# Gene Expression Profiling of Human Melanoma Cell Lines with Distinct Metastatic Potential Identifies New Progression Markers

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**Abstract.** *Background: Identification of markers associated with melanoma progression is crucial to identify new prognostic and/or therapeutic targets. Materials and Methods: By using DNA microarrays, two human melanoma cell lines, M4Be and Tw12, derived from the same tumor, but with different metastatic potential, were profiled. Western blot of cell lines, immunohistochemistry on melanoma biopsies and in silico analyses validated and extended our results. Results: Thirty-six clones were differentially-expressed between the two cell lines, representing 33 named genes and 2 expressed sequence tags. The most up-regulated gene in the strongly metastatic clone Tw12 was CD10. Protein analysis with anti-CD10 antibody confirmed this finding in cell lines and clinical samples with expression being more frequent in metastatic compared to primary tumors. Many up-regulated genes were involved in angiogenesis, invasion, growth and apoptosis. Down-regulated genes included tumor suppressor genes and those were involved in differentiation. Conclusion: We identified several genes the expression of which is associated with metastatic progression in human melanoma cells. Although further analyses are warranted to clarify their exact role in tumor progression, they might lead to new prognostic markers and/or molecular therapeutic targets in metastatic melanoma.*

Melanoma is an important health problem in the world with an increasing incidence in recent years. Fortunately, diagnosis is more frequently being made at an early stage. However, the heterogeneity of the disease remains poorly reflected by its clinicopathological features. The most reliable and significant prognostic factor is the thickness of the primary tumor (1). However, regardless of the thickness, clinical outcome varies greatly, ranging from poor to disastrous. Despite many efforts to improve the treatment of the disease, no efficient treatment is currently available once the tumor has become metastatic (2, 3). Metastatic melanoma represents a major therapeutic challenge because of its notorious resistance to chemotherapy and its association with a high rate of mortality. The mechanisms that lead to melanoma and metastastic progression involve a series of molecular alterations resulting in changes in expression of many genes. However, only few genes have been implicated in the metastatic process (4). A major challenge is to define accurate predictors of metastatic relapse and to identify new therapeutic targets (5).

DNA microarrays (6) provide the opportunity to clarify the molecular mechanisms involved in cancer progression and to identify novel genes potentially important for prognosis and treatment. Several retrospective studies on pretreatment tissue samples have suggested the power of RNA expression profiles in prognostic classification of solid tumors (7-13). Profiling of cancer cell line variants with different metastatic potential has also shown the capacity of DNA microarrays for identifying gene sets associated with metastasis in breast (14) and lung cancer (15), and osteosarcoma (16). The strategy has also been applied to melanoma cell lines (17-21), and actually represents most expression profiling studies of melanoma (22). Clark *et al.* compared the profile of a human melanoma parental cell line with weak metastatic potential with that of different daughter clones with strong metastatic potential: they identified genes involved in the metastatic process such as *RHOC* (17). Bagheri *et al.* profiled cell models to

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investigate the mechanisms by which telomerase promotes tumor invasion and metastasis (18). Bittner *et al.* profiled melanoma cell lines and tumors and identified subclasses of disease and genes associated with progression such as *WNT5A* (19). Seftor *et al.* compared parental cell lines to study the molecular determinants of uveal melanoma invasion and metastasis (20). More recently, two prognostic studies profiled clinical samples (23, 24). Winnepenninck *et al.* identified a gene expression signature associated with metastatic relapse in a series of 58 patients (24).

In this study, we profiled two clonally-related melanoma cell lines, M4Be and Tw12 (clone 4), using DNA microarrays. These cell lines are derived from the same tumor and have been previously characterized on the basis of their metastatic potential (25). The parental cell line M4Be gives a low number of metastases in the lung of immunosuppressed newborn rats, whereas Tw12 gives a high number of lung metastases. Our aim was to identify gene determinants of melanoma metastasis. We validated and extended the results at the protein level using Western blot of cell lines and immunohistochemistry (IHC) on clinical samples. Finally, we applied our gene signature to publically available expression data to estimate its potential prognostic impact.

