EUROPEAN JOURNAL OF CANCER 47 (2011) 1267–1275

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Gene expression profiling of human angiotropic primary melanoma: Selection of 15 differentially expressed genes potentially involved in extravascular migratory metastasis

Claire Lugassy [a,g,i], *Vladimir Lazar* [c], *Philippe Dessen* [c], *Joost J. van den Oord* [d], *Véronique Winnepenninckx* [e], *Alan Spatz* [f], *Martine Bagot* [a], *Armand Bensussan* [g], *Anne Janin* [b], *Alexander M. Eggermont* [h], *Raymond L. Barnhill* [a,b,*]

[a] *Department of Dermatology, Hôpital Saint-Louis, University of Paris VII, Paris, France*
[b] *Department of Pathology, Hôpital Saint-Louis, University of Paris VII, Paris, France*
[c] *Division of Functional Genomics, Gustave-Roussy Institute, Villejuif, France*
[d] *Departments of Morphology and Molecular Pathology, University Hospitals, Katholieke Universiteit Leuven, Leuven, Belgium*
[e] *Department of Pathology, Maastricht University Medical Center, Maastricht, The Netherlands*
[f] *Department of Pathology, Jewish General Hospital, McGill University, Montreal, Canada*
[g] *Unité INSERM 976, Paris, France*
[h] *Department of Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands*
[i] *Department of Pathology, University of Miami Miller School of Medicine, Miami, FL, USA*

ARTICLE INFO

Article history:
Received 24 October 2010
Received in revised form 6 January 2011
Accepted 20 January 2011
Available online 19 February 2011

Keywords:
Melanoma
Angiotropism
Extravascular migratory metastasis (EVMM)
Gene expression
Neural crest migration

ABSTRACT

Background and aim: Angiotropism and extravascular migratory metastasis (EVMM) are an important alternative means of melanoma spread. In EVMM, melanoma cells migrate along the external surfaces of vascular channels to distant sites and demonstrate microscopic angiotropism, i.e. melanoma cells arrayed along the external surfaces of vascular endothelium. Pertinent to EVMM are the origin of melanocytes from the neural crest (NC) and strong analogies of EVMM with NC cell migration. Our aim is to elucidate the molecular mechanisms underlying angiotropism and EVMM.

Methods: Frozen primary melanomas, previously utilised for gene expression profiling, were analysed for angiotropism as a differential marker. From the results of this new microarray analysis, we sought to identify genes which were directly relevant to the basic mechanisms underlying EVMM.

Results: Among 66 melanomas from patients who developed metastases or remained disease-free at 4 years of follow-up, 26 melanomas were angiotropic while 35 were not, and five were equivocal. The new microarray analysis identified 128 genes differentially expressed in angiotropic versus non-angiotropic melanomas. Among these 128 genes, 15 genes were potentially directly involved in EVMM, based their respective expressions in the NC (seven genes), in other malignant tumours of NC origin (three genes), in cell motility and/or migration (four genes) and in neurotropism (one gene).

Conclusion: The detection of these 15 genes provides additional support for the importance of angiotropism and the mechanism of EVMM in melanoma. Ongoing studies on this new profile of 15 genes may potentially identify new targets for controlling melanoma metastasis.

* *Corresponding author:* Address: Department of Pathology, Hôpital Saint-Louis, 1, avenue Claude-Vellefaux, 75475 Paris Cedex 10, France. Tel.: +33 1 42 38 50 18; fax: +33 1 42 38 50 67.
E-mail address: raymond.barnhill@sls.aphp.fr (R.L. Barnhill).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2011.01.009

1. Introduction

The fundamental problem of human melanoma is its capacity for metastasis which often proves fatal.[1] A better understanding of the mechanisms of metastasis and their molecular basis would facilitate new therapeutic strategies. As distinct from intravascular dissemination, we have described that an important mechanism of melanoma metastasis may be the migration of tumour cells along the external surfaces of vascular channels (or other anatomical tracks): "extravascular migratory metastasis" (EVMM). Thus by this mechanism of EVMM, melanoma cells spread to nearby or more distant sites.[2,3] Angiotropism has been defined histologically as the presence of melanoma (or other tumour) cells disposed along the external surfaces of vascular endothelium without intravasation. Angiotropism thus is a microscopic marker of EVMM, both in vitro and in vivo, especially as demonstrated in a modified shell-less chick chorioallantoic (CAM) model, using human GFP melanoma cells.[4,5]

Clinical and experimental data strongly suggest that angiotropism is a prognostic factor in local, regional and distant metastasis.[6] EVMM along vessels via melanoma angiotropism is particularly striking between the primary tumour and subsequent in transit melanoma metastases.[7] In a recent independent study, angiotropism has been confirmed as a prognostic factor for primary melanomas associated with local and in transit metastases.[8]

Interestingly, another independent study recently demonstrated perivascular cuffing of malignant tumour cells around the coeliac trunk in a patient with presumed pancreatic cancer, consistent with EVMM.[9]

Finally, the concepts of angiotropism and EVMM are now recognised by the international scientific community as an important alternative means of melanoma dissemination and as a key subject of future melanoma research.[8–11] Therefore, understanding the molecular basis of EVMM is a new research field of critical interest with the goals of defining new markers of angiotropism and new targets for melanoma treatment. This translational research on EVMM is complementary to other areas of melanoma research.

