Cancer Treatment and Research Communications 25 (2020) 100262

Contents lists available at ScienceDirect

Cancer Treatment and Research Communications

journal homepage: www.sciencedirect.com/journal/cancer-treatment-and-research-communications

Minimal Residual Disease in Melanoma:molecular characterization of *in transit* cutaneous metastases and Circulating Melanoma Cells recognizes an expression panel potentially related to disease progression

Maria Cristina Rapanotti [a,b,*], Tara Mayte Suarez Viguria [a,b,1], Giulia Spallone [c,1], Alessandro Terrinoni [b], Piero Rossi [d], Gaetana Costanza [e], Elena Campione [c], Paolo Lombardo [c], Cristine Don Pathirannehalage [d], Augusto Orlandi [e], Sergio Bernardini [b], Luca Bianchi [c]

[a] *Department of Onco-Haematology, University of "Tor Vergata", Rome, Italy*
[b] *Department of Experimental Medicine, University of "Tor Vergata", Rome, Italy*
[c] *Department of Dermatology, University of "Tor Vergata", Rome, Italy*
[d] *Surgery Division, University of "Tor Vergata", Rome, Italy*
[e] *Anatomic Pathology Department, University of "Tor Vergata", Rome, Italy*

ARTICLE INFO

Keywords:
Circulating Melanoma Cells (CMCs)
Cutaneous *in transit* metastases (CTM) MCAM/MUC18/CD146
ABCB5
Gene-expression-panel
Melanoma-disease-progression

ABSTRACT

Isolating circulating melanoma cells (CMCs) represents a powerful method to monitor minimal residual disease. We documented that MCAM/MUC18/CD146 expression is strongly associated with disease progression. ABCB5 is melanoma-stem antigen with self-renewal, proliferation, differentiation, tumorigenicity capabilities. These findings supported us to improve CMC detection, investigating MCAM/MUC18/CD146 and ABCB5 as enrichment targets in MM progression. Moreover, we decided to compare possible molecular diversity of these CMC fractions with metastatic tissue expression, collecting concomitantly cutaneous *in transit* metastases (CTM). We enriched CMCs from eight melanoma patients staged ≥pT1b AJCC, who developed CTMs at baseline or during follow up. We assessed a gene expression panel comprising *ABCB5*, the differentiation markers (*Tyrosinase, MART1*), angiogenic factors (*VEGF, bFGF*), the cell-cell adhesion molecules (*MCAM/MUC18/CD146 5′-portion, Long, and Short isoforms, E-Cadherin, N-—Cadherin, VE–Cadherin*) and matrix-metallo-proteinases (*MMP2 and MMP9*) *via* high-sensitive RT-PCR. Preliminary findings defined three distinct sub-populations: "endothelial" CD45-CD146+CMCs, "stem" CD45-ABCB5+CMCs and a "hybrid- stem-endothelial"- CD45-MCAM+ABCB5+CMCs. The expression panel documented that – almost high expression found in CTMs – like in 73.5% of CMCs resulted positive for at least one transcript at baseline, showing gene-expression variability. Longitudinal monitoring documented shut-down of all gene-expressions in "endothelial"- and "hybrid stem-endothelial"-subsets, whilst persistency or acquisition of *MCAM/MUC18/CD146, VE-CADH* and *MMPs* was documented in disease-progression *status.*Conversely, a drastic expression shut-down was documented when patients achieved clinical remission. The "stem"- CMCs fraction" showed quite lower gene expression frequencies. MCAM/MUC18/CD146 and ABCB5 as melanoma-specific-targets are effective in the selection of highly primitive CMCs and highlights those putative genes associated with disease spreading progression.

Introduction

Circulating Tumour Cells (CTCs) are released from primary tumours that spread through the blood stream and are considered responsible of metastatic progression [1]. Fortunately, only a small percentage of released cells are efficient as well as capable of colonizing and forming distant lesions, since survival can be limited by immune surveillance or hemodynamic forces, [2,3,4].

Nevertheless, few CTCs acquire activating changes that lead to their extravasation into the surrounding tissue, degradation of basement

* Corresponding author at:Department of Onco-Haematology& Department of Experimental Medicine, University of "Tor Vergata"- Rome, Viale Oxford 81, 00133, Rome, Italy.
E-mail address: cristinarapanotti@yahoo.it (M.C. Rapanotti).
[1] The two authors have equally contributed to this work.

https://doi.org/10.1016/j.ctarc.2020.100262

Available online 2 December 2020

membrane and extracellular matrix, capacity to migrate, adhere and propagate *via* the lymphatic and circulatory systems and establish new colonies at distant sites that will lead to metastatic disease, [5,6,7]. Epithelial-mesenchymal transition (EMT), which is indispensable for tumor metastasis, is a multi-step process involving many molecular and cellular changes, including the downregulation of epithelial proteins and the upregulation of mesenchymal proteins, endowing the cells with increased motility and invasiveness [8,9,10]. Recent studies have revealed that the EMT phenotype in CTCs may facilitate tumor metastasis. Characterizing the epithelial *vs* mesenchymal phenotypes of CTCs may be useful to identify the most aggressive CTC subpopulations and establish an appropriate therapy [11,12,13,14,15,16,17].

In Malignant Melanoma (MM), CTCs are detectable in the peripheral blood either soon after the surgical resection of the primary tumor - regardless of the thickness - in late stage disease, or even in clinically disease-free patients [18,19,20,21,22,23,24]. Measuring circulating melanoma cells (CMCs) before they become clinically detectable, also defined "Liquid biopsy", represents a potentially powerful method to monitor patients with malignancies that have a minimal morbidity. For melanoma, two studies showed that the number of "2 CMCs per 7.5 ml of blood" is prognostic and associated with shorter survival [25, 26]. In tumours as carcinomas, immuno-magnetic enrichment is conventionally, performed by targeting epithelial cell surface markers such as EpCAM, and cytokeratin antigens, used as "positive" selection. The FDA (Food and Drug Administration) has approved the CellSearch® Circulating Tumor platform for the collection and isolation of CTCs of these carcinomas. Currently, only EpCAM has been recognised (Janssen Diagnostic, LLC Raritan, NJ).

CTCs derived from epithelial cancers commonly express cytokeratins and/or EpCAM (epithelial cell adhesion molecule) [27,28,29], which are used for CTC isolation and detection. However, CMCs lack of a ubiquitous marker and do not express the classical epithelial cell surface marker EpCAM, since melanocytes originate from the neural crest. Nevertheless, a variety of markers, not well formalised in literature, associated with some melanoma-specific-cell-surface epitopes, have been proposed such as MCAM/MUC18/CD146 and MSCP/NG2, (melanoma-associated Chondroitin Sulphate) together with stem cell markers, such as ABCB5 (ATP-binging cassette subfamily member 5) and CD271, [26,30,31,32].

It has been shown that the cell-cell-adhesion molecule MCAM/MUC18/CD146 expressed up to 80% in MM is a key oncogene driving melanoma progression and metastasis [33,34,35]. We previously documented that MCAM/MUC18/CD146 expression predicts clinical relapse, whereas absence or persistent loss is related to stable disease or to disease-free status, revealing its possible role as "molecular warning of progression" [36,37,38,39]. Recently, two MCAM/MUC18/CD146 isoforms have been described, a Long and a Short variant due to alternative splicing; the short isoform is widely expressed by the endothelium, while the long isoform is expressed by melanoma cells. In addition to the membrane-anchored MCAM/MUC18/CD146, a soluble form - sCD146/MCAM/MUC18 - generated by metalloproteases proteolytic cleavage is mainly involved in tumor angiogenesis. Expressing MCAM/MUC18/CD146-positive tumors secrete soluble CD146 that, in turn, would be responsible for their growth and vascularization, [40,41,42].[2]

Another potential melanoma-associated antigen is an ATP-binding cassettesub-family B member 5 also known as P-glycoprotein ABCB5,a plasma membrane-spanning proteinthat behaves as "stem cell" marker of a slow-cycling population of tumor cells with self-renewal, proliferation, differentiation, and tumorigenicity capabilities [43,44,45]. Moreover, it has been found that melanomatumor cells expressing ABCB5 are able to avoid chemotherapy and MAPK inhibition, possibly due to the drug-efflux function [46,47]. Human tumorigenic melanoma reveals that few melanoma cells express ABCB5. These cells tend to display a primitive molecular profile and correlate with clinical melanoma progression as determined by high-density tissue microarrays that allow one to screen many melanomas representing progressive evolution from Radial (RGP) to Growth Phase (VGP) [48] and metastatic disease. Thus, this plasma membrane-spanning protein that behaves as 'stem cell' marker of a slow-cycling population of malignant cell subpopulationswith "clinical virulence resides" as consequence of unlimited self-renewal,resulting in inexorable tumor progression and metastasis 49,50,51,52,53] could represent a melanoma biomarker of early metastatic stage.

All these findings strongly supported us to improve CMC detection in order to investigate MCAM/MUC18/CD146 [35,36,37,38,39] and ABCB5 [43,44,45,46,47], as enrichment markers in MM progression.

In a preliminary recent project on melanoma liquid biopsy, [54] we enriched and isolated three distinct subpopulations from a total of sixty-one blood-samples from 21 AJCC staged≥ pT1b melanoma patients, (i.e. transition from radial to vertical phase). Then, we assessed a robust selected biomarker qualitative expression panel, contemplating the angiogenic-potential, melanoma-initiating and melanoma-differentiation drivers, cell-cell adhesion molecules, matrix-metallo-proteinases performed on MCAM and ABCB5 enriched CMCs, aimed at identifying those putative genes involved in early melanoma spreading and disease progression.

At first, a significant differential expression of the specific transcripts was documented between and within the CMC fractions enriched with MCAM, ABCB5 and both MCAM/ABCB5-coated beads, when analyzing two distinct groups: early AJCC (stage I-II) and advanced- staged patients (stage II-IV). Moreover, in the early-AJCC staged-group, we could distinguish "endothelial", $CD45^{-}MCAM^{++}$enriched-, "stem" S-CMCs, $CD45^{-}ABCB5+$ enriched- and a third hybrid bi-phenotypic $CD45^{-}MCAM+/ABCB5+$ enriched-fractions, due to three distinct gene-expression profiles. In particular, the endothelial-CMCs were characterized by positive expression of genes involved in migration and invasion, whilst the stem CMC-fraction only expressed stem and differentiation markers. The third subpopulation isolated based on concurrent MCAM and ABCB5 protein expression showed an invasive phenotype. All three distinct CMCs sub-populations revealed a "specific picture" at onset: while exhibiting a primitive "stem-mesenchymal" profile, suggesting a highly aggressive and metastasizing phenotype, they displayed distinct gene expressions and exhibited distinct roles in early local invasion and metastases.

