was established by Dr. M. Hadem (University of Hanover, Germany) and further characterized by Dr. J. P. Johnson (Institute of Immunology, Munich, Germany) and our group (Jacob et al. 1995; Jacob et al. 1998). The cells were maintained in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. Highly and weakly invasive cell clones were selected using commercially available Matrigel-coated six-well plates (Becton Dickinson, Heidelberg, Germany) as described previously (Jacob et al. 1995).

Invasion assay

Invasion assays were performed in Boyden chambers containing polycarbonate filters with a pore size of 8 μm, essentially as described previously (Jacob et al. 1995). Filters were coated with a commercially available reconstituted basement membrane (Matrigel) and the lower compartment was filled with fibroblast-conditioned medium as a chemoattractant. Mel Im melanoma cells were harvested by trypsinization, resuspended in DMEM without FCS at a density of 2×10^5 cells/ml and placed in the upper compartment of the chambers. After incubation for 4 h at 37 °C, filters were removed. The cells adhering to the lower surface were fixed, stained and counted.

cDNA expression array

Commercially available cDNA expression arrays (Atlas human cancer cDNA expression array; Clontech, Palo Alto, Calif.) were used to compare the gene expression of the two cell clones. Arrays were screened according to the manufacturer's protocol. Total RNA (10 μg) from each cell clone was radiolabelled using MMLV reverse transcriptase and a specific primer set (10 × CDS primer mix) supplied by the manufacturer. Probes were purified on Chroma Spin-200 columns and the specific radioactivity was then measured. Hybridization was performed overnight at 68 °C in a solution of ExpressHyb, sheared salmon testes DNA and labelled cDNA (final concentration $0.5–1 \times 10^6$ cpm/ml). Blots were washed three times for 20 min in 2 × SSC/1% SDS at 68 °C, followed by two additional washes for 20 min in 0.1 × SSC/0.5% SDS at 68 °C. The damp membrane was wrapped immediately and exposed both to an X-ray film and to a phosphorimager screen. For interpretation of the results two different computer programs were used (Molecular Devices, Clontech).

RT-PCR analysis

For verification of the differentially expressed cDNAs, RT-PCR analysis was performed. First strand cDNA was synthesized using 2 μg of the isolated total RNA of the highly invasive and weakly invasive cell clones, respectively, 1 μg random primer (Pharmacia), 4 μl 5 × first strand buffer (Gibco), 2 μl 10 m*M* DTT, 1 μl 10 m*M* dNTPs and 1 μl Superscript Plus (Gibco) in a total of 20 μl. To prescreen for mRNA expression semiquantitative PCR was performed (PTC-200, Biozym) using the primer sequences designed by Clontech for the chosen genes with β-actin and GAPDH for standardization. Volumes of 1–5 μl of the cDNA preparation were applied to the PCR reaction. The following PCR program was used: 25 to 40 cycles of 30 s at 94 °C and 2 min at 68 °C, and final extension for 5 min at 68 °C. PCR products were separated on a 1.8% agarose gel, stained with ethidium bromide and documented.

To precisely quantify the expression of cDNAs the real-time PCR LightCycler system (Roche) was used. For PCR 1–3 μl cDNA preparation, 0.5–2.4 μl 25 m*M* $MgCl_2$, 0.5 μ*M* forward and reverse primer and 2 μl SybrGreen LightCycler Mix in a total of 20 μl was applied. The following PCR program was performed: 600 s 95 °C (initial denaturation); 20 °C/s temperature transition rate up to 95 °C for 15 s, 10 s 58–68 °C, 22 s 72 °C, 10 s 82 °C acquisition mode single, repeated 40 times (amplification). $MgCl_2$ concentration and annealing temperature were optimized for each primer set. The PCR reaction was evaluated by melting curve analysis following the manufacturer's instructions and checking the PCR products on 1.8% agarose gels. Each quantitative PCR was performed at least in duplicate for two sets of RNA preparations.

Results

As described previously (Jacob et al. 1995), two cell clones were selected from the melanoma cell line Mel Im using a Boyden chamber model. The cell clones, referred to as Mel Im hi for the highly invasive cell clone and Mel

Im si for the weakly invasive cell clone, showed a fivefold difference in their invasive potential in vitro and differences in tumorigenicity in an in vivo model. Of note is that the rate of cell proliferation and apoptosis remained unchanged after selection. As these two cell clones had been derived from one cell line and differ specifically in their invasive potential, a comparison of the two should reveal changes involved in acquisition of the highly invasive phenotype.

