Vemurafenib resistance selects for highly malignant brain and lung-metastasizing melanoma cells

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Abstract
V600E being the most common mutation in BRAF, leads to constitutive activation of the MAPK signaling pathway. The majority of V600E BRAF positive melanoma patients treated with the BRAF inhibitor vemurafenib showed initial good clinical responses but relapsed due to acquired resistance to the drug. The aim of the present study was to identify possible biomarkers associated with the emergence of drug resistant melanoma cells. To this end we analyzed the differential gene expression of vemurafenib-sensitive and vemurafenib resistant brain and lung metastasizing melanoma cells. The major finding of this study is that the in vitro induction of vemurafenib resistance in melanoma cells is associated with an increased malignancy phenotype of these cells. Resistant cells expressed higher levels of genes coding for cancer stem cell markers (JARID1B, CD271 and Fibronectin) as well as genes involved in drug resistance (ABCG2), cell invasion and promotion of metastasis (MMP-1 and MMP-2). We also showed that drug-resistant melanoma cells adhere better to and transmigrate more efficiently through lung endothelial cells than drug-sensitive cells. The former cells also alter their microenvironment in a different manner from that of drug-sensitive cells. Biomarkers and molecular mechanisms associated with drug resistance may serve as targets for therapy of drug-resistant cancer.

Keywords:
Vemurafenib resistance
Cancer stem cells
Metastatic microenvironment
Biomarkers

Introduction

The MAPK signaling pathway involves activation of BRAF which phosphorylates and activates MEK which in turn phosphorylates and activates ERK. These reactions result in activation of transcription factors that regulate cell survival, proliferation and differentiation [1]. BRAF mutations have been found in different malignancies including melanoma. V600E is the most common mutation in BRAF leading to constitutive activation of the MAPK signaling pathway [2]. Several small molecule inhibitors targeting the V600E BRAF mutation such as vemurafenib were developed [3]. Treatment of V600E BRAF positive metastatic melanoma with vemurafenib showed initial good clinical responses. However most of the patients relapsed due to acquired resistance [4].

Acquired drug resistance is one of the major obstacles in cancer treatment and management [5,6]. Several approaches have been adopted to overcome drug resistance, among them attempts to detect novel markers that can be targeted on resistant cells [7–10]. We have previously generated xenograft human melanoma brain metastasis models, consisting of local, cutaneous variants as well as of brain and lung-metastasizing variants yielding either dormant micrometastasis or overt metastasis. These cell lines comprise BRAF V600E mutation. All the variants originated from single melanomas thus sharing a common genetic background. Genes that are differentially expressed by these variants can, thus, be assigned to the differential malignancy phenotype of the different variants [11]. Using these models we demonstrated that brain-metastasizing melanoma variants expressed a set of genes whose expression pattern differed from that of cutaneous melanoma variants [11]. In this study we analyzed the differential gene expression of vemurafenib-sensitive brain and lung metastasizing melanoma cells and corresponding cells in which resistance to this bio-drug was
induced by repeated cycles of in vitro exposure to the drug. The vemurafenib sensitive melanoma cells and their resistant counterparts originated from a single melanoma tumor having therefore a common genetic background [11]. Any difference in gene expression between these metastatic variants can therefore be attributed to the difference in the metastatic microenvironment they originated from (brain versus lungs) and their drug sensitivity/resistance status.

Materials and methods

Cells

All human melanoma cells (YDFR.CB3, YDFR.SB3, YDFR.CB3CSL3) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mMol/ml L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 12.5 units/ml nystatin and 1% Heps (Biological Industries, Beit-Haemek, Israel). Medium of melanoma cells resistant to Vemurafenib was supplemented with 1 μM PLX-4032 (Vemurafenib) (Selleck, Houston, TX) dissolved in Dimethyl Sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO). Medium of the non-resistant melanoma cells was supplemented with the same amount of DMSO. Human embryonic kidney 293T cells were maintained as described by Izraely et al. [12]. Immortalized human brain microvascular endothelial cells (hCMEC/D3) were maintained as described by Weksl et al. [13]. Immortalized human pulmonary endothelial cells (hPMEC) were maintained as previously described by Unger et al. [14]. Cells were routinely cultured in humidified air with 5% CO2 at 37 °C. The cultures were tested and determined to be free of Mycoplasma.