In order to identify genes potentially involved in EVMM, we have made use of a previously constructed melanoma gene expression microarray.[12]

A large bank of frozen primary melanomas was employed to correlate gene expression profiles with either metastasis or death, or distant metastasis-free survival at 4 years. 254 genes were identified that distinguished primary melanomas with and without metastases.[12] A more recent study analysing the differential gene expression of whole biological pathways rather than individual genes demonstrated an association between high expression of DNA repair pathways and metastases in the same melanoma study subjects.[13] In the present work, we have utilised the same bank of melanomas for the specific evaluation of angiotropism as a differential marker in melanoma specimens. Thus herein we report in a new independent analysis of these microarray data, the identification of a unique profile of 128 genes differentially expressed in angiotropic versus non-angiotropic melanoma. From this list of 128 genes, we have selected 15 genes potentially directly involved in EVMM.

2. Material and methods

2.1. *Selection of melanoma patients, methods of gene expression profiling and results from the initial study*[12]

In brief, primary melanomas from patients with available fresh frozen tissue were identified from the files of the Departments of Pathology at the University Hospitals, Leuven, Belgium and at the Gustave Roussy Institute, Villejuif, France.

The 83 primary melanomas from patients who either developed metastases or remained disease-free during the 4 years following the diagnosis of their primary melanomas were included in this study. The data of genomic expression published by Winnepenninckx et al. were used in the current study.

All the data are available on the Array Express data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under the following accession numbers: ETABM- 1 IGR_ MELANOMA_STUDY and E-TABM-2IGR_MELANOMA_VALID (validation set).

2.2. *Histological evaluation for angiotropism*

After microscopic review of the glass slides from the original 83 primary melanomas referred to above, paraffin-embedded, haematoxylin and eosin-stained glass slides from 66 primary melanomas were judged to be satisfactory for inclusion in the study and were examined for angiotropism. Thus, 15 melanomas were excluded from the current study.

The histological sections from the latter 15 melanomas were unsatisfactory and excluded for the following reasons: (1) either only frozen sections were available for review and thus the histology was of poor quality, (2) the paraffin-sections were of poor quality for microscopic study, or (3) the tissue sections did not allow for an adequate analysis of either the advancing front of the melanoma and/or adjoining non-tumoural tissue for the scoring of angiotropism. In general only one glass slide from each melanoma was available for review, and this slide was comprehensively assessed for angiotropism and vascular/lymphatic invasion without knowledge of patient outcome. All tissue sections on each microslide were systematically studied microscopically for evidence of angiotropism (as defined below) at the advancing front of the melanoma and/or in nearby tissue beyond the main tumour mass. Angiotropism was defined as two or more melanoma cells singly disposed along or in aggregates closely opposed to the external surfaces of (and not within) microvessels and/or lymphatics at the advancing front and/or some distance (1–2 mm or more) away from the main part of the melanoma. Angiotropism was scored as follows: 1 = absent: absence of melanoma cells clearly disposed along or encircling microvessels, 2 = equivocal: a single focus of melanoma cells near but not clearly disposed along or cuffing a microvessel), or 3 = present: at least one focus of two or more

Table 1 – Pertinent clinical and histologic characteristics of the 66 cases examined for angiotropism.

ID	Angiotropism	Sex	Age	Site	Type of recurrence	Status	Breslow	Ulceration	Mitotic rate
P001	3.00	m	70	2	diffuse	DOD	5.76	0	1
P002	1.00	f	78	2	new skin M+ (tr?)	reg tum	1.34	0	2
P003	3.00	f	65	4	sat	pall	3.74	0	2
P004	1.00	f	54	1	reg N	diff M+	5.44	1	2
P005	1.00	f	75	3	reg nodes	tum	7.58	1	2
P006	1.00	f	42	2		NED	1.00	0	0
P007	1.00	f	40	3		NED	1.03	1	1
P008	1.00	f	66	3		NED	3.32	0	1
P009	1.00	f	67	3	node	DOD	6.40	0	2
P010	3.00	m	72	2	LR, reg N and M+	DOD	3.52	1	2
P012	3.00	f	89	4	LR + reg N	DOD	7.04	1	2
P014	3.00	m	76	2	in transit	DOD	2.21	1	1
P015	2.00	f	57	3	sat	DOD	1.79	0	1
P016	3.00	f	47	3		NED	1.60	0	1
P017	3.00	f	35	3	reg N	DOD	4.48	1	2
P018	1.00	m	40	3	reg N	DOC	4.32	0	2
P020	1.00	f	78	3	reg N	DOD	3.30	1	1
P021	1.00	f	56	3	LR + in tr	DOD	8.16	1	2
P022	1.00	f	37	1	reg N	DOD	1.20	0	1
P023	1.00	m	58	3	in transit	NED	2.42	0	1
P024	3.00	m	63	2	LR	NED	4.16	0	2
P025	3.00	m	38	2	reg N	DOD	5.02	0	2
P026	1.00	m	64	1	reg N	DOD	1.18	0	1
P027	3.00	F	9	2	sat	NED	4.70	1	2
P028	3.00	m	78	4	M+	DOD	14.18	1	2
P030	3.00	f	68	3	reg N	DOD	2.75	0	1
P031	1.00	f	64	3	in tr	NED	17.12	1	2
P032	1.00	f	79	2	M+	tum	7.52	1	1
P033	1.00	f	74	3	reg N	DOD	4.09	1	1
P035	3.00	m	78	3	sat	DOD?	3.04	1	1
P036	3.00	f	81	2	LR + reg N	NED	4.86	0	1
P038	3.00	m	33	2		NED	1.54	0	1
P039	1.00	f	55	4	reg N + M	DOD	2.30	0	2
P040	1.00	f	53	3		NED	1.67	0	1
P041	3.00	m	22	3	reg N	DOD	3.36	0	2
P042	1.00	f	64	3		NED	1.44	0	1
P043	3.00	m	50	2		NED	1.02	0	1
P044	1.00	f	72	3	nodes and M+	DOD	7.97	1	2
P045	3.00	f	73	4	M+	DOD	3.36	1	2
P047	1.00	f	43	2		NED	1.73	0	1
P048	3.00	f	63	3	LR	tum	5.47	0	2
P049	3.00	m	52	2	reg N	NED	3.84	0	1
P051	1.00	m	41	2		NED	3.30	0	2
P053	1.00	m	69	2	diffuse	DOD	2.9	0	1
P054	2.00	m	59	3	in tr + reg N	NED	6.59	1	2
P057	1.00	f	47	3		NED	4.61	0	1
P060	3.00	f	48	3		NED	3.68	1	2
P061	3.00	f	92	3		NED	4.70	1	2
P062	3.00	f	74	1		NED	1.59	0	2
P064	1.00	f	54	3		NED	3.65	1	1
P065	3.00	f	58	3		NED	1.92	0	0
P066	1.00	f	56	3		NED	2.08	0	2
P067	2.00	f	66	2		NED	0.88	0	1
P068	1.00	f	34	2		NED	0.94	0	1
P069	1.00	m	61	3		NED	0.93	0	1
P070	1.00	f	57	3		NED	0.96	0	1
P072	1.00	f	57	2	reg N	DOD	0.98	1	2
P077	3.00	m	63	1		DOC	5.95	0	2
P078	1.00	M	47	3			0.66	0	0
P079	1.00	f	92	3		DOC	6.81	1	1
P080	1.00	f	46	3		LOF	2.08	0	1
P081	2.00	m	66	1		DOC	5.23	1	1
P082	1.00	m	64	2		NED	1.41	0	1