To detect genes differentially expressed in the two cell clones, total RNA was extracted, radiolabelled by reverse transcription and hybridized to cDNA microarrays (Atlas human cancer cDNA expression array, Clontech). Both filters were first compared visually by three independent observers for strong differences in gene expression. To improve sensitivity and to allow quantification, the analysis was then supported by two computer programs. A summary of the 38 genes analyzed in this study is shown in Table 1. In total 24 genes were more than twofold differentially expressed based on evaluation of the array data. Since small changes in expression can also result in phenotypic differences, another 12 genes with differences of more than 1.5-fold were also selected for further analysis. Puf was analyzed as a control in further assays as an equally expressed gene. E-cadherin was added to the study as it was not represented on the array. Previously determined expression patterns including loss of p16 (Bogenrieder et al. 2001) and HLA I, or equal expression of p73 (Kroiss et al. 1998) and MMP2 (Jacob et al. 1995), and RAR α, β and γ (Jacob et al. 1995) were supported by the array data.

To confirm the changes in expression pattern, all 38 genes were analyzed by semiquantitative and quantitative RT-PCR (Table 1) using three sets of independently prepared RNAs. Of the 38 genes, 19 (50%) were shown by quantitative RT-PCR to be consistently different in expression. For a further 12 genes (32%) differential expression, but opposite to that expected, was found. For seven genes the PCR data showed an equal expression pattern.

Semiquantitative RT-PCR was not sufficiently sensitive and reproducible in most cases to define changes in strength of expression less than twofold. However, real-time PCR was able to reproducibly detect even small changes in expression pattern. In Fig. 1 eight of the quantitative RT-PCR results from different genes are shown.

Genes consistently less expressed in the highly invasive cell clone Mel Im hi as determined by both array and real-time PCR were p21, Caspase 2, biglycan, vimentin, IGF, ILK, DVL1, Semaphorin V, wnt 5A, wnt 10B, E2F-1 and desmin. Real-time PCR showed downregulation of TRK-T3, PTN, cyclin C, tenascin C, c-Erb B2, RSPA, RBBP and rhoA in the highly invasive cell clone. Both array and real-time PCR showed phospholipase D, jagged 2, tPA, STAT1 and Rad to be upregulated by in Mel Im hi, and real-time PCR further showed c-myc, cdk-5 activator and E-cadherin to be overexpressed in the highly-invasive clone. Differential expression of Trip, Daxx, notch 4, integrin β7, b-raf, SPARC and Pig3 could not be confirmed by RT-PCR. Further, strong downregulation of cytokeratin 8 expression was found in the highly invasive cell clone, and consistently proven by immunohistochemistry (data not shown).

Table 1 Expression patterns obtained by array hybridization and confirmation of differential gene expression by quantitative RT-PCR. First the genes that were downregulated in the highly invasive cell clone are listed, followed by those that were upregulated and finally those that were equally expressed. The results of the array experiment are presented as fold expression (Mel Im hi/Mel Im si). The RT-PCR results are presented as the means and SD of *n* experiments. The genes are arranged according to differences in expression as measured by RT-PCR. The last column indicates whether the array data were confirmed by quantitative RT-PCR

Gene	Array	RT-PCR			Confirmed by RT-PCR
		Mean	*n*	SD	
Caspase 2	0.16	0.33[a]	3	0.176	✓
cyclin C	1.58	0.70[a]	4	0.091	
Wnt 5A	0.39	0.59[a]	3	0.120	✓
Wnt 10B	1.02	0.25[a]	4	0.132	
Desmin	0.09	0.46[a]	3	0.035	✓
DVL	0.29	0.16	4	0.372	✓
Tenascin C	1.61	0.25	4	0.389	
p21	Just si[a]	0.26	3	0.077	✓
Semaphorin V	0.36	0.27	3	0.100	✓
Vimentin	0.46	0.32	4	0.362	✓
RBBP	1.48	0.32	3	0.098	
TRK-T3	Just hi[a]	0.45	3	0.459	
RhoA	1.44	0.61	3	0.215	
E2F	0.33	0.65	4	0.314	✓
c-ERB B2	2.48	0.67	4	0.314	
PTN	7.98	0.70	5	0.405	
RPSA	2.46	0.72	3	0.360	
IGF-1	0.81	0.73	4	0.356	✓
ILK	0.81	0.75	4	0.327	✓
Emmprin	1.77	0.82	3	0.170	
Biglycan	0.70	0.85	3	0.226	✓
jagged 2	15.4	9.49[a]	5	3.450	✓
STAT 1	1.58	3.52	3	0.657	✓
E-cadherin	n.d.	2.14	3	1.137	
TPA	1.67	1.99	3	0.847	✓
Interleukin 13	2.60	1.70	3	0.100	✓
c-myc	2.40	1.64	3	0.144	✓
Phospholipase D	3.96	1.60	3	0.590	✓
Rad	2.29	1.45	4	0.706	✓
CDK-5activator	0.35	1.44	3	0.450	
Pig 3	0.20	1.20	3	0.458	
Puf	1.00	0.86	3	0.216	✓
Trip	0.52	0.92	5	0.397	
Daxx	0.47	1.02	4	1.080	
Notch 4	24.1	1.07	6	0.350	
Integrin β7	0.25	1.08	3	0.330	
b-raf	0.31	1.13	4	0.623	
SPARC	0.42	1.12	4	0.622	