## Materials and Methods

*Melanoma cell lines.* The two melanoma cell lines, M4Be and Tw12 (clone 4), were kindly provided by Dr. C.P. Thomas (Lyon, France) (26) and grown as recommended.

*DNA microarrays.* Gene expression profiling was performed with home-made cDNA-spotted nylon microarrays containing 1,039 human cDNA and control clones. Clones were obtained from the IMAGE consortium (Hinxton, UK). They were chosen to represent genes with proven or putative implication in cancer progression and/or in immune reactions, including genes involved in transcription, the cell cycle, cell adhesion, invasion, and angiogenesis. The 1,039 clones represented 845 unique genes/Expressed Sequence Tags (762 named genes and 83 ESTs, according to the Unigene Build #200). The use of control clones, PCR amplification and robotical spotting of PCR products onto Hybond-N+ membranes (Amersham Pharmacia Biotech, Quebec, Canada) were carried out as previously described (27).

Total RNA was extracted from frozen cell lines by standard methods (28) and integrity was controlled by 28S Northern blots. Before RNA hybridization, the quality of spotting, including the determination of target DNA amount accessible for each spot, was controlled by hybridization with a $^{33}$P-labeled oligonucleotide sequence common to all PCR-products. After stripping, DNA microarrays were hybridized with probes made from 5 μg of total RNA from each cell line by simultaneous reverse transcription (oligodT priming) and [α-$^{33}$P] dCTP labeling as described at http://tagc.univ-mrs.fr/pub/Cancer/. After washes, arrays were exposed to phosphor-imaging plates, which were then scanned with a FUJI BAS 1500 machine (Raytest, Paris, France).

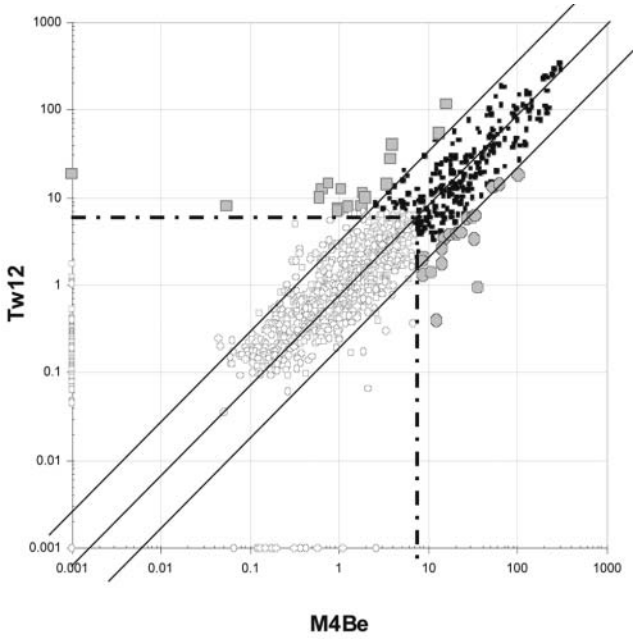


Figure 1. *Comparison of mRNA expression levels in metastatic melanoma Tw12 cells and the parental M4Be cell line. Comparison of quantified and normalized mRNA expression levels for the two cell lines displayed as a log/log diagram. The majority of expression levels remain similar within a factor of four (black and white dots, respectively above and under the retained expression threshold), although some differential expression is obvious (large grey squares and grey points represent genes up-regulated and down-regulated respectively in Tw12). Dashed lines indicate the threshold of the filter applied in analysis, and black lines the threshold of differential expression.*

Signal intensities were quantified by a modified version of the BioImage software (Millipore, USA). Hybridization images were inspected for artifacts and aberrant spots were excluded from analyses. Intensities were then normalized for the amount of spotted DNA (27) and the variability of experimental conditions (29). Expression data will be submitted to the Gene Expression Omnibus (GEO) database in a format compliant with MIAME guidelines. Before analysis, a filtering process removed genes/ESTs from the dataset which had low and poorly measured expression as defined by an expression value inferior to 7 units in both cells lines. The previously reported reproducibility of our method was sufficient enough to consider a 4-fold expression difference as significant (27).