Animals

Male athymic nude mice (BALB/c background) were purchased from Harlan Laboratories (Jerusalem, Israel). Mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in the animal quarters of Tel Aviv University and in accordance with current regulations and standards of the Israel Ministry of Health. The mice were used in accordance with institutional guidelines when they were 7 to 10 weeks old.

Orthotopic inoculation of tumor cells and in-vivo tumorigenicity assays

An orthotopic sub-dermal inoculation of nude mice and measurements of the tumorigenic properties were performed as described previously by Izraely et al. [12]. Mice were sacrificed 6 weeks after inoculation and brain, lungs and liver were harvested. The organs were immediately stored at −80 °C, until used for RNA extraction.

Drug resistance assessment

1.5 × 10^6 human melanoma cells comprising BRAF^{V600E} mutation were plated in normal growth medium until adherent. The medium was then removed and replaced with 5% FCS medium containing 5 μM Vemurafenib for 72 hrs. Melanoma cells grown in 5% FCS medium containing the same amount of DMSO served as control. Following inoculation, cells were rinsed with fresh growth medium and cultured in a drug-free medium for a week. This process was repeated 3 times, then the concentration of vemurafenib was elevated to 10 μM for two more cycles. At the end of each cycle total cell death was examined using a MEBCYTO® Apoptosis Kit (MBL, Woburn, MA) according to the manufacturers’ instructions. Melanoma cell variants were considered resistant when more than 70% of the cells survived the treatment.

Flow cytometry

Cells were detached with trypsin-EDTA (Biological Industries) into single cell suspension. 5 × 10^6 cells/sample were incubated for 1 hr at 4 °C with primary antibodies: α-CCR4 (1 μg/sample, R&D systems, Minneapolis, MN), α-CD271 (0.5 μg/sample, BioLegend, San Diego, CA), α-CD133 (0.5 μg/sample, Miltenyi Biotec, Bergisch Gladbach, Germany), α-VCAM1 (2 μg/sample, BioLegend, San Diego, CA), α-CD271 (0.5 μg/sample, Miltenyi Biotec, Bergisch Gladbach, Germany), α-CCR4 (1 μg/sample, R&D systems, Minneapolis, MN) and α-CD133 (0.5 μg/sample, Miltenyi Biotec, Bergisch Gladbach, Germany). Following incubation, cells were rinsed with fresh growth medium and cultured in normal growth medium until adherent. The medium was then removed and replaced with the same amount of DMSO. Human embryonic kidney 293T cells served as control. Following incubation, cells were rinsed with fresh growth medium and cultured in 5% FCS medium containing the same amount of DMSO. Human embryonic kidney 293T cells served as control.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using EZ-RNA Total RNA Isolation Kit (Biological Industries) and processed to cDNA with the M-MLV Reverse Transcriptase (Ambion Inc., Austin, TX). For qDNA amplification, primers were designed based on the GenBank Nucleotide Database of the NCBI website (Table 1). Amplification reactions were performed with SYBR Green 1 (Thermo Fisher Scientific, Waltham, MA) in triplicates in Rotor-gene 6000™ (Corbett life science, Hilden, Germany). PCR amplification was performed over 40 cycles, 95 °C for 15 s, 59 °C for 20 s, 72 °C for 15 s. Detection of