[a] The PCR product was found only in the weakly or highly invasive cell clone at least once

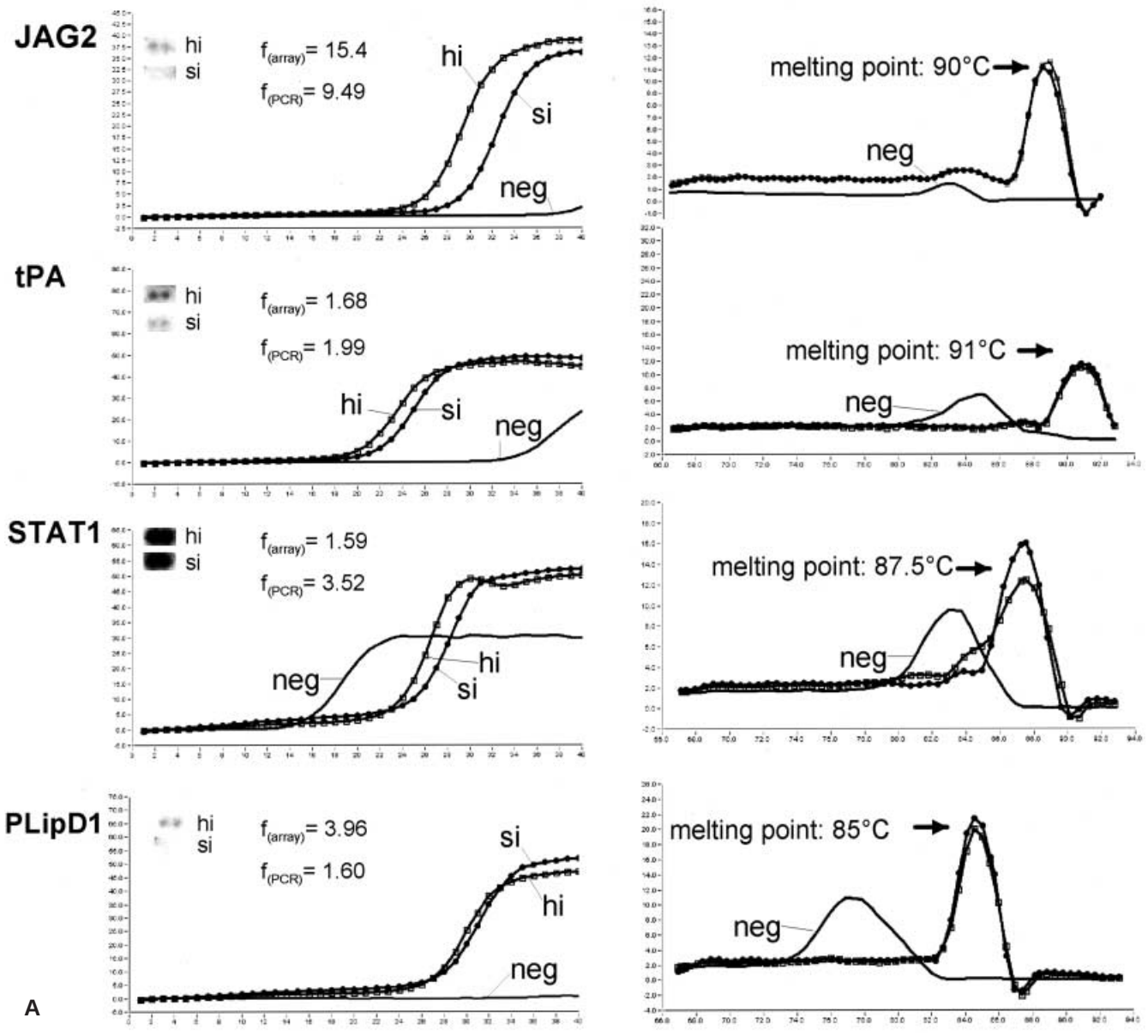
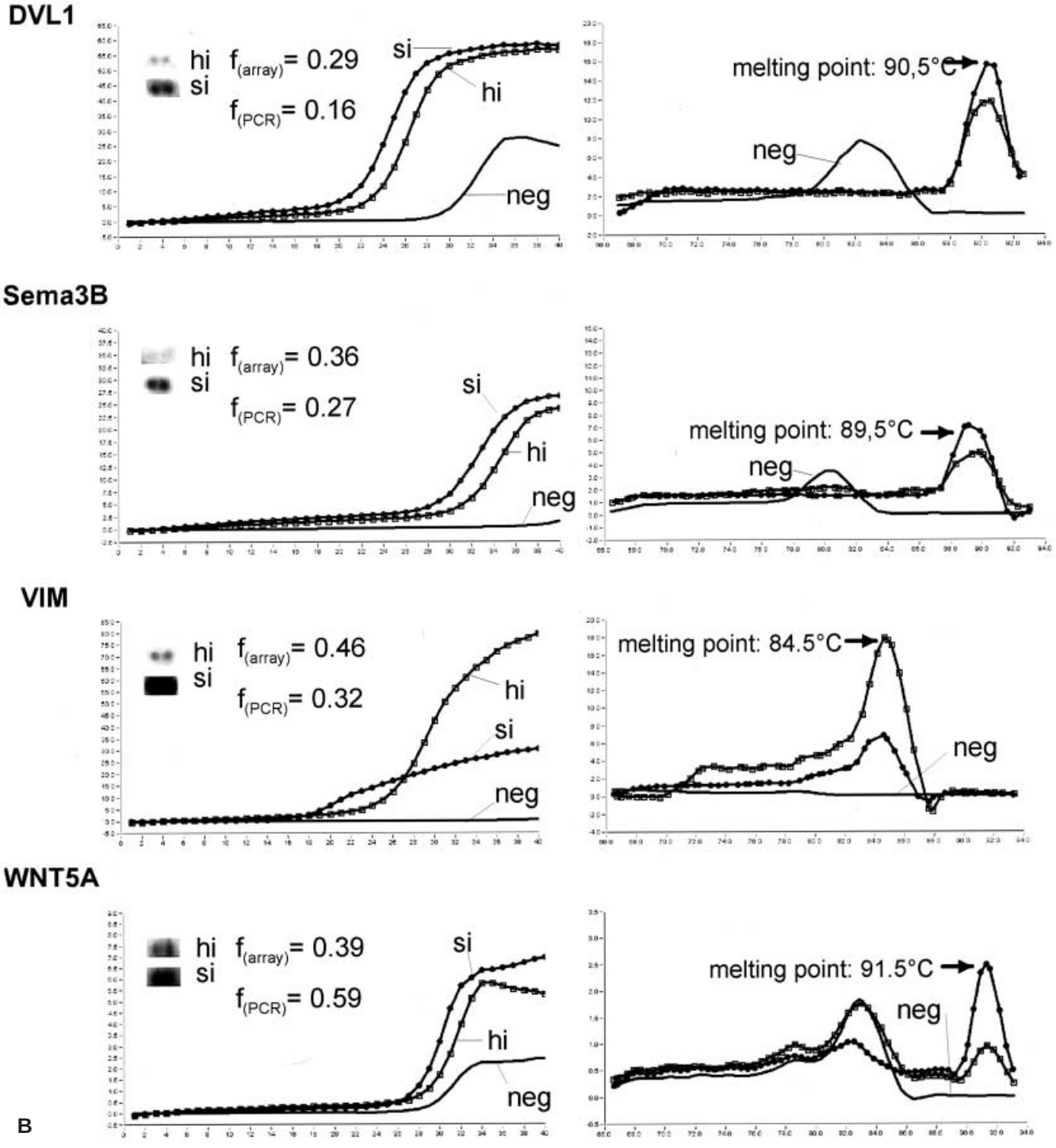


Fig. 1 A, B Examples of quantitative RT-PCR analysis and melting curves of differentially expressed genes. The array data is shown in the upper left corner for each gene. The factors of the array and the quantitative RT-PCR results are given. **A** Genes overexpressed in the highly invasive cell clone (jagged 2, tPA, STAT1, Phospholipase D1); **B** genes downregulated in the highly invasive cell clone (DVL1, Semaphorin 3B, vimentin, Wnt5a)