(*continued on next page*)

Table 1 – *continued*

ID	Angiotropism	Sex	Age	Site	Type of recurrence	Status	Breslow	Ulceration	Mitotic rate
P083	1.00	f	65	3		DOD	1.19	0	1
P084	2.00	f	92	1		DOC	1.76	0	1
P087	3.00	f	77	1		DOC	2.52	0	2

Among 66 melanomas from patients who developed metastases or remained disease-free at 4 years, 26 melanomas were angiotropic while 35 were not. Five were equivocal and eliminated from the micro-array analysis.
Angiotropism scores: 1 = absent, 2 = equivocal, and 3 = present.
DOD – dead of disease; DOC – dead of other cause; new skin M+ (tr?) – new skin metastasis (treatment?); reg tum – regional tumour; sat – satellite; pall – palliative care; reg N – regional lymph node; diff M+ – diffuse metastases; NED – no evidence of disease.
Site numbers: 1 = head and neck, 2 = trunk, 3 = limbs, 4 = acral sites.
ID (identification of patients, first column) refers to data in the Array Express data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under the following accession numbers: ETABM-1 IGR_ MELANOMA_STUDY.

melanoma cells clearly disposed along or encircling microvessels. Angiotropic melanoma cells were recognised by their unequivocal similarity to nearby melanoma cells; if there was any question about the identity of the cells cuffing vessels the case was scored as negative for angiotropism. Equivocal cases were eliminated from the microarray analysis.

2.3. *Pertinent clinical and histological data including angiotropism*

These data are available in Table 1.

2.4. *Statistical analysis*

The data used have been previously published[12] and are publicly available on the Array Express repository with the E-TABM-1 identifier (http://www.ebi.ac.uk/arrayexpress/experiments/E-TABM-1). The data correspond to a study of 83 primary melanomas which were analysed with 44K Agilent Human genome in dual colour. The data were normalised with Feature Extraction software. Normalised data of expression of 61 melanomas selected for study were analysed with the Class Comparison method integrated in BrB Array Tools [http://www.linus.nci.nih.gov/BRB-ArrayTools.html[14]] with the two angiotropism classes 1 and 3 (1 = angiotropism absent, 3 = angiotropism present).

p-values of correlation with other factors such as, distant metastasis at 4 years, Breslow thickness, etc., were obtained with DNA-Chip Analyzer (dChip) software [http://www.dchip.org[15]].

The Benjamini and Hochberg algorithm was applied to the selected probes to control for multiple tests.

2.5. *Strategy for selecting genes potentially involved in EVMM*

From the results of this new microarray analysis, we sought to identify genes which were of biological interest, i.e., which were directly relevant to the basic mechanisms or pathogenesis of EVMM. Genomic databases (Gene) and published work from the scientific literature (PubMed) were used to select genes involved in: (1) motility and migration in embryogenesis, with particular reference to neural crest development; (2) motility and migration of other malignant tumours which are of neural crest derivation, such as glioblastoma; (3) cell motility and/or migration in general; and (4) non-vascular EVMM such as neurotropism.

In addition, the list of 128 probes has been analysed with Ingenuity Pathways Analysis, GSEA and BrB Array Tools.

2.6. *Immunohistochemical validation of selected genes*

Among the seven genes implicated in neural crest cell migration, two genes considered relevant to the process and mechanisms of EVMM: TCOF1 and ANHAK were selected for preliminary gene validation studies.

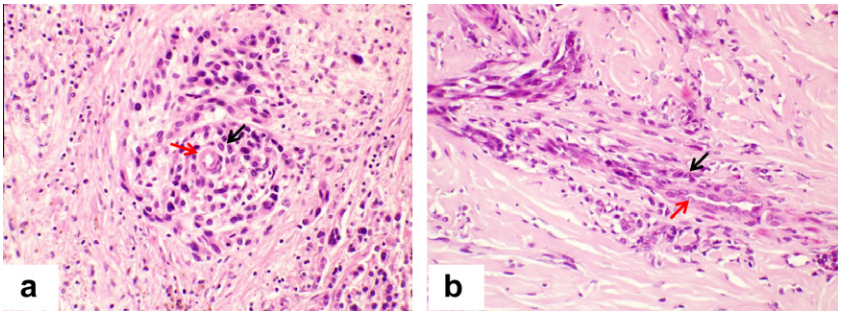

Fig. 1 – Histology of two primary human melanomas with angiotropism. (a) Microscopic image at the advancing front of the tumour shows angiotropic melanoma cells arrayed about a microvascular channel (red arrows indicate microvessels, black arrows melanoma cells). (b) This microscopic field shows melanoma cells disposed along and about a microvascular channel with linear orientation.

