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The Melanoma Vascular Mimicry Phenotype Defined in Gene Expression and Microsome Sequencing Analysis

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Abstract. *The phenomenon of vasculogenic mimicry in melanoma has been recently described to be an important factor relating to melanoma progression. Large scale gene expression profiling by real-time quantitative RT-QPCR of a panel of 40 normal tissues and 54 cancer cell lines revealed that two genetically related melanoma cell lines, one derived from a primary lesion Hs.688(A) and one derived from a lymph node metastasis Hs.688(B), displayed a unique expression pattern when compared to other cancer cell lines and tissue samples in the panel. Quantitative-RT-PCR data indicated that these melanoma cells expressed a number of activated endothelial cell-associated genes such as tissue inhibitors of matrix metalloproteinases TIMP-2, matrix metalloproteinase (MMP-1, MMP-2), thrombospondin 1 (TSP1), proto-oncogene c-MET and vascular endothelial growth factor (VEGF). To examine the gene expression profile of these unique melanoma cells in greater depth, cDNA libraries were made from isolated microsome complexes to enrich those transcripts that were destined to be translated into cell surface or secreted proteins. High throughput sequencing analysis revealed that this library contained over 7000 cDNAs and was enriched by over 80% of secreted or membrane-bound proteins. The presence in the cDNA library of genes such as acetyl LDL receptor, tumor endothelial markers-1, 5 and 8 (TEMs), flow-induced endothelial G protein coupled receptor-1 and VEGF-related protein (VRP), all of which are known to be expressed uniquely by endothelial cells, supported the hypothesis that Hs.688(A) and Hs.688(B) cells were mimicking an activated vascular phenotype. Ultimately the goal is to investigate the biological roles of endothelial cell-associated genes in the behavior of Hs.688(A) and Hs.688 (B) melanoma cells.*

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The phenomenon of vasculogenic mimicry has recently received much support for its potential role in tumor progression as vascular mimicry observed in a number of tumor types, including melanoma, breast, prostate and ovarian cancer (1-5). Previous studies have mainly focused on the role of tumor angiogenesis, the recruitment of new vessels into a tumor from pre-existing vessels (6). However, Folberg *et al.* have described a novel process by which uveal melanomas develop a highly patterned microcirculation that is independent of angiogenesis (7). In aggressive primary and metastatic melanomas, the tumor cells generate microcirculatory channels composed of extracellular matrix lined externally by tumor cells. Due to the fact that the *de novo* generation of vascular channels by aggressive and metastatic tumor cells is not strictly a vasculogenic event, a new name, "vasculogenic mimicry", was given to the process by which aggressive tumor cells generate non-endothelial cell-lined channels delimited by extracellular matrix (8, 9). The identification of vasculogenic mimicry in melanoma as a unique event from angiogenesis suggests that aggressive tumor cells may acquire their blood supply by different mechanisms. In fact, nearly 50% of patients with uveal melanoma die from metastatic melanoma. Cutaneous melanoma may disseminate through lymphatics or blood vessels. In contrast, the interior of the eye lacks lymphatics and uveal melanoma, which develops in one of the most capillary-rich tissues of the body, is a paradigm of pure hematogeneous dissemination of cancer (10). Therefore, the development of a tumor microcirculation is an important step for tumor metastasis.

Several studies have attempted to classify melanoma using molecular profiling approaches (11, 12). These studies have revealed a number of potential molecular markers for the classification of the disease. To understand the mechanism of vasculogenic mimicry at the molecular level, we have attempted to characterize the vascular mimicry phenotype by utilizing large scale, unbiased, gene expression analysis to characterize two melanoma cell lines. These melanoma cells are unique in that one cell line is derived from a primary tumor Hs.688(A) and one is derived from a

lymph node metastasis Hs.688(B). We chose some of the unique endothelial marker genes and analyzed their expression patterns using quantitative RT-PCR analysis. Analysis in a panel of 40 normal tissues and 54 cancer cell lines revealed that these two cell lines showed patterns of gene expression characteristic of endothelial cells.

To further analyze the pattern of gene expression in these cells, mRNAs of secreted or membrane bound proteins were isolated with microsomes preparation and the identities of the cell surface and secreted molecules were analyzed by high throughput sequencing. Secreted and cell surface-bound genes were successfully enriched up to 80% with a microsome isolation approach. Our results indicate that both the Hs.688(A) and Hs.688(B) cell lines expressed many cell surface and secreted marker genes similar to endothelial cells. In addition, numbers of endothelial marker genes were expressed at a higher level compared to endothelial cells. Several genes related to endothelial cell markers are also regulated by nuclear factor Î B (NFÎ B), which has been shown to play a key role in the vascular progression of melanoma (13). Further, this study has identified additional target genes that may be associated with the vascular mimicry phenotype in human melanoma.

Materials and Methods

Cell culture. Tissue culture media and serum were purchased from Invitrogen (Carlsbad, CA, USA). Human metastastic melanoma cell lines Hs.688 (A) and Hs.688 (B) were purchased from the American Type Culture Collection (ATCC). Both cells were grown in DMEM media containing 10% fetal bovine serum, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose with 4 mM L-glutamine at 37˚C in a humidified incubator.