So, in light of this evidences, we felt that it was necessary to better understand and characterize the molecular "behavior"of these three enriched CMC subpopulations analyzing their molecular expression at the time of the cutaneous*in transit* metastases, CTM, development (considered as the baseline-time-zero) and during the follow up, taking as reference control the molecular expression of CTMs.

Therefore, we collected a small cohort of eight AJCC staged≥ pT1b melanoma patients with concurrent CTM and we could assessa longitudinal monitoring at different time points of their follow-up, in seven of the 8 patients. More in detail, the selected biomarker qualitative expression panel included the following thirteengenes in our panel: vascular endothelial growth factor (*VEGF);* basic fibroblast growth factors (*bFGF*), cell-cell-adhesion molecules *MCAM/MUC18/CD146* isoforms Long, Short and 5′-portion, vascular endothelial cadherin (*VE-Cadh, CDH5*) epithelial cadherin (*E-Cadh, CDH1),* neuronal cadherin (*N—Cadh CDH2),* matrix-metallo-proteinases 2 and 9 (*MMP-2, MMP-9*), melanoma-differentiation marker yrosinase (*TyrOH*), melanoma antigen recognized by T cells *MelanA/MART1* and melanoma-initiating marker *ABCB5*.

The molecular gene comparison-analysis performed on CTM and on MCAM+ and/or $ABCB5^{-+}$enriched CMC-subpopulations collected at baseline and during clinical-course, revealed two interesting aspects: the first, showed that the CMC-fractions display distinct gene-profiles; the second that we could define an expression panel potentially related to

[2] melanoma tumor

disease progression.

Methods

Patients and healthy donors

Patients' demographic (pts) and clinical characteristics are shown in Table 1. A small cohort of eight melanoma patients entered prospectively this study. Information and consent forms, previously approved by ethical local Institutional Review Board (code: prot.0013157/2015) were provided at diagnosis, together with the permission to collect cutaneous *in transit* metastases (CTM) and baseline- and follow-up-blood samples for research purposes. Patients were considered eligible if they had a histological and immunohistochemical (S-100, HMB-45 and MART-1) confirmed diagnosis of malignant melanoma and if staged AJCC≥pT1b with subsequent development of cutaneous *in transit* metastases(CTM). The incurrence of CTM occurred at variable time intervals, starting from the date of melanoma diagnosis or from the date of the first visit after the diagnosis of distant metastases for occult melanoma, (more in detail at diagnosis for patient UPN8-ZF; +1 year for patients UPN1-AV, UPN5-VM, UPN7-PN; +3 years for patient UPN4-FM; +5 years for patient UPN2-MU; +11 years for patient UPN3 CAb; +13 years for patient UPN6-CAd). In any case, we considered the development of CTM as the baseline-time-zero and at the same time, we collected the first blood draw and we evaluated the AJCC stage.

We collected peripheral blood during follow-up in seven out of 8 patients and performed two (UPN4-FM, UPN6-CAD, UPN7-PN, UPN8-ZF) or three controls (UPN1-AV, UPN2-MU, UPN5-VM). A patient (UPN3-CAB) died before the first follow-up blood draw (+6months) due to disease progression. Two out of 7 were treated with targeted therapy anti-BRAF and anti-MEK (UPN4-FM, UPN7-PN) while five patients received anti-PD1/PD1ligand checkpoint-immune-therapy and achieved a stable-disease *status* for a period of 6 to 36 months (UPN1-AV, UPN2-MU, UPN6-Cad, UPN8-ZF), one patient (UPN5-VM) at the moment of this study in stable-disease, did not received immune-therapy (+2 years). Clinical history of patients is briefly summarized in Table 1. All patients were cured at the Dermatology Department of the University of Rome "*Tor Vergata*" (Italy). Twenty healthy donors from our Transfusion Center were included in the study as negative control.

Cell lines

We previously performed in several tumor-cell lines sensitivity tests and assessment of molecular qualitative expression (angiogenic-potential, melanoma-initiating and melanoma-differentiation drivers, cell-cell adhesion molecules, matrix-metallo-proteinases) on cell lines, making reference to our already reported data, to prove the quality of our cellular and molecular controls [54, 55]. We included to this previous molecular assay panel the melanoma-initiating and melanoma-differentiation drivers, such as Tyrosinase (*TyrOH*), melanoma antigen recognized by T-cells *MelanA/MART1* and *ABCB5* [54, 55]. Primary tumor cell lines were as follows: LnCap, DU145 (prostate cancer); MB 231, MCF7 (breast cancer); C33A, HeLa (cervix cancer); Mel 10, Mel 14, FO 1, Colo 38 (Malignant Melanoma); SH-Sy5 (neuroblastoma); U87 (glioma); U266, Arp 1 (multiple myeloma). The fibroblast cell line EDS and the endothelial cell line HUVEC were included as

Table 1
Patients' demographic, histological characteristics and clinical history.

UPN	sex	Age at first observation	Primarytumor site	Histology	Breslow grade (mm)	AJCC status at first observation	Incurrence of CTM from diagnosis	Therapy after CTM incurrence	Follow-up and Clinical Status
UPN1-AV	f	80	Unknown	/	/	IV	+1 year	Checkpoint Inhibitors (anti-PD1-PD1L)	At +2 years: Disease Progression
UPN2-MU	m	47	Trunk	SSM	1.8	IIIA	+5 years	Targeted Therapy (Anti-BRAF and anti-MEK)	At +2.6 years: Disease Progression
UPN3-CAB	f	68	Extremity	SSM	1.6	IV	+11 years	Checkpoint Inhibitors (anti-PD1-PD1L)	At +6 months: Death
UPN4-FM	m	40	Trunk	SSM	1.25	IIB	+3 years	Not yet started-Checkpoint Inhibitors (antiPD1-PD1L)	At +1 year: Stable Disease
UPN5-VM	m	60	Trunk	NM	4.5	IIB	+1 year	Checkpoint Inhibitors (anti-PD1-PD1L)	At +2 years: Clinical Remission
UPN6-CAD	f	82	Extremity	NM	2.4	IV	+13 years	Checkpoint Inhibitors (anti-PD1-PD1L)	At + 1 year: Disease Progression
UPN7-PN	m	64	Trunk	NM	4.0	IV	+1 year	Targeted Therapy (Anti-BRAF and anti-MEK)	At + 6 months year: Disease Progression
UPN8-ZF	m	42	Head	NM	2.2	IB	onset	Checkpoint Inhibitors (anti-PD1-PD1L)	At +6 months: Stable Disease

positive and negative controls.

Cutaneous in transit metastasis

We recovered from our eight melanoma patients fragments of skin lesions which resulted to be cutaneous *in transit* melanoma metastasis. Histopathological diagnosis and post-surgical staging were routinely performed according to international criteria, [56].

CMCs enrichment

Eighteen samples from 8 melanoma patients underwent CMC enrichment. Fifteen ml of blood were collected one week after sentinel lymphadenectomy and during selected follow-up time points. A homemade immuno-magnetical FITC-conjugated anti-CD146, anti-ABCB5, anti-CD146/anti-ABCB5 antibodies selection preceded by CD45 immuno-depletion allowed us to enrich three subpopulations based on expression of "endothelial" E-CMCs (CD45-MCAM+), "stem" S-CMCs (CD45-ABCB5+), and "hybrid stem-endothelial" E/S-CMCs (CD45-MCAM+/ABCB5+) markers [48]. Before performing CD45 depletion, plateletes and erithrocytes were removed by using density-gradient (HetaSep-StemCell Technologies); enriched leukocytes were then conjugated with Whole Blood CD45 Depletion Cocktail containing monoclonal CD45 antibody and magnetic nanoparticles (EasySep Magnetic Nanoparticles StemCell Technologies) using manufactory procedure, with several stoichiometric and volumes adjustments. After CD45 depletion, cell suspension was split in three separate vials and then underwent CMC enrichment, using manual immune-magnetic beads conjugated to anti-MCAM/CD146 (BD Pharmingen™), anti-ABCB5 (Biorbyt™) and to coupled anti-MCAM/CD146-ABCB5 monoclonal antibodies. A total of 54 enriched samples was recovered: twenty-four collected at baseline and thirty at time-course-follow-up. The enrichment protocol was designed in-house. The stoichiometry and experimental conditions for antibody-cell conjugation were established after having carried out serial dilutions of the melanoma cell lines into blood drawn sample from 1000 to 1cell/ml considering the expected rarity of CMCs in the blood stream (1–3 CMC/~5 billion blood cells). Patients were defined as CMCs-positive when MCAM+ or ABCB5+ but CD45-negative nucleated cells were detected. We included blood samples from 20 healthy donors from our Transfusion Center as negative control population.

Selection of reference gene panel

We report here the robust reference gene panel suitable for qualitative gene expression analysis of enriched CMC subpopulations and CTMs, above-mentioned in our previous preliminary report [54]. A qualitative test such as OneStep RT-PCR or RT-nested-PCR was assessed for the following 13 selected genes: vascular endothelial growth factor (*VEGF*), basic fibroblast growth factors (*bFGF*), vascular endothelial cadherin (*VE-Cadh, CDH5*), endothelial antigen *MCAM/MUC18/CD146* isoforms Long, Short and 5-portion, epithelial cadherin, (*E-Cadh, CDH1),* neuronal cadherin, (N-—*Cadh, CDH2),* matrix-metallo-proteinases (*MMP-2, MMP-9),* Tyrosinase (*TyrOH*), melanoma antigen recognized by T-cells *MelanA/MART1*and *ABCB5*. Selection was based on the best known and recognized papers available in the Pubmed databases and known to be major key players in the regulation of EMT, early melanoma spreading and finally disease progression [35, 36, 37, 38, 39, 4344, 52, 57, 58, 59, 60, 61, 62,63, 64].

RNA isolation and RT-PCR methods

Total RNA was isolated from primary tumor cell lines and CMC subpopulations, using a homemade protocol based on Chomczyńsky and Sacchi protocol modified for poorly cellular samples [65]. RNA integrity was measured for RNAs extracted from the 51 enriched melanoma patients subpopulations, the sixteen cell lines and the 20 healthy donors using the NanoDrop 2000 (ThermoFisher) according to the manufacturer's instructions. RNA integrity was also checked electrophoretically.