*Western blot analysis.* Western blot analysis was carried out on protein extracts from the two cell lines with anti-CD10 monoclonal antibody (Clone 56C6; Novocastra, Newcastle, UK) using standard protocols (30).

*Immunohistochemistry.* The same anti-CD10 antibody was tested on formalin-fixed paraffin-embedded sections of 54 primary cutaneous melanoma and 21 metastatic tumors as described elsewhere (31). The cut-off for positivity was the presence of staining in more than 10% of the cells examined.

Table I. *List of genes differentially expressed between metastatic melanoma Tw12 cells and the parental M4Be cell line. Clones are ordered according to decreasing ratio ($log_2$).*

| Clone ID | Up* | Gene symbol | Gene name | Refseq transcript ID | Chrom. loc. | Ratio** |
|---|---|---|---|---|---|---|
| 204702 | Tw12 | MME/CD10 | Membrane metallo-endopeptidase | NM_007289 | 3q25.1-q25.2 | 14.17 |
| 182610 | Tw12 | MGST1 | Microsomal glutathione S-transferase 1 | AK058030 | 12p12.3-p12.1 | 7.18 |
| 1341716 | Tw12 | VEGFA | Vascular endothelial growth factor A | NM_001025366 | 6p12 | 4.26 |
| 1677822 | Tw12 | CDH19 | Cadherin 19, type 2 | NM_021153 | 18q22-q23 | 4.23 |
| 136821 | Tw12 | TGFβ1 | Transforming growth factor, beta 1 | X02812 | 19q13.2-q13.1 | 4.05 |
| 753105 | Tw12 | WNT10A | Wingless-type MMTV integration site family, member 10A | BC052234 | 2q35 | 3.54 |
| 1687125 | Tw12 | CDKN1A | Cyclin-dependent kinase inhibitor 1A | NM_078467 | 6p21.2 | 3.30 |
| 1651203 | Tw12 | EST | - | - | - | 2.90 |
| 301950 | Tw12 | XBP1 | X-box binding protein 1 | AK093842 | 22q12.1-q12 | 2.88 |
| 2163351 | Tw12 | CASP2 | Caspase 2 | AB209640 | 7q34-q35 | 2.86 |
| 1678953 | Tw12 | IL8 | Interleukin-8 | NM_000584 | 4q13-q21 | 2.85 |
| 156213 | Tw12 | JUNB | Jun B proto-oncogene | CR601699 | 19p13.2 | 2.62 |
| 154997 | Tw12 | KIAA1949 | KIAA1949 | AK124880 | 6p21.3 | 2.61 |
| 132711 | Tw12 | KLF5 | Kruppel-like factor 5 | AF132818 | 13q22.1 | 2.12 |
| 726144 | Tw12 | ITGα3 | Integrin, alpha 3 | AB209658 | 17q21.33 | 2.03 |
| 340754 | Tw12 | TEGT | Testis enhanced gene transcript | AK225804 | 12q12-q13 | 2.00 |
| 136821 | Tw12 | TGFβ1 | Transforming growth factor, beta 1 | X02812 | 19q13.2-13.1 | 2.33 |
| 49439 | M4Be | NRG1 | Neuregulin 1 | NM_013960 | 8p21-p12 | -2.11 |
| 148638 | M4Be | IGFBP5 | Insulin-like growth factor binding protein 5 | NM_000599 | 2q33-q36 | -2.01 |
| 203518 | M4Be | RXRβ | Retinoid X receptor, beta | NM_021976 | 6p21.3 | -2.16 |
| 138788 | M4Be | PRLR | Prolactin receptor | BX538149 | 5p14-p13 | -2.16 |
| 205314 | M4Be | TP53 | Tumor protein p53 | DQ186648 | 17p13.1 | -2.16 |
| 488548 | M4Be | PBEF1 | Pre-B-cell colony enhancing factor 1 | NM_005746 | 7q22.2 | -2.22 |
| 490005 | M4Be | TUBβ6 | Tubulin, beta 6 | AK092677 | 18p11.21 | -2.23 |
| 267657 | M4Be | MAGEH1 | Melanoma antigen family H, 1 | AF143235 | Xp11.21 | -2.41 |
| 158347 | M4Be | ETV4 | Ets variant gene 4 | U18018 | 17q21 | -2.43 |
| 246170 | M4Be | GSN | Gelsolin | AK125819 | 9q33 | -2.44 |
| 486001 | M4Be | VEGFB | Vascular endothelial growth factor B | BM546651 | 11q13 | -2.45 |
| 266361 | M4Be | MLANA | Melan-A | NM_005511 | 9p24.1 | -2.49 |
| 265875 | M4Be | KIT | V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog | BC071593 | 4q11-q12 | -2.55 |
| 234357 | M4Be | ITGαX | Integrin, alpha X | NM_000887 | 16p11.2 | -2.75 |
| 78100 | M4Be | IGFBP2 | Insulin-like growth factor binding protein 2, 36 kDa | AB209509 | 2q33-q34 | -2.94 |
| 838611 | M4Be | APOD | Apolipoprotein D | BF790155 | 3q26.2-qter | -3.02 |
| 503874 | M4Be | NBL1 | Neuroblastoma, suppression of tumorigenicity 1 | NM_001032363 | 1p36.13-p36.11 | -3.29 |
| 155345 | M4Be | EST | - | - | - | -4.95 |
| 183641 | M4Be | S100B | S100 calcium binding protein B | BC041935 | 21q22.3 | -5.25 |