Microsome isolation. To isolate membrane-bound cell surface and secreted genes from Hs.688 (A) and Hs.688 (B), 2 x 10^8 culture cells were incubated with 100 μg/ml cycloheximide for 10 min. The culture media was removed and cells were harvested in 10 ml ice-cold 1x phosphate buffered saline (PBS) containing 100 μg/ml cycloheximide. The cells were pelleted by centrifuging at 1500 rpm for 4 min and washed twice with (30 ml) ice-cold 1xPBS containing 100 μg/ml cycloheximide. The cells were allowed to swell for 5 min in 1 ml ice-cold RSB buffer (10 mM KCl, 1.5mM $MgCl_2$ and 10 mM Tris-HCl at pH 7.4) plus 1 mg/ml heparin. The cells were then ruptured with 10 strokes of a dounce glass homogenizer. The homogenate was then transferred to a new set of RNase-free eppendorf tubes and centrifuged at 3000 rpm for 2 min at 4˚C. One ml of supernatant was pipetted into a Falcon tube containing 5.5ml of 2.5M sucrose and vortexed. Two ml of 2.5 M sucrose in TK150 M (150 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl at pH 7.4) solution was added to a 17-ml centrifuge tube and the sample extract (at the final concentration of 2.1 M sucrose) was laid on the top of the 2.5 M sucrose phase. 6.5 ml of 2.05 M sucrose (in TK150M) solution was carefully added on top and another 2 ml layer of 1.3 M sucrose (in TK150 M) was added. The sample tubes were loaded on the brackets of a SW28 rotor and centrifuged at 25,000 rpm for 5 h at 4˚C (Beckerman-Coulter XP100, CA, USA). One ml of each fraction was removed from the sample tube to a new set of pre-chilled RNase-free eppendorf tubes. Absorbance was measured at 260 nm from 10 μl of sample aliquoted from each fraction.

mRNA isolation and cDNA synthesis. The mRNA templates bound to microsomes were isolated using CuraGen's standard isolation protocol (27). In brief, microsome fractions from the sucrose gradient were mixed with three volumes of Tri-Reagent-LS (Molecular Research Center, Cincinnati, OH, USA). The tubes were incubated for 10 min at 23˚C with intermittent gentle agitation. The RNA was extracted following the manufacturer's specifications. The RNA pellets were resuspended in diethyl-pyrocarbonate (DEPC)-treated water, and the RNA content was quantified spectroscopically at 260 nm. RNA samples were stored at -20˚C. For the quantitative expression analysis, contaminating DNA was removed by treatment of the isolated RNA with DNase I (Promega, Madison, WI, USA). PolyA+ RNA was prepared by fractionation of total RNA with an mRNA purification kit that uses the biotinylated oligo-dT-streptavidin magnetic bead method (MPG, Lincoln Park, NJ, USA), followed by cDNA synthesis by reverse transcription of oligo-dT-primed mRNA (Superscript II; Life Technologies) and second-strand synthesis. Terminal phosphate removal was achieved by treatment with arctic shrimp alkaline phosphatase (Amersham Life Sciences, Piscataway, NJ, USA), followed by purification of cDNA by phenol-chloroform extraction. The yield of cDNA was quantitated by fluorometry using PicoGreen dye (Molecular Probes, Eugene, OR, USA).

Sequencing analysis. cDNAs from Hs.688(A) and Hs.688(B) were digested with 96 pairs of restriction enzymes in a 96-well plate. The fragments were then PCR-amplified using PCR primers (Amitof, Boston, MA, USA) and cDNA polymerase (Clontech, Palo Alto, CA, USA) for 25 cycles of 30 sec at 96˚C, 60 sec at 57˚C, 2 min at 72˚C. Subsequently, a 3 μl aliquot was ligated to pCR2.1 cloning vector (Invitrogen) using the Fast-Link DNA ligation kit (Epicenter, Madison, WI, USA). Vectors were electroporated into DH10B *E. coli* with 1.8 mV pulses and the cells were plated on LB plates containing ampicillin, kanamycin, and X-gal (Northeast Laboratories, Waterville, MA, USA). Colonies with inserts were selected for PCR amplification using 5 M betaine (Sigma, St. Louis, MO, USA), DYN-A and DYN-RE primers (Amitof) and *Taq* polymerase (Clontech) for 29 cycles of 1 sec at 96˚C, 1 min at 57˚C and 1 min at 72˚C. The PCR reaction products were then submitted to sequencing for clone identification. PCR templates were then added to 6 μl of SPRI beads (Bangs Laboratories, Fishers Ind.) in 0.5 M EDTA pH 8.0 (Amresco, Solon, OH, USA) and 30 μl hybridization buffer (2.5 M NaCl, 20% PEG 8000 (Sigma)) in 96-well plate format. The plates were shaken for 5 min at 600 rpm and then allowed to settle for 2 min on a magnet. The beads were washed a total of 4 times with 200 μl of 70% EtOH (AAPER, Louisville, KY, USA) and air dried for 2 min. Thirty-six μl of Nanopure water was then added to the beads. The plates were again shaken for 5 min at 600 rpm and the supernatant was collected for sequencing. Three μl of purified product was then transferred to: A (JOE-fluor), G (TAMRA-fluor), C (FAM-fluor) and T (ROX-fluor) reaction mixes (2 μl DYEnamic Direct Cycle sequencing kit: DYEnamic-M13-40ET primers, premixed dGTP, Taq polymerase (Amersham) and 1.8 μl dNTP mix (Amersham) in 384-well format. The plates were then placed in a thermocycler for 15 cycles of: 5