One of the genes – TCOF1 – was over-expressed, while the other – ANHAK – was under-expressed in the gene analysis (see Section 3). The protein products of these two genes TCOF1 and ANHAK were analysed by immunohistochemical evaluation of a frozen primary melanoma which exhibited both angiotropic and non-angiotropic areas. This melanoma was selected from the same melanoma tissue bank in Leuven, Belgium (for description, see below). Antibodies against Treacle (TCOF1 gene) and the ANHAK protein were the generous gifts of Profs. Michael Dixon (School of Dentistry, University of Manchester, Manchester M13 9PT, United Kingdom) and Jacques Baudier (Laboratoire de Transduction du Signal INSERM EMI-0104, Grenoble, France), respectively. In addition, this specific antibody against Treacle has been previously utilised to study gene product expression during neural crest cell migration in the embryo.[15]

The melanoma selected for immunohistochemistry referred to above was from the upper leg of a 72 year-old woman. Three samples from this lesion were fresh frozen in liquid nitrogen-cooled isopentane and stored at −80 °C. Routine histology of the formalin-fixed, paraffin-embedded material demonstrated a non-ulcerated malignant melanoma of conventional superficial spreading type with Breslow thickness of 2.5 mm, Clark level IV, in radial and mitogenic vertical growth phases, with extensive angiotropism in some parts of the lesion, as confirmed by double immunostaining with CD31 and melan-A.

Frozen sections from the melanoma also showed angiotropism, and were subjected to immunohistochemistry using the rabbit antibodies directed against TREACLE and AHNAK. Acetone-fixed cryoslides were incubated for 60 min at room temperature with the primary antibodies, diluted 1:100, followed by three washes in phosphate-buffered saline (PBS), pH 7.2. Prediluted peroxidase-conjugated goat-antirabbit IgG (ImmunoLogic, Duiven, The Netherlands) was incubated for 30 min, followed by three washes in PBS. Peroxidase activity was developed using 3-amino-9-ethylcarbazole and H_2O_2, and the slides were counterstained with Harris' haematoxylin.

3. Results

3.1. *Histological evaluation for angiotropism*

Among the 66 melanomas reviewed, 26 melanomas demonstrated unequivocal angiotropism (Fig. 1), 35 did not show angiotropism at the periphery of the melanoma or in nearby