*Up-regulated in; Chrom. loc., chromosomal location; **ratio is the $log_2$ ratio between expression level in Tw12 *vs.* M4Be.

*Statistical analyses.* Associations between IHC staining and histoclinical factors were calculated with the Fisher's exact test. Expression and clinical data published by Winnepenninck *et al.* (24) were downloaded from the Array Express data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under the following accession number: E-TABM-1 IGR_MELANOMA_STUDY. The metastasis-free survival (MFS) was calculated from the date of diagnosis until the date of first distant metastasis. Survival curves were derived from Kaplan-Meier estimates and compared by log-rank test. A $p$-value $<0.05$ was considered significant. Analyses were carried out with SPSS software (version 10.0.5; Chicago, Illinois, USA).

## Results

*Gene expression profiling of melanoma cell lines.* We first established the gene expression profile of the two clonally-related cell lines with different invasive potential on DNA microarrays containing ~1,000 genes selected for a putative role in cancer progression. Thirty-six clones were differentially expressed between the two cell lines (Figure 1), 17 of which were up-regulated in Tw12 and 19 in M4Be; these are listed in Table I. They represented two ESTs and

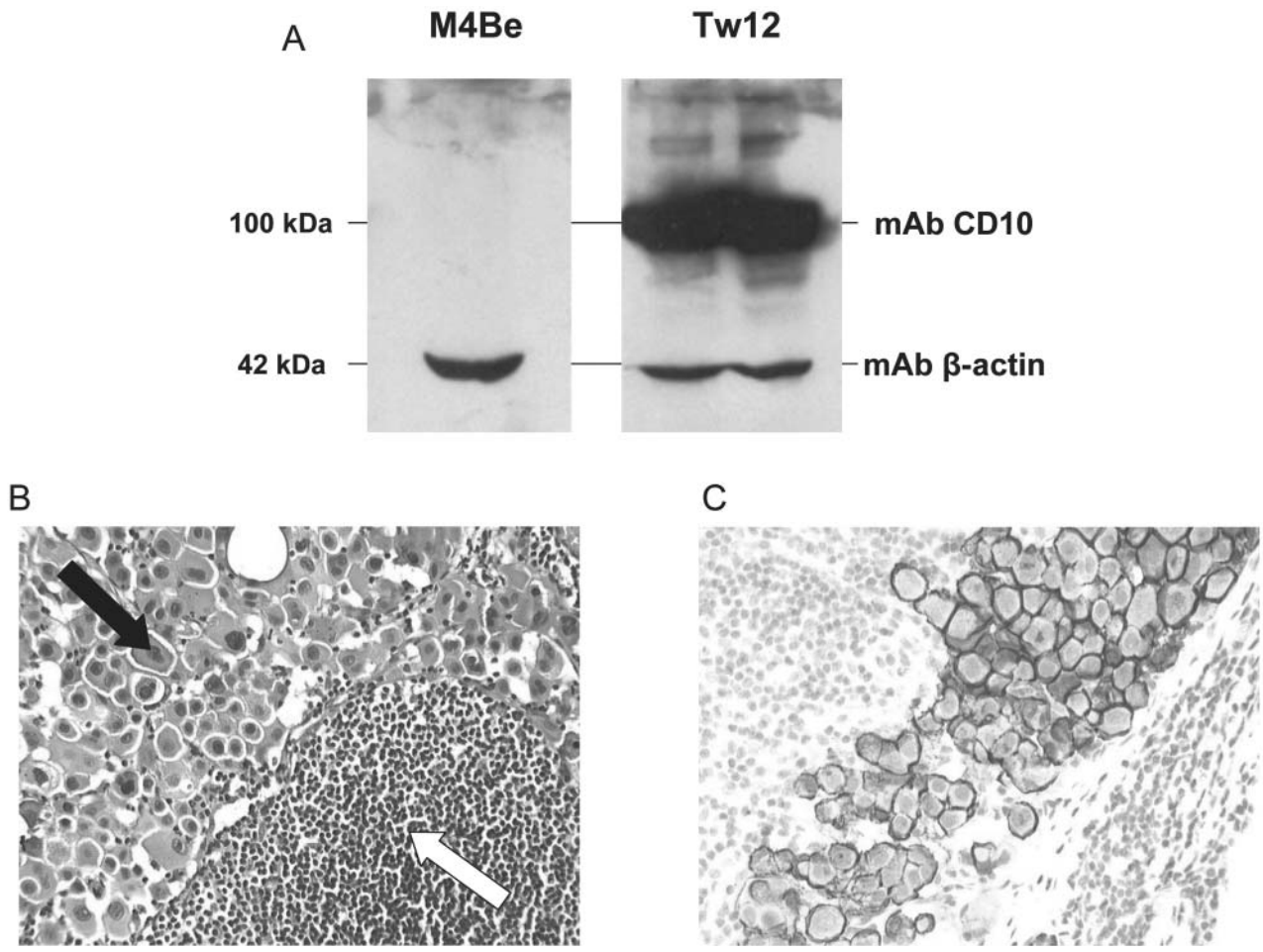


Figure 2. *Protein expression of CD10 in melanoma. A) Western blotting analysis of CD10 in the two cell lines. Note that CD10 (100 kDa) is strongly expressed in Tw12 and is not expressed in the parental M4Be cell line. Expression of CD10 in melanoma lymph node metastasis: B) Hematoxylin-eosin staining: note infiltration of lymph node sinus by melanoma cells (black arrow) and residual lymphocytes (white arrow). C) Immunostaining with anti-CD10 antibody: note the strong membrane positivity of neoplastic melanoma cells. (x350).*

33 named genes. The most up-regulated gene in the Tw12 as compared to the M4Be cell line was *CD10* and the most down-regulated was *S100B*.