Discussion

In the present study, we employed highly and weakly invasive cell clones selected from the melanoma cell line Mel Im to screen for invasion-associated genes using a cDNA microarray system. As demonstrated previously, the two cell clones represent genetically stable phenotypes with a fivefold difference in their invasive capacity in vitro (Jacob et al. 1995). Using hybridization of cDNA microarrays, 588 selected cancer genes were compared with respect to changes in their expression pattern. Analysis of the expression of previously evaluated genes such as p16, p73, HLA I, RARs and MMP2 validated the array results (Jacob et al. 1995).

Expression of genes which were shown by the array to be differentially expressed was further verified by quantitative RT-PCR. Consistent with previously reported results (Backert et al. 1999), not all of the differences found by the array were reproducible by RT-PCR. Therefore, data obtained by array analysis require confirmation by other methods such as real-time RT-PCR. In some cases even strong differences in expression pattern (e.g. TRK-T3, Notch4, Pig3) could not to be reproduced by RT-PCR. The discrepancies could be explained by methodological limitations of the array technology. Complex mixtures of diverse cDNAs differing exponentially in expression levels are compared in a single hybridization re-



action irrespective of the optimal linear range of reaction conditions. In contrast, real-time RT-PCR uses measurements optimized for each reaction. RT-PCR would therefore be expected to provide data with higher validity.

Reduced expression of several cytoskeletal proteins such as vimentin, γ-actin, desmin and cytokeratins as well as extracellular matrix proteins such as biglycan and tenascin C in the highly invasive cell clone was detected in our analysis. Invasive cancer cells seem to regulate their capacity to migrate and invade partly by changes in expression of intermediate filaments and actin. Some groups have shown a correlation between reduction in the amount of certain cytoskeletal proteins and invasive potential (Katagata and Kondo 1997), whereas others (Hendrix et al. 1996) have found evidence linking upregulation of keratins with increased invasive activity.

Biglycan belongs to the group of small proteoglycans, molecules known to bind to matrix macromolecules and

to stabilize the matrix (Buckwalter and Mankin 1998; Roughley and Lee 1994). A downregulation of biglycan expression leading to a higher metastatic potential of melanoma cells has not been described previously, although it could reasonably play a role. Tenascin C is an extracellular matrix hexameric glycoprotein expressed in the dermis (Mackie 1994). Generally a high expression of tenascin C has been found during carcinogenesis (Vollmer 1994) as in melanoma development (Tuominen and Kallioinen 1994; Tuominen et al. 1997). Our study identified for the first time a direct correlation between invasive potential in vitro and tenascin C expression.

Other genes strongly upregulated in the invasive cell clone were jagged 2, STAT1, tPA, E-cadherin and c-myc, whereas mda-6 (p21), DVL, cyclin C, caspase 2, wnt 5A, wnt 10B, RBBP, E2F-1 and semaphorin V showed a significantly reduced expression pattern. Jagged 2 was the gene most strongly upregulated in the highly invasive cell clone in this study. Jagged 2 is a protein originally identified in *Drosophila* cell fate determination (Luo et al. 1997) as a ligand for members of the notch gene family (Lanford et al. 1999). A direct correlation in tumor progression and invasion has not been reported previously.

STAT1 is a transcription factor involved in interleukin, interferon, and also growth hormone signalling (Heinrich et al. 1998; Pestka 1997). In acute leukemia STAT1 has been found to be overexpressed and constitutively active (Gouilleux-Gruart et al. 1997). STAT phosphorylation and DNA binding is important for activity and needs to be evaluated in further studies to show the functional consequence of overexpression. The c-myc gene is a transcription factor known to be involved in regulation of proliferation and apoptosis. Increased c-myc expression during melanoma progression has been shown by immunohistochemistry (Bodey et al. 1996). TPA is known as the main plasminogen activator in blood with a strong restriction of activity by PAI-1 and PAI-2. In melanoma tPA expression has been suggested to be correlated with invasion, and some in vitro studies have shown tPA function in melanoma invasion (Alizadeh et al. 1995).

Interestingly, E-cadherin was upregulated in the highly invasive cell clone, which is in contrast to previously reported findings. Several groups (Hsu et al. 1996; Johnson 1999; Sander et al. 1999; Silye et al. 1998) have reported a loss or a strongly reduced expression of E-cadherin during melanoma progression. In our model system other molecules, members of the wnt family, which lead into the same signalling pathway, are consequently changed. Due to these changes, changes in E-cadherin are no longer important. Further, ongoing sequencing analyzes E-cadherin in the cell clones.