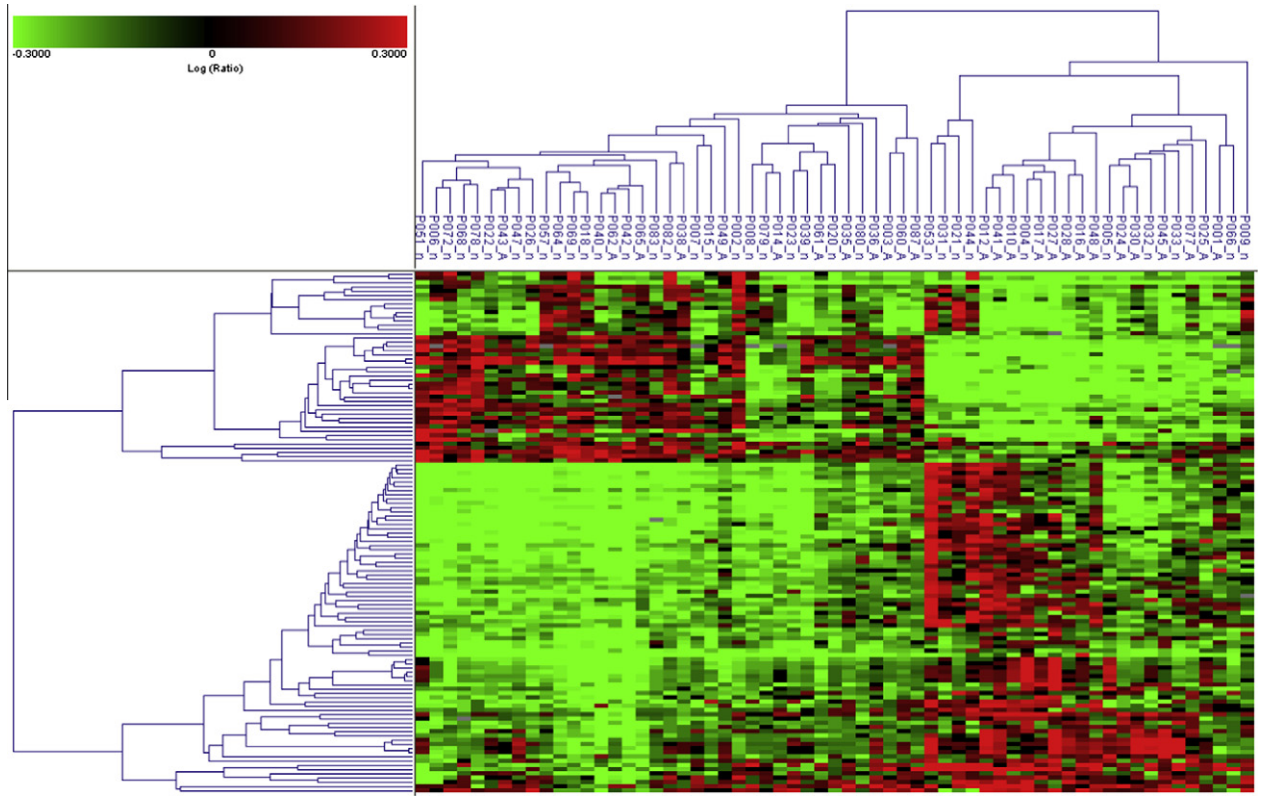

Fig. 2 – Hierarchical clustering of 61 primary melanomas. Hierarchical clustering of log ratios of the 128 probes selected by the Class Comparison method (plotted on the ordinate or vertical axis) for 61 primary melanomas (plotted on the abscissa or horizontal axis). The 61 samples are designated as "*A*" for angiotropism index = 3 (26 samples) and "*n*" for non-angiotropism index = 1 (35 samples). Data were obtained by using the average linkage for both genes and tumours; the distance metric used was 1 minus mean centred correlation. Each row represents one gene and each column represents a tumour sample. The colour bar at the top indicates the relative level of mRNA expression. Red = higher level of expression of mRNA in the tumour than the reference level of mRNA (pool of all primary tumours); green = lower level of expression than the reference; black = no change from the reference. The black bar divides the left cluster of data from the right cluster.

Table 2 – List of the 15 selected genes, probes, and sequence descriptions.

Symbol	Probes	Accession #	Sequence description
KIF14	A_23_P149668	NM_014875	Homo sapiens kinesin family member 14
DBF4	A_23_P254612	NM_006716	Homo sapiens DBF4 homologue (*Sacchromyces cerevisiae*)
FNBP1L	A_24_P366967	NM_017737	Homo sapiens formin binding protein 1-like, transcript variant 2
ECT2	A_23_P9574	NM_018098	Homo sapiens epithelial cell transforming sequence 2 oncogene
GLS	A_23_P308800	AF158555	Homo sapiens glutaminase C mRNA, complete cds. [AF158555]
TCOF1	A_23_P310317	NM_001008656	Homo sapiens Treacher Collins-Franceschetti syndrome 1, transcript variant 1
HMMR	A_23_P70007	NM_012484	Homo sapiens hyaluronan-mediated motility receptor (RHAMM), transcript variant 2
NEIL3	A_23_P155711	NM_018248	Homo sapiens nei endonuclease VIII-like 3 (*Escherichia coli*)
FGD3	A_24_P153840	NM_033086	Homo sapiens FYVE, RhoGEF and PH domain containing 3, transcript variant 2
AGAP2	A_24_P244495	D79989	Centaurin-gamma-1 (PIKE) [ENST00000257897]
KCTD11	A_23_P354027	NM_001002914	Homo sapiens potassium channel tetramerisation domain containing 11
F10	A_23_P205177	NM_000504	Homo sapiens coagulation factor X
CEBPA	A_23_P375494	NM_004364	Homo sapiens CCAAT/enhancer binding protein (C/EBP), alpha
AHNAK	A_23_P21363	NM_024060	Homo sapiens AHNAK nucleoprotein, transcript variant 2
AQP3	A_23_P112482	NM_004925	Homo sapiens aquaporin 3 (Gill blood group)

tissue as specified by the criteria above, and five were equivocal for angiotropism (Table 1). The five equivocal cases were eliminated from the study at this point, and thus 61 primary melanomas were available for the microarray analysis in this study. Thus these 61 samples represent the current study cohort.

3.2. *Statistical analysis*

The study set comprised 61 melanomas among which 26 melanomas were angiotropic and 35 were non-angiotropic. Using these two classes (3 = angiotropism present and 1 = angiotropism absent), the number of genes that passed the filtering criteria was 6766. A class comparison (two-tailed *t*-test samples) of the study set of the 63 melanomas with the two angiotropism indexes 1 (absent) and 3 (present) yielded 128 probes with a level of probability of 0.001 (the probability of obtaining this list by chance is 0.004). A class prediction yielded values of good prediction of 69–86% with an ROC index of 0.89. These results clearly indicated that these 128 genes are differentially expressed in the angiotropic versus non-angiotropic melanomas. The Benjamini and Hochberg algorithm was applied to the 128 selected probes, and the maximum value of False Discovery Rates (FDR) (computed in BrB Arrays Tools) was 0.052.

The list of the 128 probes (and genes) is shown in Supplementary Table 1. A heatmap of the log ratios of the 128 Human Agilent probes after class comparison between angiotropism indexes 1 and 3 is illustrated in Fig. 2.

From Ingenuity Pathways Analysis, GSEA and BrB Array, no obvious pathways have been detected.

The expression profiles of these 128 probes have been further analysed with dCHIP software. From the statistical analysis of clusters, the correlation between angiotropism and distant metastasis at 4 years yielded a *p*-value of 0.0008; the correlation between angiotropism and ulceration was significant (*p*-value = 0.0004). Finally, the correlation between angiotropism and Breslow melanoma thickness was highly significant (*p*-value = 0.000001).

3.3. *Detection of genes potentially involved in EVMM*

By combining genomic databases and published work from the scientific literature, we identified, among the 128 differentially expressed genes, 15 genes potentially involved in EVMM (Tables 2 and 3). These 15 genes were classified according to their expression in relationship to neural crest (NC) cell migration (seven genes: TCOF1, NEIL3, AHNAK, KCTD11, HMMR, CEBPA and AQP3); cell migration of other malignant tumours with NC origin (three genes: ECT2, AGAP2 and GLS); cell motility and/or migration (four genes: FGD3, F10, DBF4 and FNBP1L); and one gene (KIF14) was related to neurotropism (Table 3). Nine of these genes were over-expressed compared to non-angiotropic melanoma, while seven were under-expressed (Tables 2 and 3). More detailed information

Table 3 – Subclassification of the 15 selected genes into four categories.