By performing RT-nested-PCR, we analyzed the molecular expression of the following genes: vascular endothelial growth factor (*VEGF*) basic fibroblast growth factors (*bFGF*) vascular endothelial cadherin (*VE-Cadh, CDH5*), endothelial antigen *MCAM/MUC18/CD146* isoforms Long, Short and 5′-portion, epithelial cadherin, (*E-Cadh CDH1),* neuronal cadherin, (*N-—Cadh CDH2),* matrix-metallo-proteinases (*MMP-2, MMP-9),* Tyrosinase (*TyrOH*), melanoma antigen recognized by T cells *MelanA/MART1*and *ABCB5*.

Three hundred nanograms of total RNA (Applied BioSystems, Roche Molecular Systems, Inc., Branchburh, New Jersey, USA) were used in all RT-PCR experiments, as foreseen by the manufacturer's instructions. First strand cDNA was generated with 2,5μM oligo $d(T)_{16}$, 5 mM $MgCl_2$, 1 mM dNTPs, 1 unit of RNase Inhibitor (Applied BioSystems) and 1 h incubation at 42 °C. Two μl aliquot of cDNA were used for single step sensitive RT PCR for all genes, with the exception of *MCAM/MUC18/CD146, ABCB5, TyrOH* and *MelanA/MART1* where nested PCR was also performed. A hot start Taq polymerase was used for amplification using the housekeeping gene beta2-microglobulin as control. All PCR experiments were performed in triplicate. Cell line total RNAs have always been included as positive or negative controls in all performed experiments. Primer sequences and PCR conditions are reported in detail in the Supplementary Information. The resulting nested products (25 μl) were analyzed on a 1.8% agarose gel. RNA integrity was checked electrophoretically, whilst the quality of cDNA was controlled by amplification of housekeeping genes such as β2-microglobulin.

Statistical analysis

For statistical evaluations, due to the small sample size ($n = 7$), we used non parametric McNemar'*stest* to analyze the accordance of the result (positive/negative) through time (baseline /follow-up) before and after therapy for small samples ($n<10$), [37]. The association analysis of reference gene-status and follow-up was analyzed by Fischer's extract test ($N<$or equal to 5).The differences were considered as statistically significant for values $p<0.05$.SPSS 20 software program (Spss inc. Chicago, IL, USA) was used for statistical analysis.

Results

Expression of melanoma-initiating and melanoma-differentiation drivers, proangiogenic, markers cell-cell adhesion factors and matrix-metallo-proteinases in cell lines

We report here the sensitivity tests of the molecular expression assay, that we previously performed in several tumor-cell lines making clear reference to our already reported data, exclusively to prove the quality of our cellular and molecular controls [37,54,55]. As previously reported, M14 melanoma cells were serially diluted to mimic in vivo conditions of occult metastatic melanoma cells in blood and to establish sensitivity of our assay, starting from 1×10^6 M14 cells mixed with 7×10^6 cells from blood of healthy donors (BHD) up to one M14 melanoma cell as already described [37]. The expression of proangiogenic, cell-cell adhesion factors and matrix-metallo-proteinases (*VEGF, bFGF, VE-Cadh, E-CADH , N-—Cadh, MCAM/MUC18/CD146, MMP-2* and *MMP-9*) resulted heterogeneous, as previously reported [54, 55]. Nevertheless, all four melanoma cell lines (M10, M14, FO1 and Colo38) expressed all above mentioned genes and the melanoma tissue stem- and differentiation markers *TyrOH, MelanA/MART1* and *ABCB5*.These molecular assays allow us to assure the efficiency, specificity and sensitivity of our amplification procedures considering the expected poorly cellular patient-samples.

Expression of melanoma-initiating and melanoma-differentiation drivers, proangiogenic, markers cell-cell adhesion factors and matrix-metallo-proteinases in cutaneous in transit metastases

Molecular expression assays performed in CTM documented a high positivity frequency of some ofthe selected references genes as follows: *MCAM/MUC18/CD146*5′-portion and Long, Short isoforms (100%, 80%, 80% respectively), *VE-Cadh* (100%), *N—Cadh* (100%), *MMP-2* (100%), *MMP-9* (80%). Pro-angiogenic factors showed middle frequency expression *VEGF (*60%), *bFGF (*60%) coupled with *MelanA/MART1* (60%), *ABCB5* (60%) *E-Cadh* (40%), while tissue differentiation marker *Tyr-OH* expression was only 10%.

Expression of melanoma-initiating and melanoma-differentiation drivers, proangiogenic, markers cell-cell adhesion factors and matrix-metallo-proteinases in immune-magnetically enriched MCAM- and ABCB5-CMC fractions from melanoma patients at baseline

All the 54 enriched CMC samples, divided in 18 $CD45^{-}MCAM^{+}$, 18 $CD45^{-}ABCB5^{+}$and 18 $CD45^{-}MCAM^{+}/ABCB5^{+}$subsets, were molecularly characterized by assessing the expressional panel of the thirteen above-mentioned genes. All 54 enriched CMC samples expressed b2-microglobulin house-keeping gene. This is a tangible demonstration of high quality cell-recovery and total RNA extraction, despite cellular rarity recovery. Analyzing the case totality, fortythree (79.6%) samples were found positive for expression of one of the thirteen transcripts at least in one fraction from their blood, “endothelial” (E-CMCs), “stem” (S-CMCs) and “hybrid stem-endothelial” (E/S-CMCs), while thirty-seven out of 54 (66.6%) were concomitantly positives in all three fractions. At first observation (baseline), we could analyze eight blood-drawn blood samples divided into three aliquots representing the three subpopulations, “endothelial” E-CMCs, “stem” S-CMCs and “hybrid-endothelial/stem”. Documented molecular expression as percentage was: *MCAM/MUC18/CD146*5′-portion and Long, Short isoforms (37.5%, 87.5%, 62.5% – 25%, 12.5%, 12.5%–50%, 87.5%, 75%), ABCB5 (25%, 12.5%, 25%),*VEGF (*12.5%), *bFGF* (25%, 25%, 37,5%), *N—Cadh* (25%, 25%, 37.5%), *VE-Cadh* (50%), *MMP2* (62.5%, 37.5% 62.5%) and *MMP9* (50%, 62,5%, 75%) respectively. Main melanoma-associated-markers *Tyr-OH, MelanA/MART1* and epithelial cell-adhesion molecule E-Cadherin, *E-Cadh*, were never detected in all CMC-fractions. More, in detail, six patients out of 8 molecularly characterized at first observation, documented detectable transcripts showing a different expression frequency in all the three CMC-fractions (UPN1-AV, UPN2-MU, UPN4-FM, UPN5-VM, UPN6-CAd, UPN7-PN). A patient (UPN8-ZF) resulted positive only in the E-CMCs and in theE/S-CMCs fractions. Peculiar case of a patient staged-AJCC IV (UPN 3-CAb), who died shortly after the baseline-blood sample, although the corresponding CTM expressed several genes, the three CMC-fractions resulted to be negative for all transcripts, (with the exception of internal control). At first observation, at least one isoform (Long, Short) or 5′-portion of the molecular *MCAM/MUC18/CD146* expression, was detected in seven out of 8 the “endothelial”- and “hybrid stem-endothelial”-fractions (87,5%) whilst the “stem” subset showed lower frequency (37,5%). Despite the monoclonal ABCB5 antibody enrichment, *ABCB5* expression was detected at very low frequency: effectively, only one patient (UPN6-Cad) was positive in all three CMC-fractions while another (UPN7-PN) showed positivity in the "hybrid stem-endothelial”-fraction respectively, (12.5%−12.5%−25%).

Expression of melanoma-initiating and melanoma-differentiation drivers, proangiogenic, markers cell-cell adhesion factors and matrix-metallo-proteinases in immune-magnetically enriched MCAM- and ABCB5-CMC fractions from melanoma patients during follow-up time course

We could assay the longitudinal monitoring in seven of the 8 patients (UPN1-AV, UPN-2MU, UPN4-FM, UPN5-VM, UPN6-CAd, UPN7-PN, UPN-8ZF) at different time points of their follow-up (+6 months-/+30 months).

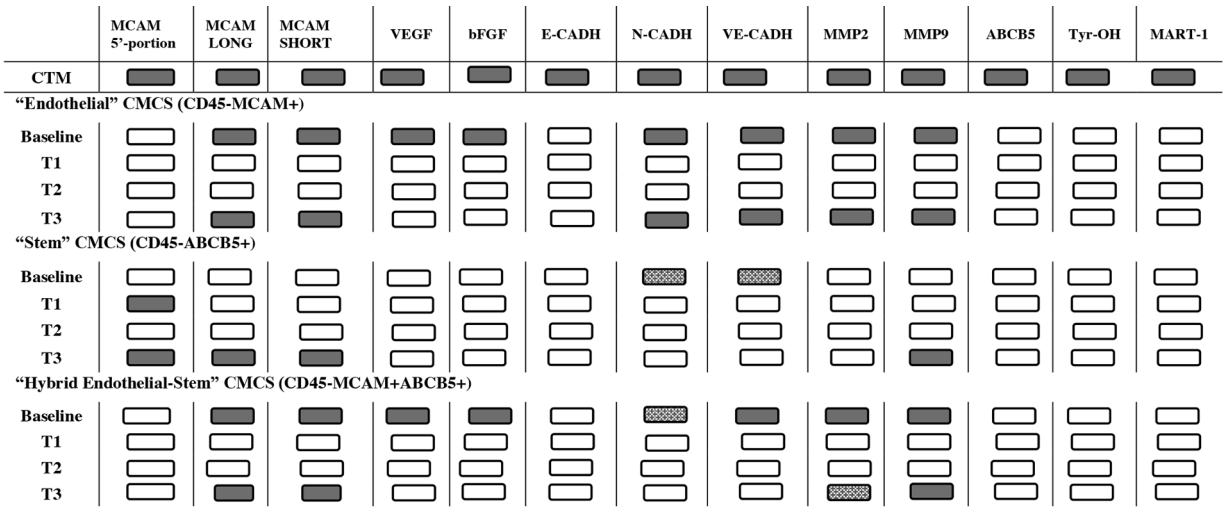
Molecular analysis performed in these CMC-enriched fractions deriving from the 30 blood samples collected at follow- up, documented a shut-down of almost all gene-expressions in all three subsets. In detail, the gene expression frequencies as follows: *MCAM/MUC18/CD146*5′-portion and Long, Short isoforms (26%%, 30%, 26%), *VE-Cadh* (23.3%), *N-—Cadh* (10%), *MMP2* (43.3%) *MMP9* (63.9%), *ABCB5* (0%), *VEGF* (0%), *bFGF*(0%). Taking in analysis the four patients (UPN1-AV, UPN2-MU, UPN4-FM, UPN5-VM) that achieved clinical remission condition-over a period of two years, (three out of 4 undergoing therapy) we documented in all CMC-fractions almost a total negativity-expression, if not for persisting MMPs-expressions ranging from (25–50%).