sec at 96˚C, 10 sec at 52˚C and 60 sec at 72˚C. Reactions were quenched at 4˚C. For each template, the four reactions were pooled into one well of a 96-well plate and 65 μl of 100% EtOH (AAPER) was added. The plates were chilled at 4˚C for 60 min and centrifuged at 4˚C for 30 min at 2000 rpm (Thermo-IEC 1L-GP, Thermo Electron Corporation, MA, USA). The supernatant was removed and the plates were air dried to completion at 25˚C. Three μl of formamide loading dye (Amersham) was added to each well. In addition, 960 μl of TAMRA-spiked loading dye (10:1 formamide; Amersham): 55-mer TAMRA (Amitof)) was added to selected wells for an electrophoresis quality control. The samples were electrophoresed on the Amersham 1000 (Amersham Biosciences Inc.) electrophoresis platform. The sequencing information was analyzed.

Quantitative RT-PCR analysis. Real-time RT–QPCR analysis was performed on the experimental mRNAs to confirm the results obtained by the microarray analysis. The PCR primers and probes (Synthegen LLC, Houston, TX, USA) for thrombospondin, TEM1, TEM 5 and GAPDH were as follows: RT–QPCR was performed on an ABI 7900HT instrument under the following conditions: 48˚C, 30 min of reverse transcription; 95˚C, 10 min; 95˚C, 15 sec; 60˚C, 1 min. The reaction was performed for up to 40 cycles.

Thrombospondin 1

Forward primer: 5'-GTGCTGCAGAATGTGAGGTTTGTC-3'
Reverse primer: 5'-ACAGTGACACTCAGTGCAGCTATC-3'
Probe: 5'-AGGACAAGCATCCGCAAAGTGACTGAA-3'

Tumor endothelial marker 1

Forward primer: 5'- CTACGTTGGTGGCTTCGAGTGTTA-3'
Reverse primer: 5'-GATCTGGATAGTTGGCTGCGATCA-3'
Probe: FAM-5'-ATGAGGAAGATGAAGACGAGGCCTGGAA-3'-TAMRA

Tumor endothelial marker 5

Forward primer: 5'-ATCACCTACATCCTCAACCACAGC-3'
Reverse primer: 5'-TCAAAGCCACAGGGATGTAGAAGG-3'
Probe: FAM-5'-TGTGGCATCACAGCTGCAGTCAACAT-3'-TAMRA

Control house-keeping gene GAPDH

Forward primer: 5'-AAAGTGGATATTGTTGCCATCA-3'
Reverse primer: 5'-GGTGGAATCATATTGG AACATG-3'.
Probe: FAM-5'-CCCCTTCATTGACCTCAACTACATGG-3'-TAMRA

Real-time quantitative RT-QPCR data were analyzed with Hierarchical cluster analysis to reveal the specific endothelial pattern of gene expression using Spotfire software.

Gene expression using an oligonucleotide array. Gene expression analysis was performed with CuraGen's proprietary oligonucleotide microarray containing over 8000 genes. RNA isolation, sample labelling and hybridization were performed as previously described (25). The results were analyzed with Spotfire Hierarchical cluster analysis.

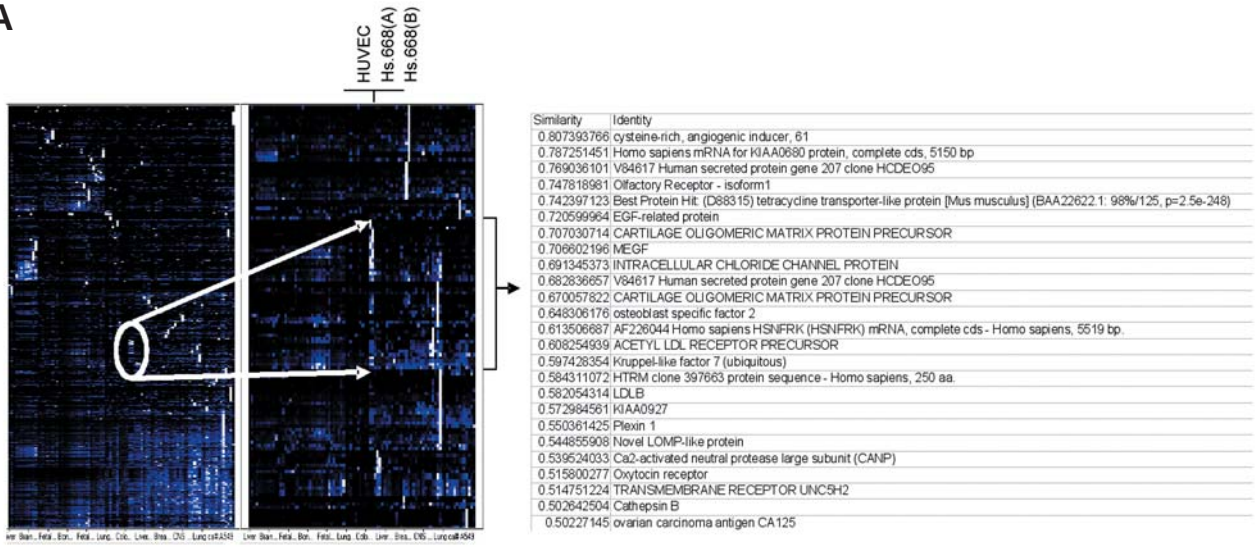
Western immunoblot analysis. To characterize microsome isolation, calnexin was used to monitor the quality of the microsome isolation procedure. Equal amounts (10 μgs) of samples from each gradient fraction were resolved by SDS-PAGE on 12.5% gels by the method of Laemmli (26). Proteins were probed with rabbit anti-calnexin polyclonal antibody (1:2000 dilution (Santa Cruz Biotech Inc, CA, USA) followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad, CA, USA) (1:1000 dilution). Proteins were visualized with a chemiluminescence detection system using the Super Signal substrate (Pierce, IL, USA).