FOLD	NC	NCDT	MIGR	NEUROT
+1.50	TCOF1			
+1.37	NEIL3			
−1.94	AHNAK			
−1.46	KCTD11			
+1.39	HMMR			
−1.78	CEBPA			
−2.07	AQP3			
+1.52		ECT2		
−1.43		AGAP2		
+1.52		GLS		
−1.36			FGD3	
−1.61			F10	
+1.68			DBF4	
+1.58			FNBP1L	
+1.81				KIF14

FOLD: fold change; NC: genes involved in neural crest cell migration; NCDT: genes involved in migration of neural crest-derived tumours; MIGR: genes involved in migration/motility; NEUROT: gene involved in neurotropism.

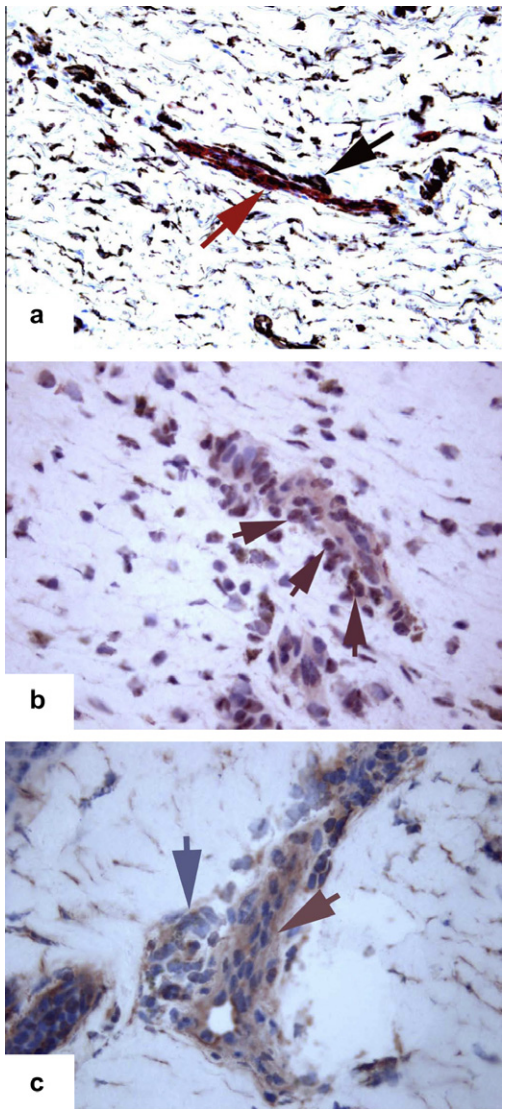

Fig. 3 – Immunohistochemistry of a primary human melanoma with angiotropism. (a) Double immunohistochemistry with melan-A and CD31 performed on formalin-fixed, paraffin-embedded tissue sections. The advancing front of the melanoma demonstrates angiotropism. Double immunostaining shows angiotropic melanoma cells expressing melan-A (red chromogen) arrayed along vascular channels which are labelled with CD31 (brown-black chromogen) (red arrow: angiotropic melanoma cell; black arrow: endothelial cell). (b) Frozen-section immunohistochemistry of the same angiotropic melanoma for the TCOF1 gene product Treacle. The angiotropic melanoma cells arrayed about a vascular channel show nuclear immunoreactivity with the treacle antibody (purple arrows: angiotropic treacle-positive melanoma cells). (c) Frozen-section immunohistochemistry for the ANHAK gene product. In general, the angiotropic melanoma cells do not show immunoreactivity with the antibody against the ANHAK protein while non-angiotropic portions of the melanoma express the protein (images not shown) (blue arrow: angiotropic Ahnak-negative melanoma cells, brown-red arrow: microvessel).

and references concerning the 15 selected genes relevant to EVMM are available in Supplementary Document 1.

3.4. *Immunohistochemical validation of selected genes*

Fig. 3 demonstrates the immunohistochemical evaluation of the primary human melanoma exhibiting an angiotropic phenotype. Double immunostaining revealed diffuse cytoplasmic expression of melan-A (red chromogen) by both angiotropic (Fig. 3a) and non-angiotropic portions of the melanoma while vascular channels were labelled with the endothelial marker CD31 (brown-black chromogen) (Fig. 3a).

The Treacle (protein product of TCOF1 gene) immunostaining of normal skin showed nuclear positivity in the epidermis and slight immunostaining of vessel walls. In the pigmented, non-angiotropic portion of the melanoma, there was very little immunostaining in the nuclei of melanoma cells and weak labelling of vessel walls. In the angiotropic portion of the melanoma, nuclear immunostaining of angiotropic melanoma cells was observed (Fig. 3b). There was immunostaining of the ANHAK protein in the radial growth phase and superficial dermal non-angiotropic portion of the melanoma; loss of immunostaining was observed in angiotropic melanoma cells associated with microvascular channels (Fig. 3c).

4. Discussion

Over the past decade accumulating evidence supports the idea that EVMM is an important biological phenomenon and means of melanoma (and other tumoural) metastasis.[3,9] Further, additional studies have provided preliminary data that angiotropism is a prognostic factor in melanoma.[6,8] Thus because of the growing significance of angiotropism and EVMM, we have undertaken the current studies to define the fundamental mechanisms responsible for EVMM. To this end, we describe this initial work of correlating particular gene expression with the *angiotropic phenotype* in primary human melanoma. Our goals in conducting this research are to recognise angiotropism more objectively, particularly via the development of more specific biomarkers, and to elucidate the molecular mechanisms underlying this migratory phenomenon.