Conversely, patients, sharing stable disease not yet submitted to clinical treatments (UPN4-FM, UPN5-VM, UPN8-ZF) or patients showing a disease progression during treatments, (UPN1-AV, UPN2-MU, UPN6-CAd, UPN7-PN), documented a persisting or acquired positive expression of *MMP2* (60%) *MMP9* (100%) *and VE-Cadh* (71%), particularly in the E-CMCS and E/S-CMCs fractions. In two patients we could detectalso *ABCB5* expression, (UPN2-MU, UPN6-CAd). Most evident finding is that at least one isoform (Long, Short) or 5′-portion of the molecular expression of *MCAM/MUC18/CD146*(100%) was documented in all four patients at the time of disease-progression, stage (UPN1-AV, UPN2-MU,UPN6-CAd, UPN7-PN), and never during clinical remission. As illustrated in Fig. 1, different expression distribution of the selected genes was observed between and within MCAM-, ABCB5- or MCAM/ABCB5-coated-CMCs suggesting the molecular heterogeneity of these three subpopulations at different stages of melanoma disease and during follow-up. Moreover, these findings suggest that CMCs can be efficiently enriched and isolated by either MCAM/CD146 and/or ABCB5 cell surface markers.

Correlation between ***melanoma-initiating factors, melanoma-differentiation drivers, proangiogenic, markers cell-cell adhesion factors, matrix-metallo-proteinases and three enriched-CMC subpopulations***

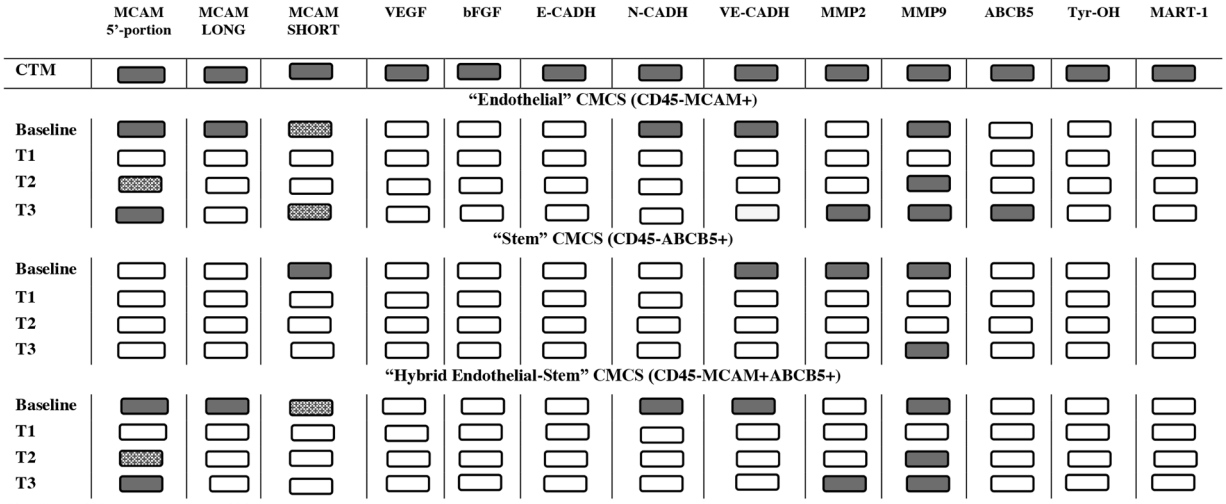
We decided to statistically analyze our small case studies, by performing McNemar’s *test* was performed to analyze the “changes of positive/negative expression”of the reference-genes selected in this study, associated with the condition “pre-and after- therapy”, in all the three CMC subpopulations, ($n = 7$). We evidenced a statistically difference for *MCAM/MUC18/CD146* long isoform expression that was lost after therapy ($p<0.05$; five patients out of 7 patients became negative at T1), both in CD45-CD146+ “endothelial”-fraction and $CD45^{-}MCAM^{+}/ABCB5^{+}$ “hybrid stem-endothelial” (E/S-CMCs), ($p<0,05$). Statistical difference was evidenced also for *MCAM/MUC18/CD146*-Short isoform in “hybrid stem-endothelial” (E/S-CMCs), where four out of 6 patients became negative undergoing to therapy($p<0,05$).

We also analyzed the changes between gene-reference-*status* (positive/negative) and follow-up ($n = 5$) at T1 by Fischer *test.* We evidenced statistical association between *MCAM/MUC18/CD146 long* negativity and clinical remission *status*, considering both $CD45^{-}CD146^{+}E^{-}$CMCs and $CD45^{-}MCAM^{+}/ABCB5^{+}$ “hybrid stem-endothelial” (E/S-CMC) fractions (five patients positive at baseline-t0 became negative; $p<0,004$). We evidenced a significantly association between *MCAM/MUC18/CD146*-Short acquired negativity and clinical remission *status* in $CD45^{-}MCAM^{+}/ABCB5^{+}$ *E*/S-CMC fraction, (four positive patients out of



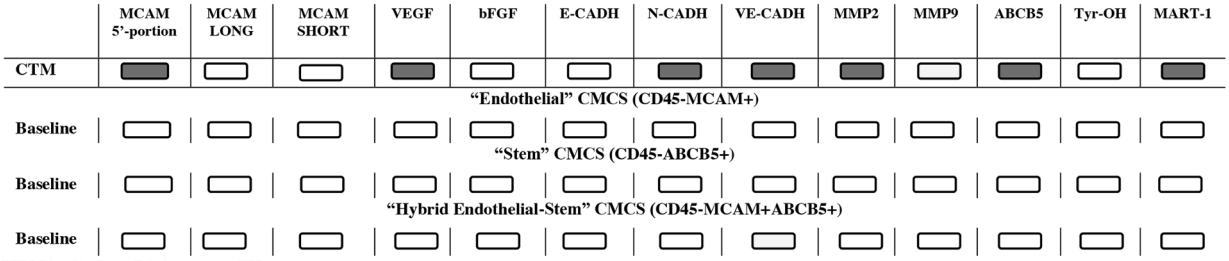
UPN1. AV AJCC- stage IV.

CTM: Cutaneous *in transit* Metastasis; **Baseline:** first observation**; T1:** + 6 months, anti-PD1- therapy, in clinical remission**; T2:**+1 year, anti-PD1- therapy, in clinical remission; **T3:** + 2 years, anti-PD1- therapy in disease-progression.



UPN2. MU AJCC- stage IIIA.

CTM: Cutaneous *in transit* Metastasis; **Baseline:** first observation**; T1:** + 1 year, anti-PD1- therapy in clinical remission**; T2:** +2 years, anti-PD1- therapy, lympho-nodal-recurrence; **T3:** + 2,6 years, post-surgery *plus* anti-PD1- therapy in disease-progression.



UPN3. CAb AJCC- stage IV.

CTM: Cutaneous *in transit* Metastasis; **Baseline:** first observation- anti-PD1- therapy, disease-progression and death.

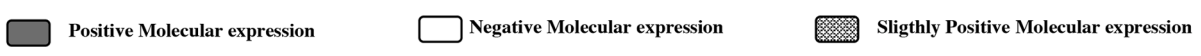
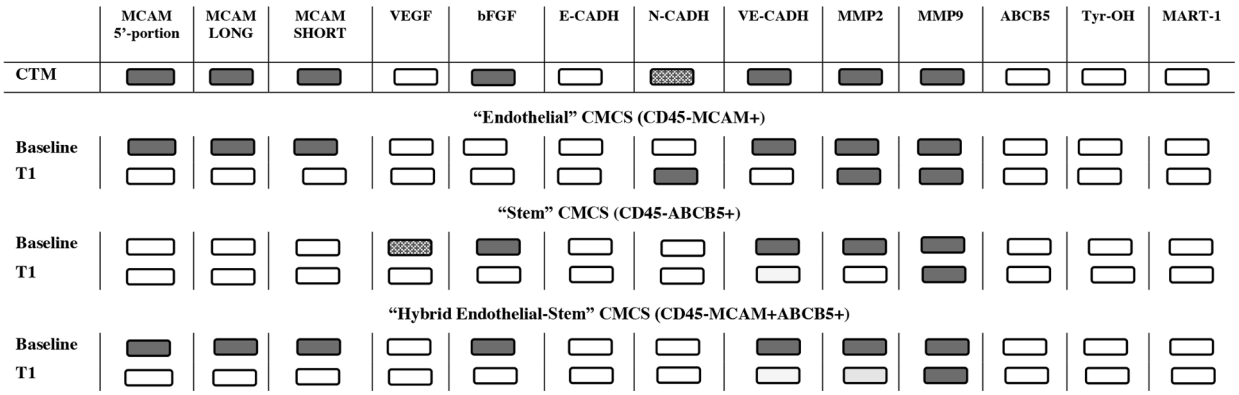
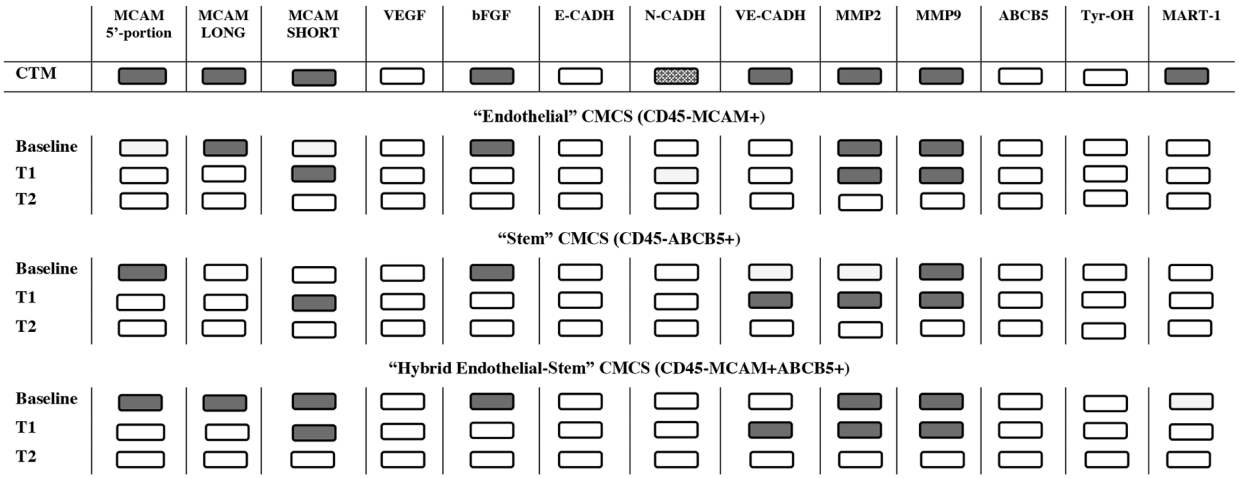