Results

In this study, we attempted to characterize the vascular mimicry phenotype of human melanoma cells Hs.688(A) and Hs.688(B) by quantitative RT-PCR analysis compared to a panel of 40 normal tissues and 54 cancer cell lines. The gene expression analysis revealed that both Hs.688(A) and Hs.688(B) cells expressed many marker genes unique to endothelial cells using Hierarchical cluster analysis (Figure 1). The Hierarchical cluster analysis allows us to group together similar expression patterns of genes from two melanoma cell lines and human umbilical vein endothelial cells (HUVEC) (Figure 1A), and these genes include thrombospondin 1 (TSP1), TEM1, TEM5, TEM8, VEGF, MMP-1 and MMP-2. The representative quantitative RT-PCR results for TSP1, TEM1 and TEM5 clearly showed that the melanoma cells expressed the unique patterns of genes similar to endothelial cells (Figure 1B).

In order to study the endothelial-like features of these two melanoma cell lines in detail, we focused on cell surface and secreted proteins by isolating the mRNA populations from microsome complexes. Using high speed ultracentrifugation and sucrose gradient separation, we were able to successfully isolate a microsome complex containing mRNAs for both secreted and cell surface molecules. The microsome isolated mRNAs were then purified and converted to a cDNA library for sequencing analysis. The rough endoplasmic reticulum (ER) marker gene calnexin was used to monitor the microsome isolation process (Figure 2). Calnexin was clearly enriched in the first two fractions by Western immunoblot analysis compared to the rest of the gradient fractions (Figure 2A). The nucleic acid contents from each fraction were also measured with absorbance at 256 nm (Figure 2B). The subsequent sequencing analysis revealed that over 7000 cDNAs were enriched for secreted and cell surface molecules. This procedure successfully enriched the secreted and cell surface genes by over 80%. Genes such as acetyl LDL receptor, TEM 1, 5 and 8, flow-induced endothelial G protein coupled receptor-1 and VEGF-related protein (VRP), all of which are known to be expressed uniquely by endothelial cells.

A



Similarity	Identity
0.807393766	cysteine-rich, angiogenic inducer, 61
0.787251451	Homo sapiens mRNA for KIAA0680 protein, complete cds, 5150 bp
0.769036101	V84617 Human secreted protein gene 207 clone HCDEO95
0.747818981	Olfactory Receptor - isoform1
0.742397123	Best Protein Hit: (D88315) tetracycline transporter-like protein [Mus musculus] (BAA22622.1: 98%/125, p=2.5e-248)
0.720599964	EGF-related protein
0.707030714	CARTILAGE OLIGOMERIC MATRIX PROTEIN PRECURSOR
0.706602196	MEGF
0.691345373	INTRACELLULAR CHLORIDE CHANNEL PROTEIN
0.682836657	V84617 Human secreted protein gene 207 clone HCDEO95
0.670057822	CARTILAGE OLIGOMERIC MATRIX PROTEIN PRECURSOR
0.648306176	osteoblast specific factor 2
0.613506687	AF226044 Homo sapiens HSNFRK (HSNFRK) mRNA, complete cds - Homo sapiens, 5519 bp.
0.608254939	ACETYL LDL RECEPTOR PRECURSOR
0.597428354	Kruppel-like factor 7 (ubiquitous)
0.584311072	HTRM clone 397663 protein sequence - Homo sapiens, 250 aa.
0.582054314	LDLB
0.572984561	KIAA0927
0.550361425	Plexin 1
0.544855908	Novel LOMP-like protein
0.539524033	Ca2-activated neutral protease large subunit (CANP)
0.515800277	Oxytocin receptor
0.514751224	TRANSMEMBRANE RECEPTOR UNC5H2
0.502642504	Cathepsin B
0.50227145	ovarian carcinoma antigen CA125

Figure 1. →

Gene expression from HUVEC and two melanoma Hs.688(A) and Hs.688(B) cells were analyzed with oligonucleotide microarray and quantitative RT-PCR analysis (Figure 3). Table I lists a number of endothelial marker genes expressed in HUVEC, the Hs.688(A) and Hs.688(B) cells. Endothelial marker genes such as fibronextin 1, galectin 1, integrin beta 1, follistatin, VEGF-c and fibulin-like protein were expressed similarly in Hs.688(A) and Hs.688(B) compared to HUVECs. Table II lists genes expressed at higher levels in Hs.688(A) and Hs.688(B) than in HUVEC cells, including LDL receptor, TEM 1, 5 and 8, semaphorin 3C, VRP, fibulin 5, syndecan-1 and adlican.