In the current work we have thus introduced angiotropism as a new biomarker in a large bank of frozen and paraffin-embedded primary melanomas, and we have identified a new list of 128 genes differentially expressed in angiotropic versus non-angiotropic melanomas. The uniqueness of this study is a new comprehensive analysis of gene expression specifically correlated with the phenomenon *angiotropism* (and *EVMM*), which has not been previously studied at the molecular level. Furthermore, in an attempt to identify the genes potentially most relevant or unique to the mechanisms of EVMM, we have examined and screened each gene for potential functions related to or showing other associations with neural crest cell migration, other neoplasms of neurocristic origin, cellular motility and cellular migration in general, and non-angiotropic forms of EVMM, such as

neurotropism in neoplasms. This comprehensive screening of the 128 genes resulted in a list of 15 genes judged to be most relevant to angiotropism and EVMM in melanoma.

Among these 15 genes, we have identified seven genes of considerable relevance to angiotropism and EVMM because of their involvement in neural crest cell (NCC) migration (Table 3). These seven genes are TCOF1[16], NEIL3[17], AHNAK[18], KCTD11[19], HMMR[20], CEBPA[21] and AQP3.[22] Indeed, the pathways of NCC migration and their regulation eventuate, among other phenotypes, in the establishment of melanocytes in the skin.[1] In addition, EVMM has striking parallels with the migration of stem cells from the neural crest in the embryo[3] and recent studies of embryonic development in mice have confirmed the migration of NCC along the external surfaces of microvascular channels.[23] In addition, a recent study has demonstrated that enteric neural crest cells follow endothelial cells during their migration within the gut of embryos and are associated with striking angiotropism.[24] Finally, neural crest cells migrate at rates of about 2 μm/min or more[25], and are, therefore, comparable to migrating tumour cells.[3] The dysregulation of such embryonic pathways may result in the reverse migratory phenomena of metastatic melanoma cells.[26]

Because of the apparently close and mechanistically important relationship between EVMM and NCC migration, we have undertaken immunohistochemical validation studies of two genes TCOF1 and ANHAK, which are directly implicated in NCC migration. Our preliminary results have specifically shown in angiotropic melanoma cells the over-expression of Treacle (the protein product of the TCOF1 gene) and the under-expression of ANHAK (the protein product of the AHNAK gene). These results correspond to the over-expression of TCOF1 and the under-expression of AHNAK in the current microarray analysis and constitute for the first time more specific and practical identification of angiotropic melanoma cells in tissue sections. These results also provide the first objective evidence that mechanisms of NCC migration appear directly relevant to (if not directly involved in) EVMM.

Thus, these preliminary data seem to indicate that only angiotropic melanoma cells are expressing Treacle, whereas the same angiotropic cells show diminished expression of AHNAK. It is well-established that TCOF1 has links with NCC migration, since strong TCOF1 expression has been observed in migrating neural crest cells in the craniofacial mesenchyme, as confirmed by the immunohistochemical detection of Treacle using the same antibody.[27] On the other hand, the AHNAK-encoded protein was originally identified in human cell lines of neural crest origin as a 700-kDa phosphoprotein. AHNAK is one of the genes whose expression is typically repressed in neuroblastoma, which represents the most primitive neoplasm originating from migratory neural crest cells.[28]

More details and references showing the relevance of the 15 selected genes to the mechanism of EVMM are provided in Supplemental Document 1.

The list of 128 probes has been also analysed with the Ingenuity Pathways Analysis, GSEA and BrB Array Tools. However, as described in Section 3, the latter studies did not yield any significant findings with respect to the potential mechanisms of EVMM.

In the current microarray analysis, the fold changes for over- and under-expression of these 15 genes were not very high. This magnitude of change may potentially be explained by a dilutional effect, i.e. the fact that angiotropic melanoma cells may constitute only a small proportion of the entire "angiotropic melanoma", for example, at the advancing front of the tumour. This hypothesis seems consonant with our preliminary immunohistochemical investigation identifying the over-expressed protein Treacle and the under-expressed AHNAK protein in angiotropic melanoma cells only, but requires more comprehensive study.

Finally, one highly pertinent goal of our research on angiotropism and EVMM is the further refinement of angiotropism as a prognostic factor and one with significant clinical utility. As we have stated, we believe that the importance of angiotropism is its function as a surrogate marker of EVMM. There is already preliminary evidence from two studies that angiotropism has significant predictive value for melanoma metastasis.[6,8] The current investigation has clearly confirmed once more that angiotropism is a prognostic factor in melanoma, showing a significant association with distant metastasis at 4 years (p-value = 0.0008). Nonetheless, a major limitation at present is the difficulty in recognising angiotropism at the microscopic level. However, via our ongoing studies we believe that the development of biomarkers for angiotropism should significantly facilitate the detection of angiotropism and augment its robustness as a prognostic factor in melanoma.

In conclusion, we are in the process of conducting a much larger and more comprehensive analysis in order to confirm the present findings. These results may potentially lead to the detection of specific biomarkers (such as Treacle) for angiotropic melanoma cells. In addition, these findings focus attention on the mechanisms of NCC migration in the embryo as the basis for a better understanding of EVMM, and potentially for the identification of new targets for controlling melanoma metastasis.

Conflict of interest statement

None declared.

Sources of support

This study was funded in part by a grant from "Société Française de Dermatologie" (French Society of Dermatology).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.01.009.

REFERENCES

1. Barnhill RL, Piepkorn M, Busam KJ. *Pathology of melanocytic nevi and malignant melanoma*. 2nd ed. New York: Springer; 2004.