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Reaction specificity</th>
<th>Accession no.</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>IL1R1</td>
<td>Human</td>
<td>NM_0008773</td>
<td>5′-GCAGATTACTATTGTTCGTGGTAC-3′</td>
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<tr>
<td>JARD1B</td>
<td>Human</td>
<td>NM_0066183</td>
<td>5′-AGCAAGCTCACCAGGCTCCTGCTG-3′</td>
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<tr>
<td>CYR61</td>
<td>Human</td>
<td>NM_0015544</td>
<td>5′-CCTAAGGAGGCTCGAGG-3′</td>
</tr>
<tr>
<td>MMP-1</td>
<td>Human</td>
<td>NM_0024213</td>
<td>5′-GTGGCTAGTGTGCTG-3′</td>
</tr>
<tr>
<td>SPINK1</td>
<td>Human</td>
<td>NM_0031224</td>
<td>5′-CAGAATATATGACCT-3′</td>
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<td>CEACAM1</td>
<td>Human</td>
<td>NM_0017124</td>
<td>5′-CTCACCTGATGCT-3′</td>
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<tr>
<td>ABCG2</td>
<td>Human</td>
<td>NM_0048272</td>
<td>5′-TGGCTTCTAGTCAAGGAAGAGGCAAGC-3′</td>
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<td>Oct4</td>
<td>Human</td>
<td>NM_0027015</td>
<td>5′-GAAGAGAAGAGGCTCACCAGA-3′</td>
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<td>E-cadherin</td>
<td>Human</td>
<td>NM_0043603</td>
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<td>CCL17</td>
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<tr>
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<td>5′-CAGGACCAGGATIATCC-3′</td>
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<td>TNF-α</td>
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<td>β2M</td>
<td>Human</td>
<td>NM_0040842</td>
<td>5′-ATGAGACCGCAACTATGC-3′</td>
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<td>β2M</td>
<td>Mouse</td>
<td>NM_0097353</td>
<td>5′-CTGGCTGTTTCTGTGCTG-3′</td>
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</table>

5′, Sense; AS, Anti-sense.

Table 1 qRT-PCR oligonucleotide primers.
human cells (micro-metastases) in mouse tissue by qRT-PCR was performed as previously described by Izraely et al. [11].

Stable expression of mCherry vector

To produce the MLV infectious viruses, the 293T packaging cell line was co-transfected using calcium phosphate method with the retroviral backbone plasmid mCherry-pQCXIP, packaging plasmid gag/pol and envelope plasmid pVSV-G (Clontech Laboratories, Mountain View, CA). After 48 hrs of incubation, the virus particles in the medium were collected and filtrated (0.45μm, Millipore, Billerica, MA). 2×10⁶ melanoma cells were infected in the presence of 8μg/ml polybrene overnight. The cells were infected for the second time with new virus particles for 4hrs and then the virus-containing medium was replaced with fresh medium. After 72hrs 1μg/ml Puromycin (InvivoGen, Toulouse, France) was added for additional 7 days in order to select for stable infection.

Table 2

<table>
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<tr>
<th>RefSec number</th>
<th>Gene name</th>
<th>Role in cancer</th>
<th>CB3 vem vs con</th>
<th>SB3 vem vs con</th>
<th>CB3CSL3 vem vs con</th>
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<tr>
<td>NM_004827</td>
<td>ABCG2</td>
<td>Cancer drug resistance [49]</td>
<td>1.91</td>
<td>1.19</td>
<td>4.27</td>
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<tr>
<td>NM_130847</td>
<td>AMOTL1</td>
<td>Angiogenesis via regulating endothelial cell function [20]</td>
<td>1.57</td>
<td>1.38</td>
<td>1.56</td>
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<tr>
<td>NM_001657</td>
<td>AREG</td>
<td>Cell proliferation, motility and invasion via EGFR binding [21]</td>
<td>1.50</td>
<td>1.24</td>
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<tr>
<td>NM_001130046</td>
<td>CCL20</td>
<td>Cancer development and metastasis [22]</td>
<td>1.71</td>
<td>1.93</td>
<td>1.92</td>
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<tr>
<td>NM_004360</td>
<td>CDH1 (E-cadherin)</td>
<td>Cell–cell adhesion and tumor suppression [23]</td>
<td>−1.84</td>
<td>−1.71</td>
<td>−4.11</td>
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<td>NM_006851</td>
<td>GLIPR1</td>
<td>In correlation with invasive potential of melanoma [24]</td>
<td>1.60</td>
<td>1.50</td>
<td>16.79</td>
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<tr>
<td>NM_003484</td>
<td>HMGA2</td>
<td>Induction of neoplastic transformation and promotion of metastasis [25,26]</td>
<td>1.31</td>
<td>1.30</td>
<td>1.48</td>
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<tr>
<td>NM_000576</td>
<td>IL1β</td>
<td>Tumor-mediated angiogenesis and stimulates tumor invasiveness [27,28]</td>
<td>8.15</td>
<td>1.36</td>
<td>7.37</td>
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<tr>
<td>NM_000877</td>
<td>IL1R1</td>
<td>IL-1β receptor, decreases the recognition of melanoma by immune cells [29]</td>
<td>2.20</td>
<td>1.22</td>
<td>2.78</td>
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<tr>
<td>NM_001199640</td>
<td>IL33</td>
<td>Contributes to invasive behavior via epithelial-to-mesenchymal transdifferentiation in cancer cells [30]</td>
<td>1.78</td>
<td>1.21</td>
<td>2.22</td>
</tr>
</tbody>
</table>