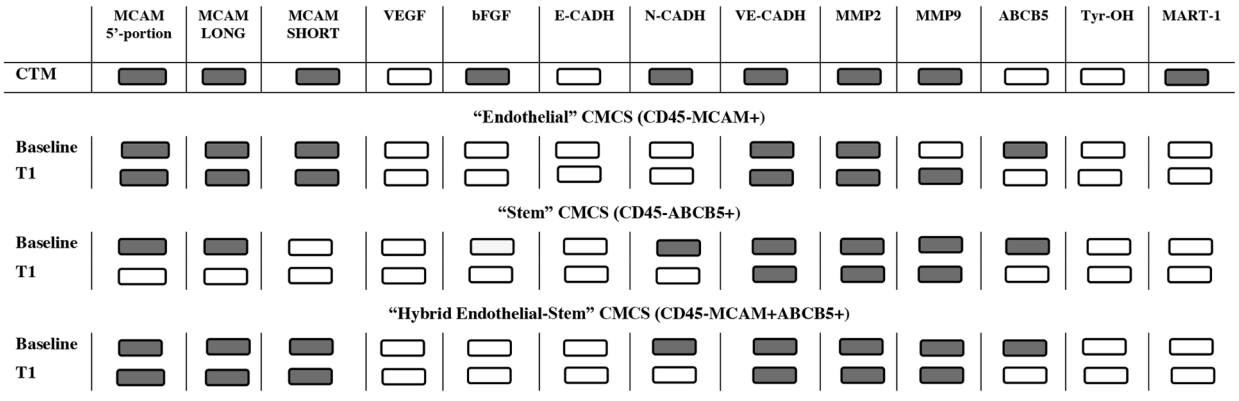
Fig. 1. Schematic graphic representation summarizing each sequential patient-specific expression profile.



UPN4. FM AJCC- stage IIB.
CTM: Cutaneous *in transit* Metastasis; **Baseline:** first observation; **T1:** + 1 year, anti BRAF and anti MEK therapy, in clinical remission



UPN5. VM. AJCC- stage IIB.
CTM: Cutaneous *in transit* Metastasis; **Baseline:** first observation; **T1:** + 1 year, pre-therapy in clinical remission**; T2:**+ 2 years, pre-therapy in clinical remission**.**



UPN6. CAd. AJCC- stage IV.
CTM: Cutaneous *in transit* Metastasis; **Baseline:** first observation; **T1:** + 1 year, anti-PD1- therapy, in disease-progression.

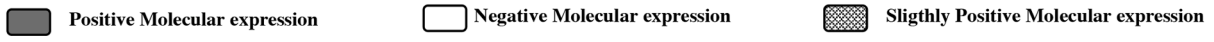
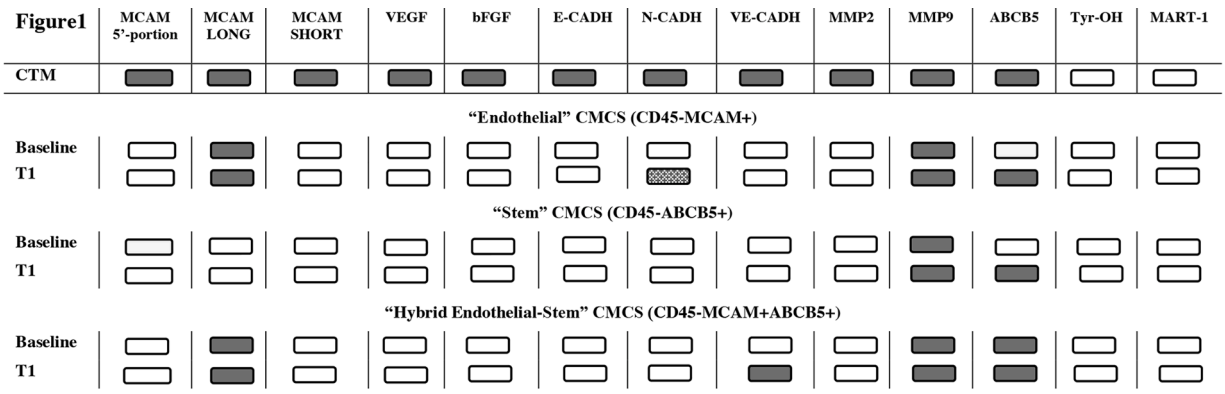


Fig. 1. (*continued*).

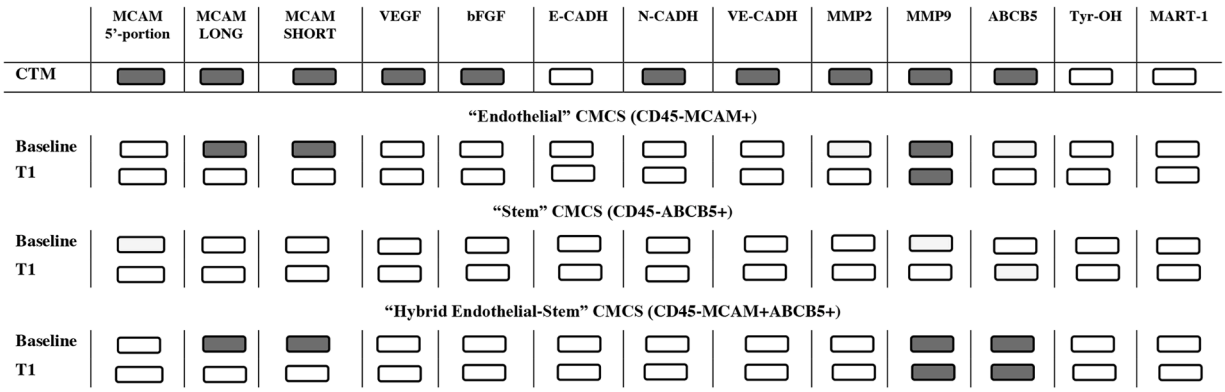
5 at baseline became negative when clinical remission *status* was achieved;$p<0,02$). When we analyzed association between reference-genes and disease progression we could only evidence a possible lost of the tissue-specific differentiation cadherin, *n-CADH* expression ($p<0.07$, Not significant).

Discussion

Various cell surface antigens have been implicated in the pathogenesis of human melanoma metastases [66,67]. Proteins, associated with stem and progenitor cells, are also detected in melanoma and



UPN7. PN. AJCC- stage IV.
CTM: Cutaneous *in transit* Metastasis; **Baseline:** first observation; **T1:** + 5 months, anti-BRAF and anti-MEK therapy, in disease-progression.



UPN8. ZF. AJCC- stage IB.
CTM: Cutaneous *in transit* Metastasis; **Baseline:** first observation; **T1:** + 6 months, anti-PD1- therapy, in stable-disease.

Fig. 1. (*continued*).

include: cancer testis antigens [68,69], bone morphogenetic proteins [70], Notch receptors [50], Wnt proteins [51] or specific stem cell-associated-markers, such as multidrug resistance transporters of the ABC family, CD133, CD166, CD34, Nestin, and c-Kit.2 [46, 44, 71-74]. Most of these studies employed melanoma cell lines and only a few analyzed expression of cell surface antigens on human melanomas [66, 67]. Detection and enumeration of two or more circulating tumor cells (CTCs) in 7.5 ml of blood from metastatic melanoma patients using the CellSearch system, has been established as an independent prognostic marker, [25, 26, 30], both prior to initiation and during/after therapy proved to be indicative of progression free survival (PFS) and overall survival [75,76,77]. The CellSearch platform has been widely used for detection of EpCAM positive CTCs in epithelial carcinomas. This system applied for melanoma identifies CD45/CD34 negative cells except MCSP (melanoma chondroitin sulfate proteoglycan)/ positive cells as CMCS. The latter expressed over 90% of melanoma tissue cells. Unfortunately, the methodologies used to identify the presence of CMCs, have not been standardized. Nevertheless, it has been documented that CMCs display a quite heterogeneous phenotype [48, 78]. Moreover, another pivotal process that facilitates tumor metastasis is EMT. Characterizing the epithelial *vs* mesenchymal phenotypes of CTCs may be helpful to identify the most aggressive CTC subpopulations and establish a valid therapy [8,9,10, 16]. It has recently defined that EMT occurs through distinct intermediate states with different invasive, metastatic and differentiation characteristics, [8,9,10, 79, 80,81,82]. Experimental model provided direct evidence that residence in a hybrid epithelial/mesenchymal (E/M) state was sufficient for maintenance of stem cell properties showing phenotypic plasticity, from epithelial to completely mesenchymal states, passing through intermediate hybrid states, that allow them to move collectively, as proposed by Jolly et al., [80] reaching efficiently the bloodstream intact, giving rise to clusters of circulating tumor cells (CTCs) and forming metastases. Moreover, all EMT "subpopulations" present similar tumor-propagating cell capacity, displaying differences in cellular plasticity, invasiveness and metastatic potential [81, 82].

In this study, we decided to analyze MCAM and ABCB5 as effective markers in order to identify and select CMCs, which are known as a heterogeneous population not uniquely definable. We selected cell-cell-adhesion MCAM/MUC18/C146, not only because it is an effective marker to capture and detect CMC - due to its high surface expression, up to 80%, [35,36,37,38, 60, 61, 83,84,85] - but also because it is considered an EMT inducer. In addition to its function as key oncogene in driving melanoma progression and metastasis, this membrane glycoprotein is a component of the inter-endothelial junction [86], and is even recognized as a mesenchymal marker [87]. CMC has an important role in the interaction with bone marrow stromal cells that allows motility and migration of hematopoietic microenviroment to heterotopic sites [86, 87]. Recently, a MCAM/MUC18/C146 stromal/mesenchymal signature associated with poor prognosis in several cancers it has been described, in particular in breast cancer. CMC's association to triple-negative receptor status promotes undifferentiated malignant cell motility [77, 88, 89].