*Analysis of CD10 expression in melanoma.* We validated and extended our results at the protein level. We focused on the membrane metallo-endopeptidase CD10/MME, for which an antibody efficient in paraffin-embedded tissues was available.

We first demonstrated the differential expression of CD10 at the protein level. In agreement with the mRNA results, Western blot analysis of protein extracts from the two cell lines (31) showed a strong CD10 expression in the highly metastatic Tw12 clone and a lack of expression in the weakly metastatic M4Be clone (Figure 2A).

We then assessed the potential clinical relevance of CD10 in human melanoma samples. We studied CD10 expression by IHC on formalin-fixed paraffin-embedded sections of 54 primary cutaneous melanoma (including 9 lentigo maligna, considered as a melanoma precursor) and 21 metastatic tumors. CD10 expression was found in 19 of 45 primary cutaneous melanoma and in 18 of 21 metastatic tumors ($p=0.001$, Fisher's exact test). None of the tumors of Clark's levels I+II (0/10) stained with anti-CD10 antibody, whereas tumors of levels III+IV+V were CD10-positive in 19/35 cases ($p=0.002$, Fisher's exact test). Only one of the nine cases of lentigo maligna was weakly positive. Results are summarized in Table II and examples of staining are shown in Figure 2B-C. This result corroborates the mRNA expression data and suggests that CD10 is a marker of aggressive melanoma.

Table II. *CD10 immunostaining in primary and metastatic melanoma.*

| | CD10 expression |
|---|---|
| Primary cutaneous melanoma | 19/45 (42%) |
| Clark's levels I and II* | 0/10 |
| Clark's levels III,IV,V** | 19/35 |
| Metastatic melanoma | 18/21 (89%) |
| Lymph nodes | 11/13 |
| Other sites | 7/8 |
| Lentigo maligna | 1/9 (11%) |
| *In situ* | 0/8 |
| Clark's level III | 1/1 |

*Three cases of acral lentiginous melanoma; **1 case of acral lentiginous melanoma (CD10+).

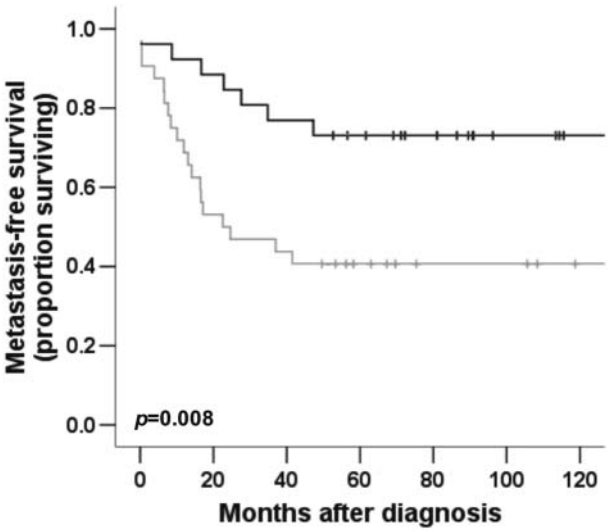


Figure 3. *Kaplan-Meier metastasis-free survival for melanoma patients. Metastasis-free survival for the 58 tumors profiled by Winnepenninckx et al.' (24) in the two prognostic groups defined according to 29 genes (46 probe sets) of our 36 gene list: black curve, predicted "good-prognosis group"; grey curve, predicted "poor-prognosis group". The p-value was calculated using the log-rank test.*

*Prognostic impact of the gene signature in melanoma.* We compared our 36-gene list with a recently published 361-gene prognostic signature obtained on 58 tumors (24). The overlap was limited to only one gene (*VEGFA*). We applied our gene list to Winnepenninckx *et al.*'s publicly available data: 32 of the 58 profiled tumors were from patients without any distant metastatic relapse within 4 years, and 26 from patients with metastatic relapse within 4 years. Twenty-nine of our 36 genes were present in their microarrays and represented by 46 probe sets. Based on these probe sets, we classified the 58 samples according to the correlation coefficient of their expression profile with the median profile of the 26 samples with metastatic relapse. With a threshold equal to 0, our discriminator gene set was able to separate the samples into two groups with different clinical outcome. Five-year MFS was 41% in the predicted "poor-prognosis group" and 73% in the predicted "good-prognosis group" ($p=0.008$, log-rank test, Figure 3).