Discussion

In this study we characterized the vascular mimicry phenotype of two human melanoma cell lines Hs.688(A) and Hs.688(B) by utilizing large scale, unbiased, gene expression analysis. These melanoma cells are unique in that one cell line is derived from a primary tumor Hs.688(A) and one is derived from a lymph node metastasis Hs.688(B). Quantitative RT-PCR analysis of a panel of 40 normal tissues and 54 cancer cell lines revealed that these two cell lines showed very similar patterns of gene expression characteristic of endothelial cells.

To further analyze patterns of gene expression in these cells, we focused on characterizing the cell surface and secreted molecules. Microsomes were isolated and the mRNAs of secreted or membrane-bound proteins were converted to cDNA for sequencing and expression analysis with proprietary oligonucleotide array. Secreted and cell surface-bound genes were successfully enriched up to 80% with a microsome isolation approach. Our results show that both cell lines expressed many cell surface and secreted molecules similar to endothelial cells, such as galectin 1, follistatin, integrin beta 1 and fibronectin 1. Furthermore, the endothelial marker genes TSP1, syndecan 1, TIG2, fibulin 5, TEM 1, semaphoring 3C and angiopoietin 1, were found to be expressed at higher levels in Hs.688(A) and Hs.688(B) cells compared to HUVEC cells.

TSP1, a homotrimeric protein, is a potent anti-angiogenic protein that promotes vascular quiescence (14). It was interesting to note the expression level of TSP1 in Hs. 688(A) and Hs.688(B) was higher compared to other types of cancer cell lines and normal cells (Figure 1B). This is consistent with the behavior of vasculogenic mimicry described by Holash *et al.* (15). They observed a process called "vessel co-option" in which tumors co-opt the existing vasculature, which regresses leading to massive necrosis. The tumor is then vascularized at the periphery by tumor angiogenesis. The high level of expression of TSP1 may be necessary for the formation of microcirculatory channels lined externally by tumor cells, which is different compared to the vasculogenesis process of endothelial cells. This could be the explanation of the controversial role of TSP1 in other studies showing that overexpression of TSP1 is associated with enhancement of tumor invasion in several types of tumors such as breast, lung and pancreatic carcinoma cell lines (16, 17). It is reasonable to speculate that TSP1 functions as an anti-angiogenetic factor for endothelial cells during angiogenesis, but that it functions as a tumour vascular promoting factor during melanoma vascular mimicry.

The higher gene expression level of fibulin-5 in these two melanoma cell lines is also interesting in that fibulin-5 protein