Several findings documented that tumorigenic heterogeneity within the melanoma vertical growth phase (VGP) is the definition of a subpopulation of human melanoma cells that expresses the multidrug resistance transporter known as adenosine triphosphate–binding cassette subfamily B, ABCB5 [42, 61]. The ABCB5 trans-membrane transporter, belonging to the superfamily of integral membrane proteins, is associated with melanomagenesis, stem cell maintenance, metastasis and chemoresistance [42, 43]. Cells expressing the ABCB5-cell membrane-associated transporter, tend to display a primitive molecular profile and correlate with clinical melanoma progression as determined by high density tissue microarrays that allowed the screening of numerous melanomas representing progressive evolution from radial growth phase (RGP) to VGP and metastatic disease, [48, 90]. The association of an ABCB5-expressing melanoma subset with tumorigenic growth - typical of the VGP - support the *rationale* that CMCs derive from rare cancer-subpopulations with the potential skill to initiate metastases [43,44,45,46,47,48].

All these reasons made us develop a highly effective homemade CMCs enrichment protocol, selecting MCAM/MUC18/CD146 and ABCB5 as melanoma-specific epitopes, followed by molecular characterization made using a biomarker expression panel. We believe that the molecular expression analysis of this MCAM/MUC18/CD146-ABCB5 enrichment could provide great potential and biological information to better define melanoma high-risk and low-risk patients. The selection of melanoma patients staged≥pT1b developing cutaneous *in transit* metastases , from whom we concomitantly collected CTMs samples, allowed us to improve cell-recovery and characterize more highly aggressive CMCs [49, 61]. The most significant finding from our study is that based on gene expression data MCAM/MUC18/CD146 and ABCB5 are suitable and effective cell-surface targets also when using our “homemade liquid biopsy procedure". The different expression of the specific transcripts documented between and within the three CMC fractions, at first observation and during clinical follow-up, confirmed the *veracity* of this approach, outlining different specific-patient expression-profiles. Absence of circulating-tumor-cell-recovery and consequent no molecular gene expression was observed in healthy donors’ blood. This gave further validating to our approach.

We could confirm [90, 91] the rare *ABCB5* expression in CMCs even in the enriched CD45-ABCB5-positive fraction with respect to CTM.

According to our previously reported findings, two distinct CMC subpopulations,“endothelial”- and “hybrid stem-endothelial”-fractions expressed *MCAM/MUC18/CD146* at least one isoform (Long, Short or 5′-portion 100%) at first observation (87,5%) and at disease progression (100%), this latter accompanied by a persisting or acquired positive expression of *MMP2* (60%) *MMP9* (100%) and *VE-Cadh* (71%). Conversely, they documented a significant statistical drastic shut-down, particularly of *MCAM/MUC18/CD146 long* isoform, when patients achieved clinical remission, considering both $CD45^{-}CD146^{+}E^{-}$CMCs and $CD45^{-}MCAM^{+}$/$ABCB5^{+}$“hybrid stem-endothelial”. We underline that *MCAM/MUC18/CD146 long* isoform appears to be associated with malignant disease, as melanoma, rather than rather than in inflammatory processes such as the *short* counterpart.

The three distinct CMC-fractions showed a not very high molecular expression of proangiogenic factors, *VEGF* and *bFGF*(25%−12.5%) even at baseline (first observation) when patients were not treated-naïve therapy, completely lost during immune-therapy or targeted-therapy. No detection of melanoma-differentiation markers *Tyr-OH, MelanA/MART*and *E-Cadh.N—Cadh* expression was not frequent, despite its well-known association to neuro-ectodermal malignant tissue-transformation,ratherwe evidencedthat their expression was lost in advanced disease progression, even if not yet significant data, ($p<0.07$).

Our previous report performed on these enriched MCAM/MUC18/CD146- and/or ABC5-CMCs at baseline, provided evidence that we selected three distinct CMC sub-populations,sharing primitive, “stem-mesenchymal” behavior, which makes them highly aggressive and able to metastasize. We decided to analyze theee minimal residual-disease *status* comparing the sequential molecular expression of follow-up-blood-liquid biopsies and cutaneous *in transit* metastases.

Each patient showed a sequential specific-gene expression profile from the first observation, at CTM development, throughout the clinical follow-up, but all profiles defined clearly the usefulness of this gene expression panel including those genes associated to *MCAM/MUC18/CD146* such as *MMP2, MMP9* and *VE-CADH.*More in detail, patients who achieved clinical remission lost all gene-expressions during follow-up. Conversely, when disease progression occurred we documented re-acquisition of *MCAM/MUC18/CD146, MMPs* and *VE-CADH* gene expression in both MCAM- “endothelial”- and MCAM/ABCB5 “hybrid stem-endothelial”-fractions. Despite our smaller case-series, we can suppose that our MCAM-, ABCB5 - and dualMCAM/ABCB5 enrichment is effective in the selection of those CMC subpopulations sharing primitive, stem-mesenchymal signature that makes them highly aggressive and able to metastasize. This data is confirmed by the documented partial overlapping of some gene molecular expressions.

This study is among the first [54, 78, 91], to characterize different cancer sub-populations, contemplating a hybrid fraction, unveiling the molecular expression and suggesting distinct biological pathways activated in these cells.

The sequential molecular monitoring using our expression panel and including those genes associated to disease progression could be able to recognize melanoma high-risk or low-risk disease patients.

We are aware that our molecular analysis performed on this case series lacks of a quantitative normalization of gene expression, (applying RT-nested-PCR instead of a Real Time qPCR with a standard curve).The initial aim was to ascertain whether through our enrichment homemade procedure we were able to identify also rare CMC subsets and to verify if they showed a specific molecular signature, by assessing a biomarker qualitative expression panel, contemplating thirteen genes (exploring angiogenic-potential, melanoma-initiating and melanoma-differentiation drivers, cell-cell adhesion molecules, matrix-metalloproteinases).

The next development of our project, given the poor recovery of cellular RNA level due to rare CMC, will be to asses a quantitative real-time polymerase chain reaction (qRT-PCR) and amplitude-based amplicon multiplexing droplet digital-PCRto define mRNA measurement detection thresholds. In particular, it would be appropriate for those reference genes, such as *MCAM/MUC18/CD146, MMPs* and *VE-Cadh*, that we found to be statistically significant associated with disease progression.

There is no extensive knowledge about the differential biology of these three CMC-subpopulations. Our observations need to be validated in a larger case-series to achieve a stronger statistical significance. Finally, quantitative real-time PCR should be assessed for at least *MCAM/MUC18/CD146, MMPs* and *VE-Cadh* tofurther validate the prognostic role of these reference genes.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors contribution

MCR conceived, designed the study and organized the database. MCR and LB wrote the original draft of the manuscript. MCR, EC, AT, PR, SB, and LB substantially contributed to the conception, designed the work, reviewed, edited the manuscript and analyzed data. MCR and TMSV performed all molecular biological experiments. EC, GS, PR, PL, CDP and AO as dermatology clinicians, anatomo-pathogists and surgeons, respectively, selected, diagnosed, cured and collected all the clinical informations. GC performed the statistical analysis and wrote the related section of manuscript. MCR, EC, GS, AT, SB, and LB revised it critically for important intellectual content. All authors contributed to

manuscript revision, read and approved the submitted version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ctarc.2020.100262.