## Discussion

The well-defined differences in metastatic behavior and the clonal relationship of the Tw12 (clone 4, highly metastatic) and M4Be cell lines allow their use to identify potentially relevant genes involved in melanoma metastasis. We used DNA microarrays containing ~900 genes with proven or putative implication in cancer progression and/or in immune reactions. We identified 36 genes with strong differential expression between the two cell lines. Results were validated and extended using western blot of cell lines, and IHC and *in silico* analyses of clinical samples.

Functional annotation of differentially expressed genes helps generate hypotheses about the biological mechanisms that sustain the metastatic process in melanoma. Of course, whether these genes are causative or only a consequence of the phenotype in a biological sense or reflect another associated phenomenon remain to be explored. However, interesting points must be highlighted. Several genes strongly overexpressed in the highly metastatic Tw12 clone are involved in invasion, angiogenesis, growth, apoptosis and drug resistance. *CD10* was the top up-regulated gene, in agreement with Seftor *et al.* (20). CD10/CALLA is a cell-surface membrane metallo-endopeptidase normally expressed on human neutrophils and several other cell types, including epithelial and lymphoid cells (32). CD10 is also expressed and functional on melanoma cell lines (33, 34). In addition, preliminary (35-37) and recent (38) data suggest that CD10 expression is a progression marker in human melanoma, particularly in metastatic tumors (20, 36-39). Even if its implication in the progression of other types of cancer has been recently reported (40-45), its role remains poorly understood, but might be related to the modulation of various signaling and invasive pathways in cancer cells. In our series, both primary and metastatic tumors were available for two patients. In one case, the metastasis was strongly stained, whereas the primary tumor was negative. In the second, both primary and metastatic tumors were positive for CD10. In the first case, tumor cells may behave like M4Be and Tw12 cell lines, *i.e.* they express CD10 when they become metastatic. In the second, the metastatic phenotype might have been acquired earlier in the primary site. Although its exact role in progression remains to be clarified, CD10 inhibitors such as phosphoramidon, thiorphan or acetorphan should be tested as potential therapies in metastatic melanoma.

Identification of other genes up-regulated in the Tw12 metastatic cell line warrants further investigation. Interleukin-8 (*IL8*) is the most potent angiogenic factor produced by melanoma cells (46, 47) and contributes to disease progression (48). Antibodies directed against IL8 inhibit angiogenesis, tumor growth and metastasis of melanoma (49, 50). TGFβ is a tumor and metastasis promoter in several types of cancer (51). It has pro-angiogenic activity and induces IL8 expression in highly metastatic melanoma cells (52). This induction could be one of mechanisms by which TGFβ1 promotes angiogenesis, growth, and metastasis of human melanoma. Hypoxia is an important feature of tumor microenvironment, contributing to cancer progression. Several genes up-regulated in Tw12 (*VEGFA, XBP1* and *CDKN1A*) have a link with hypoxia. XBP1 is an essential survival factor under hypoxic stress and is required for tumor growth (53). *VEGFA* codes for vascular endothelial growth factor A, the expression of which is associated with tumor thickness and/or metastatic relapse in melanoma ((54) for review). *JUNB* is a regulator of VEGF transcription (55) and was up-regulated in Tw12. Overxepression of *VEGFA* and *JUNB* involved in angiogenesis and vasculogenesis could support the concept of "vasculogenic mimicry" of aggressive melanoma cells recently described (56). Importantly, *VEGFA* and *XBP1* (57) are also potential therapeutic targets: anti-angiogenic drugs, such as bevacizumab, thalidomide or sorafenib, are being assessed in melanoma clinical trials ((58) for review). Like *VEGFA, CDKN1A* expression is increased by hypoxia (59). CDKN1A/P21 is involved in the control of the G1/S transition of the cell cycle; its protein expression has been associated with shorter survival in a series of melanoma patients (60). *ITGα3* encodes the alpha-3 subunit of human alpha-3/beta-1 integrin (VLA-3), a receptor for fibronectin, laminin, and collagen, involved in the migration and invasion of melanoma cells (61). Kruppel-like factor (KLF5) is a growth mediator of various epithelial cells with angiogenic activity. Resistance to apoptosis has been associated with the acquisition of the metastatic phenotype of melanoma. *TEGT*, which codes for the anti-apoptotic protein BAX inhibitor-1 (BI-1), was overexpressed in Tw12, as in prostate (62) and breast (63) cancers. *MGST1* is involved in anticancer drug resistance (64), and is overexpressed in prostate cancer (65). *CDH19* shares strong homology and co-clusters on 18q22-q23 with *CDH7*, which is up-regulated in melanoma (66).