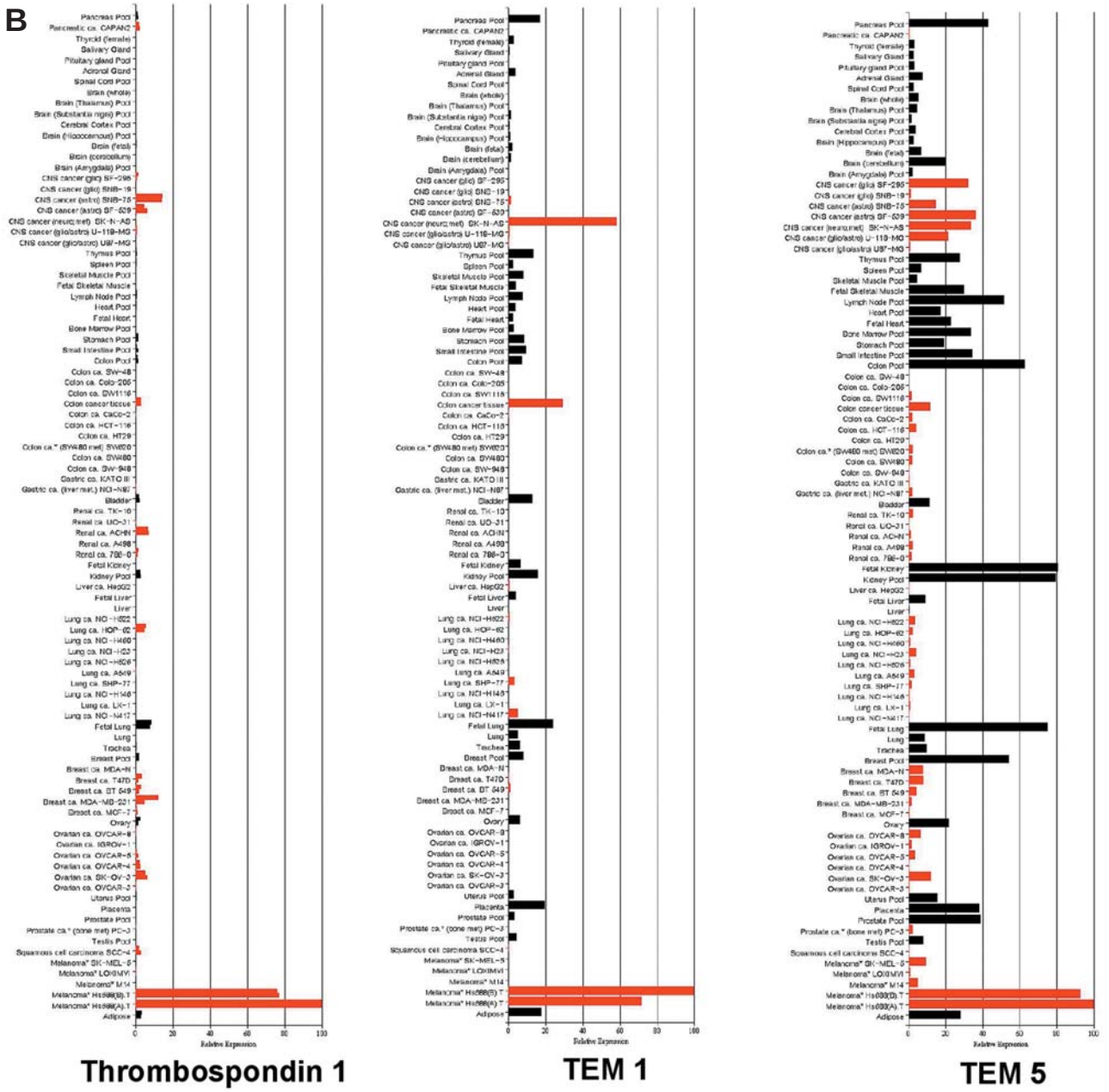


Figure 1. *(1A) Hierarchical clustering quantitative RT-PCR expression analysis with a panel of 40 normal tissues and 54 cancer cell lines using Spotfire software and the clustering patterns were generated with tree view program. Genes that are highly expressed by Hs.688(A) and Hs.688(B) and human endothelial cell HUVEC are indicated with the circled region on the left panel and the same region magnified is on the right side panel. The blue color grids indicate overexpressed genes and white color grids indicate no change in gene expression level. A partial list of endothelial marker genes is summarized on the right hand side. (1B) Thrombospondin 1, TEM1 and TEM5 were shown as examples of Hierarchical clustering quantitative RT-PCR analysis across a panel of 40 normal tissues and 54 tumor samples.*

mediates the development of elastic fiber. Studies have shown that fibulin-5 can promote cell attachment through cell surface integrins (18). Fibulin may stabilize the attachment of cells to elastic fibers, which could contribute to the organization of nascent elastic fibers during vascular development.

Syndecan-1 was also expressed at higher level in Hs. 688(A) and Hs. 688(B) cells than in normal human HUVEC cells in the oligonucleotide microarray analysis. Syndecan-1 is a transmembrane protein that binds both growth factors and extra cellular matrix (ECM) components, acting as a

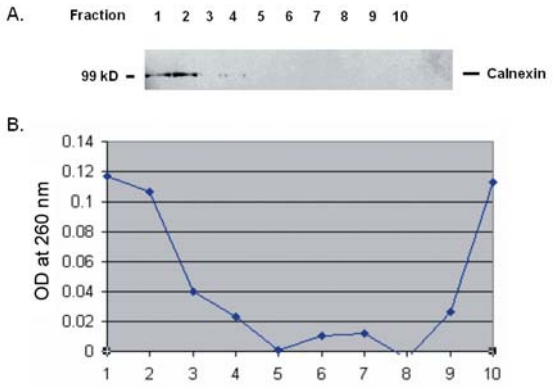


Figure 2. *(2A) Characterization of microsome isolated from Hs.688(A) and Hs.688(B) using linear sucrose gradient. Proteins were extracted from each fraction of the sucrose gradient and an equal amount of proteins from each fraction were denatured and resolved using 12.5% SDS-PAGE analysis. Western immunoblot analysis was performed to determine the presence of the microsome complex marker protein calnexin. The positive signal of the calnexin band in fraction 1 and 2 indicates the presence of microsome complex. (2B) The amount of bound RNAs were quantitated by measuring absorbance at 260 nm.*

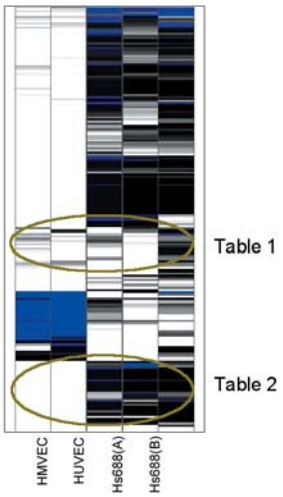


Figure 3. Magnified view of Hierarchical cluster microarray expression analysis with Spotfire software. Expression analysis of Hs.688(A); Hs.688(B) and endothelial cells in culture reveals a subset of genes that is expressed in the melanoma and endothelial cells in culture (Table I). Gene expression levels were indicated with different color schemes as black>blue>grey>white. A second subset is expressed by the melanoma cells at a higher level than HUVECs or dermal endothelial cells (HMVEC) in vitro (Table II).