References

[1] P. Paterlini-Brechot, N.L Benali, Circulating tumour cells (CTC detection: clinical impact and future directions), Cancer Lett. 253 (2007) 180–204.
[2] S. Mocellin, U. Keilholz, C.R. Rossi, D Nitti, Circulating tumor cells: the 'leukemic phase' of solid cancers, Trends Mol. Med. 12 (2006) 130–139.
[3] R.A. Ghossein, S. Bhattacharya, J Rosai, Molecular detection of micrometastases and circulating tumour cells in solid tumors, Clin. Cancer Res. 5 (1999) 1950.
[4] K.J. Luzzi, I.C. MacDonald, E.E. Schmidt, N. Kerkvliet, V.L. Morris, et al., Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases, Am. J. Pathol. 153 (3) (1998) 865–873.
[5] N.K. Haass, K.S. Smalley, L. Li, M Herlyn, Adhesion, migration and communication in melanocytes and melanoma, Pigment Cell Res. 18 (2005) 150–159.
[6] S. Mocellin, D. Hoon, A. Ambrosi, D. Nitti, C.R Rossi, The prognostic value of circulating tumor cells in patients with melanoma: a systematic review and meta-analysis, Clin. Cancer Res. 12 (2006) 4605–4613.
[7] R.R. Braeuer, I.R. Watson, C.J. Wu, A.K. Mobley, T. Kamiya, E. Shoshan, M Bar-Eli, Why is melanoma so metastatic? Pigment Cell Melanoma Res. 27 (2014) 19–36.
[8] R. Kalluri, R.A. Weinberg, The basics of epithelial-mesenchymal transition, J. Clin. Invest. 119 (2009) 1420–1428.
[9] J.P. Thiery, H. Acloque, R.Y. Huang, M.A Nieto, Epithelial-mesenchymal transitions in development and disease, Cell 139 (2009) 871–890 [PMID:19945376 DOI:10.1016/j.cell.2009.11.007].
[10] M. Yu, A. Bardia, B.S. Wittner, S.L. Stott, M.E. Smas, et al., Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition, Science 339 (2013) 580–584 [PMID: 23372014 DOI:10.1126/science.1228522].
[11] H. Okabe, T. Ishimoto, K. Mima, S. Nakagawa, H. Hayashi, et al., CD44s signals the acquisition of the mesenchymal phenotype required for anchorage-independent cell survival in hepatocellular carcinoma, Br. J. Cancer 110 (2014) 958–966 [PMID: 24300972DOI:10.1038/bjc.2013.759].
[12] A. Satelli, Z. Brownlee, A. Mitra, Q.H. Meng, S Li, Circulating tumor cell enumeration with a combination of epithelial cell adhesion molecule- and cell-surface vimentin-based methods for monitoring breast cancer therapeutic response, Clin. Chem. 61 (2015) 259–266 [PMID: 25336717].
[13] P. Lembessis, P. Msaouel, A. Halapas, A. Sourla, Z. Panteleakou, et al., Combined androgen blockade therapy can convert RT-PCR detection of prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) transcripts from positive to negative in the peripheral blood of patients with clinically localized prostate cancer and increase biochemical failure-free survival after curative therapy, Clin. Chem. Lab. Med. 45 (2007) 1488–1494.
[14] A. Tsouma, C. Aggeli, N. Pissimissis, P. Lembessis, G.N. Zografos, et al., Circulating tumor cells in colorectal cancer: detection methods and clinical significance, Anticancer Res. 28 (2008) 3945–3960.
[15] Z. Panteleakou, P. Lembessis, A. Sourla, N. Pissimissis, A. Polyzos, et al., Detection of circulating tumor cells in prostate cancer patients: methodological pitfalls and clinical relevance, Mol. Med. 15 (2009) 101–114.
[16] P.A. Theodoropoulos, H. Polioudaki, S. Agelaki, G. Kallergi, Z. Saridaki, et al., Circulating tumor cells with a putative stem cell phenotype in peripheral blood of patients with breast cancer, Cancer Lett. 288 (2010) 99–106.
[17] M. Ignatiadis, G. Kallergi, M. Ntoulia, M. Perraki, S. Apostolaki, et al., Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2 in early breast cancer, Clin. Cancer Res. 14 (2008) 2593–2600.
[18] B. Gaugler, B. Van den Eynde, P. van der Bruggen, P. Romero, J.J. Gaforio, et al., Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes, J. Exp. Med. 179 (1994) 921–930.
[19] K. Tsukamoto, I.J. Jackson, K. Urabe, P.M. Montague, V.J Hearing, A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase, EMBO J. 11 (1992) 519–526.
[20] B. Mellado, L. Gutierrez, T. Castel, D. Colomer, M. Fontanillas, et al., Prognostic significance of the detection of circulating malignant cells by reverse transcriptase-polymerase chain reaction in long-term clinically disease-free melanoma patients, Clin. Cancer Res. 5 (1999) 1843–1848.
[21] G. Palmieri, M. Strazzullo, P.A. Ascierto, S.M. Satriano, A. Daponte, et al., Polymerase chain reaction-based detection of circulating melanoma cells as an effective marker of tumor progression. Melanoma Cooperative Group, J. Clin. Oncol. 17 (1999) 304–311.
[22] T.M. Rose, G.D. Plowman, D.B. Teplow, W.J. Dreyer, K.E. Hellström, et al., Primary structure of the human melanoma-associated antigen p97 (melanotransferrin) deduced from the mRNA sequence, Proc. Natl. Acad. Sci. U. S. A. 83 (5) (1986) 1261–1265.
[23] U. Keilholz, P. Goldin-Lang, N.E. Bechrakis, N. Max, A. Letsch, et al., Quantitative detection of circulating tumor cells in cutaneous and ocular melanoma and quality assessment by real-time reverse transcriptase-polymerase chain reaction, Clin. Cancer Res. 10 (2004) 1605–1612.
[24] R.A. Ghossein, J. Rosai, Polymerase chain reaction in the detection of micrometastases and circulating tumor cells, Cancer 78 (1996) 10–16.
[25] D. Klinac, E.S. Gray, J.B. Freeman, A. Reid, S. Bowyer, et al., Monitoring changes in circulating tumour cells as a prognostic indicator of overall survival and treatment response in patients with metastatic melanoma, BMC Cancer 14 (2014) 423.
[26] C. Rao, T. Bui, M. Connelly, G. Doyle, I. Karydis, et al., Circulating melanoma cells and survival in metastatic melanoma, Int. J. Oncol. 38 (2011) 755–760.
[27] M.1. Trzpis, P.M. McLaughlin, L.M. de Leij, M.C Harmsen, Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule, Am. J. Pathol. 171 (2) (2007) 386–395.
[28] L. Yang, J.C. Lang, P. Balasubramanian, K.R. Jatana, D. Schuller, et al., Optimization of an enrichment process for circulating tumor cells from the blood of head and neck cancer patients through depletion of normal cells, Biotechnol. Bioen. 102 (2009) 521–534, https://doi.org/10.1002/bit.22066.
[29] P.1. Chu, E. Wu, L.M Weiss, Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases, Mod. Pathol. 13 (2000) 962–972.
[30] L. Khoja, P. Lorigan, C. Zhou, M. Lancashire, J. Booth, et al., Biomarker utility of circulating tumor cells in metastatic cutaneous melanoma, J. Invest. Dermatol. 133 (2013) 1582–1590.
[31] A.D. Boiko, O.V. Razorenova, M. van de Rijn, S.M. Swetter, D.L. Johnson, et al., Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271, Nature 466 (2010) 133–137.
[32] X. Luo, D. Mitra, R.J. Sullivan, B.S. Wittner, A.M. Kimura, et al., Isolation and molecular characterization of circulating melanoma cells, Cell Rep. 7 (2014) 645–653.
[33] T. Ishikawa, Z. Wondimu, Y. Oikawa, G. Gentilcore, R. Kiessling, et al., Laminins 411 and 421 differentially promote tumor cell migration via alpha6beta1 integrin and MCAM (CD146), Matrix Biol. 38 (2014) 69–83.
[34] T. Ishikawa, Z. Wondimu, Y. Oikawa, S. Ingerpuu, I. Virtanen, et al., Monoclonal antibodies to human laminin alpha4 chain globular domain inhibit tumor cell adhesion and migration on laminins 411 and 421, and binding of alpha6beta1 integrin and MCAM to alpha4-laminins, Matrix Biol. 36 (2014) 5–14.
[35] V.O. Melnikova, M. Bar-Eli, Bioimmunotherapy for melanoma using fully human antibodies targeting MCAM/MUC18 and IL-8, Pigment Cell Res. 19 (2006) 395–405.
[36] M. Rapanotti, I. Ricozzi, E. Campione, A. Orlandi, L Bianchi, Blood MUC-18/MCAM expression in melanoma patients: a suitable marker of poor outcome, Br. J. Dermatol. 169 (2013) 221–222.
[37] M.C. Rapanotti, T.M. Suarez Viguria, G. Costanza, I. Ricozzi, A. Pierantozzi, et al., Sequential molecular analysis of circulating MCAM/MUC18 expression: a promising disease biomarker related to clinical outcome in melanoma, Arch. Dermatol. Res. 306 (2014) 527–537.
[38] M.C. Rapanotti, E. Campione, G. Spallone, A. Orlandi, S. Bernardini, et al., Minimal residual disease in melanoma: circulating melanoma cells and predictive role of MCAM/MUC18/MelCAM/CD146, Cell Death Discov. 3 (2017) 17, https://doi.org/10.1038/cddiscovery.2017.5, 7005.
[39] A. De Luca, D. Carpanese, M.C. Rapanotti, T.M. Viguria, M.A. Forgione, et al., The nitrobenzoxadiazole derivative MC3181 blocks melanoma invasion and metastasis, Oncotarget 9 (2017) 15520–15538, https://doi.org/10.18632/oncotarget.14690.
[40] S. Alais, N. Allioli, C. Pujades, J.L. Duband, O. Vainio, et al., "HEMCAM/CD146 downregulates cell surface expression of beta1 integrins, J. Cell Sci. 114 (2001) 1847–1859.
[41] J. Stalin, L. Vivancos, N. Bardin, F. Dignat-George, M Blot-Chabaud, MCAM and its isoforms as novel target in angiogenesis research and therapy. In: Intech editors. Physiologic and Pathologic Angiogenesis-Signaling Mechanism and Targeted Therapy, SE19SG –, LondonUnited Kingdom, 2017, pp. 429–450.
[42] J.1. Stalin, K. Harhouri, L. Hubert, C. Subrini, D. Lafitte, et al., Soluble melanoma cell adhesion molecule (sMCAM/sCD146) promotes angiogenic effects on endothelial progenitor cells through angiomotin, J. Biol. Chem. 288 (2013) 8991–9000, https://doi.org/10.1074/jbc.M112.446518.
[43] T. Schatton, G.F. Murphy, N.Y. Frank, K. Yamaura, A.M. Waaga-Gasser, et al., Identification of cells initiating human melanomas, Nature 451 (2008) 345–349, https://doi.org/10.1038/nature06489.
[44] B.J. Wilson, K.R. Saab, J. Ma, T. Schatton, P. Pütz, et al., ABCB5 maintains melanoma-initiating cells through a proinflammatory cytokine signaling circuit, Cancer Res. 74 (2014) 4196–4207, https://doi.org/10.1158/0008-5472.CAN-14-0582.
[45] V. Kupas, C. Weishaupt, D. Siepmann, M.L. Kaserer, M. Eickelmann, et al., RANK is expressed in metastatic melanoma and highly upregulated on melanoma-initiating cells, J. Invest. Dermatol. 131 (2011) 944–955, https://doi.org/10.1038/jid.2010.377.
[46] E.S. Gray, A.L. Reid, S. Bowyer, L. Calapre, K. Siew, et al., Circulating melanoma cell subpopulations: their heterogeneity and differential responses to treatment, J. Investig. Dermatol. 135 (2015) 2040–2048, https://doi.org/10.1038/jid.2015.127.
[47] M. Chartrain, J. Riond, A. Stennevin, I. Vandenberghe, B. Gomes, et al., Melanoma chemotherapy leads to the selection of ABCB5-expressing cells, PloS ONE 7 (2012) e36762, https://doi.org/10.1371/journal.pone.0036762.