Genes underexpressed in the strongly metastatic Tw12 clone include two melanocytic markers, *S100B*, a calcium-binding protein, and *MLANA*, a melanoma antigen, as previously reported in highly invasive uveal melanoma cell lines (20). Their down-regulation, like that of *MAGEH1*, is associated with the absence of melanin in Tw12 suggesting a dedifferentiation process associated with increased metastatic potential. High expression of MLANA mRNA and/or protein is associated with reduced motility and invasive ability (19) and with improved disease outcome (67, 68). *KIT* was also down-regulated in the Tw12 cell line in agreement with loss of protein expression during melanoma progression (69). The loss of expression of *NBL1*, which encodes a known tumor suppressor gene involved in the retinoic acid-induced differentiation of neuroblastoma cells (70), of *NRG1*, which encodes neuregulin 1, and of *APOD* (20), which encodes apolipoprotein D (71), may reflect the loss of neural crest-derived differentiation markers as aggressive melanoma cells become less differentiated. Down-regulated genes also included *RXRB*, involved in differentiation, and two other tumor suppressor genes *TP53* and *GSN*. *GSN* encodes gelsolin, an actin-binding protein implicated in cell motility with reported metastasis suppressor function in melanoma cell lines (72). Down-regulation of *IGFBP2* and *IGFBP5* is in agreement with the underexpression of *IGFB5* in melanoma *vs.* naevi and *IGFBP7* in metastases *vs.* primary melanoma (73).

The small overlap between our 36-gene list and the 361-gene prognostic signature reported by Winnepenninck *et al.* may be linked to several methodological differences with respect to the microarray technology used (nylon *vs.* glass solid support, radioactive *vs.* fluorescent RNA labeling, single *vs.* double labeling and hybridization, cDNA clones *vs.* long oligonucleotides, analytical method), genes tested and samples profiled, homogeneous cell lines *vs.* whole tumor tissue samples that include melanoma cell lines as well as non-melanoma cells such as stromal and infiltrating immune cells. However, the clinical samples could be classified by our gene list into two different prognostic groups, further suggesting the potential role of the genes that we have identified *in vitro* in metastatic process.

## Conclusion

We provide a list of genes potentially involved in the metastatic process of melanoma. Genes up-regulated in the highly metastatic cell line included genes involved in angiogenesis, invasion, growth and apoptosis. Down-regulated genes included tumor suppressor genes and genes involved in cell differentiation. For most of these genes, the prognostic impact on survival needs to be clarified in large series of patients. Their exact role in tumor progression remains to be clarified by experimental models, not only in melanoma but also perhaps in other solid tumors with high rates of pulmonary metastasis. Importantly, several of these genes are targetable by drugs under development, which should be tested as potential therapies in metastatic disease. In this context, the two cell lines investigated in this study may serve as models for testing such compounds and other inhibitors of the invasive phenotype.

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