co-receptor for multiple growth factor receptors (19). High levels of soluble syndecan-1 expression in tumors have been directly associated with increased invasion and cancer progression (20). Hs.688(A) and Hs.688(B) also express another gene, tazarotene-induced gene 2 (TIG2), recently

Table I. *Genes expressed at similar levels between two melanoma cell lines Hs.688(A), Hs.688(B) and endothelial HUVEC cells.*

Gene Name	Fold change
Follistatin	2.3
Collagen, type VIII, alpha	5.5
KIAA0965	1.8
Pentaxin-related protein PTX3	3.3
Integrin beta 1	2.7
Vascular endothelial growth factor C	4.4
Beta-2 microglobulin	3.5
Interleukin 18 receptor 1	2.2
Pescadillo	3.8
Galectin 1	6.1
Olfactory receptor	3.1
STK3	2.6
Glycogen synthase kinase 3 alpha	3.4
Endoglin (CD105 antigen)	2.5
TR3 beta	2.1
Butyrophilin-like	4.9
Protein tyrosine phosphatase, receptor type, O	2.2
Alpha-5-integrin	3.5
Procollagen C-endopeptidase enhancer	4.1
Norch 3	3.7
Fibulin-like protein	2.2
Fibronectin	4.5

Note: Gene expression levels of Hs.688(A) and Hs.688(B) are similar with HUVEC cells.

Table II. *Genes were up-regulated in two melanoma cell lines Hs.688(A) and Hs.688(B) compared to HUVEC cells.*

Gene Name	Fold change
Collagen, type III, alpha	2.9
TEM-1	4.6
Heat-shock protein	3.7
Collagen, type XVI, alpha 1	2.1
Proenkephalin	3.2
Insulin-like growth factor binding protein 3	2.5
Collagen, type XI, alpha	3.1
Semaphorin 3C	2.2
IGFBP3	2.3
Adican	2.6
Fibulin 5	2.9
Cadherin-11	3.5
Interleukin-6	2.1
Cadherin-11	2.4
Angiopoietin 1	3.3
TIG2	2.1
Syndecan-1	2.8

Note: Up-regulated gene expression of Hs.688(A) and Hs.688(B) compared to HUVEC cells.

identified as a ligand for the retinoid receptor ChemR23 (21). TIG2 is a novel retinoid-responsive gene in skin, which is not expressed in primary keratinocyte and fibroblast cultures, but found highly expressed in non-lesional psoriatic skin (22).

We also found some of the TEMs such as TEM1 were up-regulated in both melanoma cell lines. TEMs were recently identified and shown to display elevated expression during tumor angiogenesis (17). TEM1 localizes on the cell surface and would be an ideal drug target for both small molecules and therapeutic antibodies. TEM1 was found expressed at high levels in colorectal cancer, and our findings are consistent with the results in colorectal cancer (24). Another marker gene up-regulated in Hs.688(A) and Hs.688(B) was adlican, the function of which has not been intensively studied. One recent report showed that adlican was overexpressed in colorectal cancer (24). It is intriguing to see that marker genes such as TEM1 and adlican are shared by colon cancer and melanoma. This may suggest that different types of tumor share some similar mechanism in terms of vasculogenesis during invasion and metastasis. Angiopoietin-1 was also found to be associated with the tumor vasculature process (23).

Conclusion

This study has characterized the gene expression patterns of two human melanoma cell lines using high throughput sequencing with microsome isolated cDNA library and gene expression analysis. A number of genes, such as acetyl LDL receptor, TEMs-1, 5 and 8, flow-induced endothelial G protein coupled receptor-1 and VRP are known to be expressed uniquely by endothelial cells. Some of these genes were expressed at higher levels in the two melanoma cell lines compared to in HUVEC cells. These findings support the hypothesis that these two melanoma cells were mimicking an activated vascular phenotype. Ultimately the goal is to investigate the biological roles of endothelial cell-associated genes in the tumor invasion and metastasis of these melanoma cells.

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References

1 Warren BA and Shubik P: The growth of the blood supply to melanoma transplants in the hamster cheek pouch. Lab Invest *15*: 464-478, 1966.

2 Goodall CM, Sanders AG and Shubik P: Studies of vascular patterns in living tumors with a transparent chamber inserted in hamster cheek pouch. J Natl Cancer Inst *35*: 497-521, 1965.

3 Timar J and Toth J: Tumor sinuses- vascular channels. Pathol Oncol Res *6*: 83-86, 2000.

4 Maniotis AJ, Folberg R, Hess A, Seftor EA, Gardner LM, Pe'er J, Trent JM, Meltzer PS and Hendrix MJ: Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry. Am J Pathol *155*: 739-752, 1999.

5 Hendrix MJ, Seftor EA, Hess AR and Seftor RE: Molecular plasticity of human melanoma cells. Oncogene *22*: 3070-3075, 2003.