Caspase 2 (CASP-2, ICH-1, NEDD2) is a protein known to be activated during apoptosis (Harvey et al. 1997). An overexpression of caspase 2 in cultured mammalian cells induces apoptosis (Kumar et al. 1994). Recently, Hiwasa and Nakagawara (1998) have further shown that caspase 2 suppresses tumor activity by affecting the expression level of ras protein. In caspase 2-deficient mice generated by Bergeron et al. (1998), the oocytes are resistant to cell death following exposure to chemotherapeutic drugs. It is therefore possible that a loss of caspase 2 expression also plays a role in reduction of apoptosis and enhancement of melanoma invasion, as suggested by our model system.

Cyclin C belongs to the group of regulatory subunits specifying the activity of cyclin-dependent kinases during cell cycle progression. Li et al. (1996) have shown that haploinsufficiency of the cyclin C protein is likely to be sufficient to promote tumorigenesis. A downregulation during acquisition of a more invasive phenotype could result in the same effect. DVL is a member of the segment polarity gene family in *Drosophila*, and plays an important role in the early development of patterning processes (Pizzuti et al. 1996). For colon and breast carcinoma no differential expression pattern has been found when comparing normal and tumor tissues (Bui et al. 1997). Overexpression of DVL has been shown to inhibit GSK-3-β kinase activity (Wagner et al. 1997), which could result in an enhanced stability of β-catenin. The role of β-catenin in colon carcinoma, and also melanoma, has lately been a matter of intensive discussion and its role has been proven in several studies.

A correlation between loss of semaphorin V expression and tumor progression has already been shown for lung cancer (Roche et al. 1996). The loss of chromosome 3p21, the locus of the semaphorin gene, is a critical event in pathogenesis of lung cancer. A reduction of expression as found in the highly invasive cell clone resembles this loss, although the functional importance has not yet been determined.

Mda-6 (p21, melanoma differentiation associated protein 6) plays a key role in regulating the cell cycle (Gartel et al. 1996). Downregulation or inactivation of mda-6, as seen in prostate cancer (Gao et al. 1999) and also in melanoma metastasis (Jiang et al. 1995; Sparrow et al. 1998; Vidal et al. 1995), proves its inverse role in cancer progression. Overexpression of mda-6 results in G_1 arrest and has been shown to effectively suppress tumor growth in vitro and in vivo (Gartel et al. 1996). Downregulation, as postulated in our model, is therefore a common finding during melanoma metastasis.

Wnt genes, e.g. wnt 5A and wnt 10B, encode a family of secreted glycoproteins which modulate cell fate and behavior in embryos through activation of receptor-mediated signalling pathways (Moon and Miller 1997; Moon et al. 1997). Wnt 5A has been described as a tumor suppressor gene by some groups (Olson et al. 1997), and moreover wnt 5A transfection into renal carcinoma cells has been shown to result in in vitro growth suppression and repression of telomerase activity (Olson et al. 1998). Other groups have described wnt 5A as well as wnt 10B as oncogenes (Iozzo et al. 1995). Wnt 10B has been shown to be genetically altered in multiple mammary tumors as a consequence of MMTV integration (Callahan 1996).

RBBP4 is a member of a highly conserved subfamily of WD repeat proteins and has been shown to interact with Rb (Qian et al. 1993). Disruption of the Rb pathway frequently occurs in malignant melanoma through inacti-

vation of p16 or activation of cdk4. Recently, downregulation of RBBP2, another protein belonging to the same subfamily as RBBP4 and involved in the Rb pathway, has been shown (Vogt et al. 1999). Obviously several members of this protein family are deregulated during melanoma metastasis. E2F-1 is a transcription factor active in late G_1 and S phase of the cell cycle (Allen et al. 1997; Muller et al. 1997). For glioma it has been shown that overexpression triggers apoptosis and suppresses tumor growth in vitro and in vivo (Fueyo et al. 1998). A similar function for melanoma can be postulated. Thus a reduction of expression as observed in our model seems likely.

In conclusion, our results confirm previously observed expression patterns of a series of cDNA and reveal new genes differentially expressed between weakly and highly invasive melanoma cells. The role of these newly identified genes should be evaluated in further studies analyzing protein expression patterns and function of the proteins in vitro and in vivo in melanoma.

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