[48] M.D. Laga, F. George, M.D. Murphy, Cellular heterogeneity in vertical growth phase melanoma, Arch. Pathol. Lab. Med. 134 (2010) 1750–1757.
[49] M.Y. Hsu, S.A. Rovinsky, C.Y. Lai, S. Qasem, X. Liu, et al., Aggressive melanoma cells escape from BMP-7 mediated autocrine growth inhibition through coordinated Noggin upregulation, Lab. Invest. 88 (2008) 842–855.
[50] K. Balint, M. Xiao, C.C. Pinnix, A. Soma, I. Veres, et al., Activation of Notch1 signaling is required for b-catenin–mediated human primary melanoma progression, J. Clin. Invest. 115 (2005) 3166–3176.
[51] A.T. Weeraratna, Y. Jiang, G. Hostetter, K. Rosenblatt, P. Duray, et al., Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma, Cancer Cell (2002) 1279–1288.
[52] N.Y. Frank, A. Margaryan, Y. Huang, A.M. Waaga-Gasser, M. Gasser, et al., ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma, Cancer Res. 65 (2005) 4320–4333.
[53] W.M. Klein, B.P. Wu, S. Zhao, H. Wu, A.J. Klein-Szanto, et al., Increased expression of stem cell markers in malignant melanoma, Mod. Pathol. 20 (2007) 102–107.
[54] M.C. Rapanotti, E. Campione, T.M. Suarez Viguria, G. Spallone, G. Costanza, P. Rossi, et al., Stem – Mesenchymal signature cell genes detected in heterogeneous circulating melanoma cells correlate with disease stage in melanoma patients, Front. Mol. Biosci. (2020), https://doi.org/10.3389/fmolb.2020.00092.
[55] M.C. Rapanotti, L. Franceschini, T.M.S. Viguria, C. Ialongo, D. Fraboni, et al., Molecular expression of bone marrow angiogenic factors, cell-cell adhesion molecules and matrix-metallo-proteinases in plasmacellular disorders: a molecular panel to investigate disease progression, Mediterr. J. Hematol. Infect. Dis. 10 (1) (2018), e2018059, https://doi.org/10.4084/MJHID.2018.059.
[56] C.M. Balch, A.C. Buzaid, S.J. Soong, M.B. Atkins, N. Cascinelli, et al., Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma, J. Clin. Oncol. 19 (2001) 3635–3648.
[57] P. Carmeliet, R.K. Jain, Angiogenesis in cancer and other diseases, Nature 407 (2000) 249–257.
[58] R.B. Hazan, R. Qiao, R. Keren, I. Badano, K. Suyama, Cadherin switch in tumor progression, Ann. N. Y. Acad. Sci. 1014 (2004) 155–163.
[59] R. Silye, A.J. Karayiannakis, K.N. Syrigos, S. Poole, S. van Noorden, W. Batchelor, et al., E-cadherin/catenin complex in benign and malignant melanocytic lesions, J. Pathol. 186 (1998) 350–355.
[60] LehmannJ.M., HolzmannB., BreitbartE.W., SchmiegelowP., RiethmullerG., JohnsonJ.P. (1987). Discrimination between benign and malignant cells of melanocytic lineage by two novel antigens, a glycoprotein with a molecular weight of 113,000 and a protein with a molecular weight of 76,000. 47:841–845.
[61] S. Xie, M. Luca, S. Huang, M. Gutman, R. Reich, J.P. Johnson, et al., Expression of mcam/muc18 by human melanoma cells leads to increased tumor growth and metastasis, Cancer Res. 57 (1997) 2295–2303.
[62] J.M. Ray, W.G. Stetler-Stevenson, The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis, Eur. Respir. J. 7 (11) (1994) 2062–2072.
[63] B.J. Curry, M.J. Smith, P Hersey, Detection and quantitation of melanoma cells in the circulation of patients, Melanoma Res. 6 (1) (1996) 45–54.
[64] B. Schittek, Y. Bodingbauer, U. Ellwanger, H.J. Blaheta, C Garbe, Amplification of Melan A messenger RNA in addition to tyrosinase increases sensitivity of melanoma cell detection in peripheral blood and is associated with the clinical stage and prognosis of malignant melanoma, Br. J. Dermatol. 141 (1999) 30–36.
[65] P. Chomczynski, N Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[66] T. Murakami, A.R. Cardones, S.T Hwang, Chemokine receptors and melanoma metastasis, J. Dermatol. Sci. 36 (2004) 71–78.
[67] G.W. Swart, P.C. Lunter, J.W. Kilsdonk, L.C Kempen, Activated leukocyte cell adhesion molecule (ALCAM/CD166): signaling at the divide of melanoma cell clustering and cell migration? Cancer Metastasis Rev. 24 (2005) 223–236.
[68] A.J. Simpson, O.L. Caballero, A. Jungbluth, Y.T. Chen, L.J Old, Cancer/testis antigens, gametogenesis and cancer, Nat. Rev. Cancer 5 (2005) 615–625.
[69] E.F. Velazquez, A.A. Jungbluth, M. Yancovitz, S. Gnjatic, S. Adams, et al., Expression of the cancer/testis antigen NY-ESO-1 in primary and metastatic malignant melanoma (MM)-correlation with prognostic factors, Cancer Immun. 7 (2007) 11–18.
[70] T. Rothhammer, P.J. Wild, S. Meyer, F. Bataille, A. Pauer, et al., Bone morphogenic protein 7 (BMP7) expression is a potential novel prognostic marker for recurrence in patients with primary melanoma, Cancer Biomark. 3 (2007) 111–117.
[71] N. Bonitsis, A. Batistatou, S. Karantima, K Charalabopoulos, The role of cadherin/catenin complex in malignant melanoma, Exp. Oncol. 28 (2006) 187–193.
[72] K.J.1. Radford, R.F. Thorne, P Hersey, CD63 associates with transmembrane 4 superfamily members, CD9 and CD81, and with beta 1 integrins in human melanoma, Biochem. Biophys. Res. Commun. 222 (1996) 13–18.
[73] E. Selzer, V. Wacheck, R. Kodym, H. Schlagbauer-Wadl, W. Schlegel, et al., Erythropoietin receptor expression in human melanoma cells, Melanoma Res. 10 (2000) 421–426.
[74] E.A. Djerf, C. Trinks, A. Abdiu, L.K. Thunell, A.L. Hallbeck, et al., ErbB receptor tyrosine kinases contribute to proliferation of malignant melanoma cells: inhibition by gefitinib (ZD1839), Melanoma Res. 19 (2009) 156–166, https://doi.org/10.1097/CMR.0b013e32832c6339.
[75] J.S. de Bono, H.I. Scher, R.B. Montgomery, C. Parker, M.C. Miller, et al., Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer, Clin. Cancer Res. 14 (2008) 6302–6309, https://doi.org/10.1158/1078-0432.CCR-08-0872.
[76] S.J. Cohen, C.J. Punt, N. Iannotti, B.H. Saidman, K.D. Sabbath, et al., Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer, J. Clin. Oncol. 26 (2008) 3213–3221, https://doi.org/10.1200/JCO.2007.15.8923.
[77] M. Cristofanilli, G.T. Budd, M.J. Ellis, A. Stopeck, J. Matera, et al., Circulating tumor cells, disease progression, and survival in metastatic breast cancer, N. Engl. J. Med. 351 (2004) 781–791.
[78] E. Quintana, M. Shackleton, H.R. Foster, D.R. Fullen, M.S. Sabel, et al., Phenotypic heterogeneity among tumorigenic melanoma cells from patients that is reversible and not hierarchically organized, Cancer Cell 18 (2010) 510–523, https://doi.org/10.1016/j.ccr.2010.10.012.
[79] X.H. Zhao, Z.R. Wang, C.L. Chen, L. Di, Z.Z. Bi, A.H. Li, et al., Molecular detection of epithelial-mesenchymal transition markers un circulating tumour cell from pancreatic cancer patients: potential role in clinical practice, World J. Gastroenterol. (2019) 25138–25150, https://doi.org/10.3748/wjgv25i1.1.138.
[80] M.K. Jolly, M. Boareto, B. Huang, D. Jia, M. Lu, E. Ben-Jacob, Implications of the hybrid epithelial/mesenchymal phenotype in metastasis, Front. Oncol. 5 (2015) 155, https://doi.org/10.3389/fonc.2015.00155.
[81] PastushenkoI., BrisebarreA., SifrimA., FioramontiM., RevencoT., BoumahdiS., et al. Identification of the tumour transition states occurring during EMT Nature. 2018; 556 (7702): 463–468. 10.1038/s41586-018-0040-3.
[82] C. Kröger, A. Afeyan, J. Mraz, E.N. Eaton, F. Reinhardt, Y.L. Khodor, et al., Acquisition of a hybrid E/M state is essential for tumorigenicity of basal breast cancer cells, Proc. Natl. Acad. Sci. U. S. A. 116 (23) (2019) 11553–11554, https://doi.org/10.1073/pnas.1907473116, 4.
[83] I.M Shih, The role of CD146 (Mel-CAM) in biology and pathology, J. Pathol. 189 (1999) 4–11.
[84] N. Bardin, F. Anfosso, J.M. Massé, E. Cramer, F. Sabatier, et al., Identification of CD146 as a component of the endothelial junction involved in the control of cell-cell cohesion, Blood 98 (2001) 3677–3684.
[85] B. Delorme, J. Ringe, N. Gallay, Y. Le Vern, D. Kerboeuf, et al., Specific plasma membrane protein phenotype of culture-amplified and native human bone marrow mesenchymal stem cells, Blood 111 (2008) 2631–2635. Epub 2007 Dec 17.
[86] B. Guezguez, P. Vigneron, N. Lamerant, C. Kieda, T. Jaffredo, et al., Dual role of melanoma cell adhesion molecule (MCAM)/CD146 in lymphocyte endothelium interaction: MCAM/CD146 promotes rolling via microvilli induction in lymphocyte and is an endothelial adhesion receptor, J. Immunol. 179 (2007) 6673–6685.
[87] B. Sacchetti, A. Funari, S. Michienzi, S. Di Cesare, S. Piersanti, et al., Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment, Cell 131 (2007) 324–336, https://doi.org/10.1016/j.cell.2007.08.025.
[88] G. Zabouo, A.M. Imbert, J. Jacquemier, P. Finetti, T. Moreau, et al., CD146 expression is associated with a poor prognosis in human breast tumors and with enhanced motility in breast cancer cell lines, Breast Cancer Res. 11 (2009) 1–14, https://doi.org/10.1186/bcr2215. R1.
[89] W. Onstenk, J. Kraan, B. Mostert, M.M. Timmermans, A. Charehbili, et al., Improved circulating tumor cell detection by a combined EpCAM and MCAM cell search enrichment approach in patients with breast cancer undergoing neoadjuvant chemotherapy, Smit. Mol. Cancer Ther. 14 (2015) 821–827, https://doi.org/10.1158/1535-7163.MCT-14-0653.
[90] A.M. Elliott, M.A. Al-Hajj, ABCB5 mediates doxorubicin resistance in melanoma cells by protecting the mitochondrial genome, Mol. Cancer Res. 7 (2009) 79–87.
[91] C. Aya-Bonilla, S. Gray, J. Manikandan, J.B. Freeman, P. Zaenker, et al., Immunomagnetic-enriched subpopulationsof melanoma circulating tumour cells (CTCs) exhinit distinct transcriptome profiles, Cancer 157 (2019) 1–15, https://doi.org/10.3390/cancers11020157.