2. Lugassy C, Barnhill RL, Christensen L. Melanoma and extravascular migratory metastasis. *J Cutan Pathol* 2000;**27**(9):481.
3. Lugassy C, Barnhill RL. Angiotropic melanoma and extravascular migratory metastasis: a review. *Adv Anat Pathol* 2007;**14**(3):195–201 [Review].
4. Lugassy C, Kleinman HK, Fernandez PM, et al. Human melanoma cell migration along capillary-like structures in vitro: a new dynamic model for studying extravascular migratory metastasis. *J Invest Dermatol* 2002;**119**(3):703–4.
5. Lugassy C, Kleinman HK, Vernon SE, Welch DR, Barnhill RL. C16 laminin peptide increases angiotropic extravascular migration of human melanoma cells in a shell-less chick chorioallantoic membrane assay. *Br J Dermatol* 2007;**157**(4):780–2.
6. Barnhill R, Dy K, Lugassy C. Angiotropism in cutaneous melanoma: a prognostic factor strongly predicting risk for metastasis. *J Invest Dermatol* 2002;**119**(3):705–6.
7. Lugassy C, Vernon SE, Busam K, et al. Angiotropism of human melanoma: studies involving in transit and other cutaneous metastases and the chicken chorioallantoic membrane implications for extravascular melanoma invasion and metastasis. *Am J Dermatopathol* 2006;**28**(3):187–93.
8. Van Es SL, Colman M, Thompson JF, McCarthy SW, Scolyer RA. Angiotropism is an independent predictor of local recurrence and in-transit metastasis in primary cutaneous melanoma. *Am J Surg Pathol* 2008;**32**(9):1396–403.
9. Levy MJ, Gleeson FC, Zhang L. Endoscopic ultrasound fine-needle aspiration detection of extravascular migratory metastasis from a remotely located pancreatic cancer. *Clin Gastroenterol Hepatol* 2009;**7**(2):246–8.
10. Zbytek B, Carlson JA, Granese J, et al. Current concepts of metastasis in melanoma. *Expert Rev Dermatol* 2008;**3**(5):569–85.
11. Payette MJ, Katz 3rd M, Grant-Kels JM. Melanoma prognostic factors found in the dermatopathology report. *Clin Dermatol* 2009;**27**(1):53–74.
12. Winnepenninckx V, Lazar V, Michiels S, et al. Gene expression profiling of primary cutaneous melanoma and clinical outcome. *J Natl Cancer Inst* 2006;**98**(7):472–82.
13. Kauffmann A, Rosselli F, Lazar V, et al. High expression of DNA repair pathways is associated with metastasis in melanoma patients. *Oncogene* 2008;**27**(5):565–73.
14. Simon R, Lam A, Li MC, et al. Analysis of gene expression data using brb-array tools. *Cancer Inform* 2007;**3**:11–7.
15. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci USA* 2001;**98**(1):31–6.
16. Passos-Bueno MR, Ornelas CC, Fanganiello RD. Syndromes of the first and second pharyngeal arches: a review. *Am J Med Genet A* 2009;**149A**(8):1853–9.
17. Hildrestrand GA, Neurauter CG, Diep DB, et al. Expression patterns of Neil3 during embryonic brain development and neoplasia. *BMC Neurosci* 2009;**10**:45.
18. Downs KM, McHugh J, Copp AJ, Shtivelman E. Multiple developmental roles of Ahnak are suggested by localization to sites of placentation and neural plate fusion in the mouse conceptus. *Mech Develop* 2002;**119**(Suppl. 1):S31–8.
19. Gallo R, Zazzeroni F, Alesse E, et al. REN: a novel, developmentally regulated gene that promotes neural cell differentiation. *J Cell Biol* 2002;**158**(4):731–40.
20. Nagy JI, Hacking J, Frankenstein UN, Turley EA. Requirement of the hyaluronan receptor RHAMM in neurite extension and motility as demonstrated in primary neurons and neuronal cell lines. *J Neurosci* 1995;**15**(1 Pt 1):241–52.
21. Calella AM, Nerlov C, Lopez RG, et al. Neurotrophin/Trk receptor signaling mediates C/EBP alpha, -beta and NeuroD recruitment to immediate-early gene promoters in neuronal cells and requires C/EBPs to induce immediate-early gene transcription. *Neural Dev* 2007;**2**:4.
22. Verkman AS, Hara-Chikuma M, Papadopoulos MC. Aquaporins – new players in cancer biology. *J Mol Med* 2008;**86**(5):523–9.
23. Schwarz Q, Maden CH, Vieira JM, Ruhrberg C. Neuropilin 1 signaling guides neural crest cells to coordinate pathway choice with cell specification. *PNAS* 2009;**106**:6164–9.
24. Nagy N, Mwizerwa O, Yaniv K, et al. Endothelial cells promote migration and proliferation of enteric neural crest cells via beta1 integrin signaling. *Dev Biol* 2009;**330**(2):263–72.
25. Drerup CM, Wiora HM, Topczewski J, Morris JA. Disc1 regulates foxd3 and sox10 expression, affecting neural crest migration and differentiation. *Development* 2009;**136**(15):2623–32.
26. Barnhill RL, Chastain MA, Jerdan MS, et al. Angiotropic neonatal congenital melanocytic nevus: how extravascular migration of melanocytes may explain the development of congenital nevi. *Am J Dermatopathol* 2010;**32**(5):495–9.
27. Dixon J, Jones NC, Sandell LL, et al. Tcof1/Treacle is required for neural crest cell formation and proliferation deficiencies that cause craniofacial abnormalities. *Proc Natl Acad Sci USA* 2006;**103**(36):13403–8.
28. Shtivelman E, Cohen FE, Bishop JM. A human gene (AHNAK) encoding an unusually large protein with 1.2-μm polyionic rod structure. *Proc Natl Acad Sci USA* 1992;**289**:5472–6.