6 Folkman J: Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. N Engl J Med *333*: 1757-1763, 1995.

7 Folberg R, Rummelt V, Parys-Van Ginderdeuren R, Hwang T, Woolson RF, Pe'er J and Gruman LM: The prognostic value of tumor blood vessel morphology in primary uveal melanoma. Ophthalmology *100*: 1389-1398, 1993.

8 McDonald DM and Foss AJ: Endothelial cells of tumor vessels: abnormal but not absent. Cancer Metastasis Rev *19*: 109-120, 2000.

9 Hess AR, Seftor EA, Gardner LM, Carles-Kinch K, Schneider GB, Seftor RE, Kinch MS and Hendrix MJ: Molecular regulation of tumor cell vasculogenic mimicry by tyrosine phosphorylation: role of epithelial cell kinase (Eck/EphA2). Cancer Res *61*: 3250-3255, 2001.

10 Folberg R, Mehaffey M, Gardner LM, Meyer M, Rummelt V, and Pe'er J: The microcirculation of choroidal and ciliary body melanomas. Eye *11 (Pt 2)*: 227-238, 1997.

11 Bittner M, Meltzer P, Chen Y, Jiang Y, Seftor E, Hendrix M, Radmacher M, Simon R, Yakhini Z, Ben-Dor A, Sampas N, Dougherty E, Wang E, Marincola F, Gooden C, Lueders J, Glatfelter A, Pollock P, Carpten J, Gillanders E, Leja D, Dietrich K, Beaudry C, Berens M, Alberts D and Sondak V: Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature *406*: 536-540, 2000.

12 Gutgemann A, Golob M, Muller S, Buettner R and Bosserhoff AK: Isolation of invasion-associated cDNAs in melanoma. Arch Dermatol Res *293*: 283-290, 2001.

13 Kashani-Sabet M, Shaikh L, Miller JR, 3rd, Nosrati M, Ferreira CM, Debs RJ and Sagebiel RW: NF-kappa B in the vascular progression of melanoma. J Clin Oncol *22*: 617-623, 2004.

14 Zabrenetzky V, Harris CC, Steeg PS and Roberts DD: Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung and breast carcinoma cell lines. Int J Cancer *59*: 191-195, 1994.

15 Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD and Wiegand SJ: Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science *284*: 1994-1998, 1999.

16 Tuszynski GP and Nicosia RF: The role of thrombospondin-1 in tumor progression and angiogenesis. Bioessays *18*: 71-76, 1996.

17 Cai XS, Jia YW, Mei J and Tang RY: Tumor blood vessels formation in osteosarcoma: vasculogenesis mimicry. Chin Med J (Engl) *117*: 94-98, 2004.

18 Jacob MP: Fibulin-5 is essential for elastic fiber development. Med Sci (Paris) *19*: 395-397, 2003.

19 Heimann H, Coupland SE, Gochman R, Hellmich M and Foerster MH: Alterations in expression of mucin, tenascin-c and syndecan-1 in the conjunctiva following retinal surgery and plaque radiotherapy. Graefes Arch Clin Exp Ophthalmol *239*: 488-495, 2001.

20 Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J and Zako M: Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem *68*: 729-777, 1999.

21 Meder W, Wendland M, Busmann A, Kutzleb C, Spodsberg N, John H, Richter R, Schleuder D, Meyer M and Forssmann WG: Characterization of human circulating TIG2 as a ligand for the orphan receptor ChemR23. FEBS Lett *555*: 495-499, 2003.

22 Nagpal S, Patel S, Jacobe H, DiSepio D, Ghosn C, Malhotra M, Teng M, Duvic M and Chandraratna RA: Tazarotene-induced gene 2 (TIG2), a novel retinoid-responsive gene in skin. J Invest Dermatol *109*: 91-95, 1997.

23 Metheny-Barlow LJ and Li LY: The enigmatic role of angiopoietin-1 in tumor angiogenesis. Cell Res *13*: 309-317, 2003.

24 Zou TT, Selaru FM, Xu Y, Shustova V, Yin J, Mori Y, Shibata D, Sato F, Wang S, Olaru A, Deacu E, Liu TC, Abraham JM and Meltzer SJ: Application of cDNA microarrays to generate a molecular taxonomy capable of distinguishing between colon cancer and normal colon. Oncogene *21*: 4855-4862, 2002.

25 Hughes TR, Mao M, Jones AR, Burchard J, Marton MJ, Shannon KW, Lefkowitz SM, Ziman M, Schelter JM, Meyer MR, Kobayashi S, Davis C, Dai H, He YD, Stephaniants SB, Cavet G, Walker WL, West A, Coffey E, Shoemaker DD, Stoughton R, Blanchard AP, Friend SH and Linsley PS: Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nat Biotechnol *19*: 342-347, 2001.

26 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*: 680-685, 1970.

27 Ju J, Huang C, Minskoff SA, Mayotte JE, Taillon BE and Simons JF: Simultaneous gene expression analysis of steady-state and actively translated mRNA populations from osteosarcoma MG-63 cells in response to IL-1 alpha *via* an open expression analysis platform. Nucleic Acid Res *31*: 5157-5166, 2003.

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