The table displays genes uniformly up or down-regulated (FC ≥ 1.25 or FC ≤ −1.25 respectively) in three melanoma cell variants (YDFR.CB3, YDFR.SB3, YDFR.CB3CSL3) comparing vemurafenib-resistant to the corresponding non-resistant cells, as obtained from GeneChip Human Transcriptome Array 2.0 analysis. FC – Fold Change, vem – vemurafenib-resistant cells, con – control vemurafenib-sensitive cells.

Fig. 1. Melanoma cells resistant to vemurafenib show increased malignant phenotype in vitro. mRNA expression of the following genes: IL1R1, ABCG2, SPINK1, JARID1B, MMP-1 and E-cadherin was tested in human melanoma cells resistant and sensitive to vemurafenib using qRT-PCR analysis. The obtained values were normalized to hβ2M. All data are means of at least three independent experiments ± SD. Significance was evaluated using Student’s t-test for each vemurafenib-resistant variant compared to its vemurafenib-sensitive counterpart, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005. Control – vemurafenib-sensitive cells, vemur resist – vemurafenib-resistant cells.
to select a stably infected cell population. After selection, Puromycin was continuously added to the cultures.

**Adhesion to brain and lung endothelial cells**

Wells of 96-well plates were coated with 100 μg/ml rat-tail collagen type I (BD Pharmingen™) for 1 hr at 37 °C. Following one wash with PBSX1, 5 × 10^4 hCMEC/D3 or hPMEC cells in 100 μl EBM2 or M-199 growth medium, respectively were cultured for 24 hrs to form a confluent monolayer. Then, endothelial cells were activated with 0.1 μg/ml IFNγ and 0.1 μg/ml TNFα (PeproTech, Rocky Hill, CT) in suitable starvation medium for additional 24 hrs. At the end of the incubation period, cells were gently washed twice with PBSX1, and 1 × 10^5 mCherry-infected melanoma cells suspended in tris-buffered saline (TBSX1), containing 2 mM CaCl2 and 1 mM MgCl2, were added onto the stimulated hCMEC/D3 or hPMEC monolayer. Cells were incubated for 30 minutes at 37 °C to allow adhesion to occur. The total fluorescence signal of melanoma cells added to each well was measured by a Synergy HT fluorescent multi-well plate reader (BioTek, Winooski, VT) at wavelength of 590/645 nm before removing the non-adherent cells. Then, the wells were washed twice with PBSX1 to remove the non-adherent cells and fluorescence of the adherent cells was measured again. The fluorescence rate of the adherent cells was normalized to the total fluorescence in the same well. Wells containing only endothelial cells served as a blank control. The activation of the endothelial cells with IFNγ and TNFα was monitored by measuring the expression of VCAM-1 using flow cytometry [15].

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**Fig. 2.** Vemurafenib-resistant melanoma cells exhibit a higher level of CCR4 and up-regulate the expression of its ligands CCL17 and CCL22 within the remote mice organs in vivo. (A) Flow cytometry analysis of CCR4 in vemurafenib-resistant and non-resistant melanoma cells. The FACS diagrams shown are of a representative experiment. The continuous line in the diagrams represents negative isotype control; the discontinuous line represents anti-CCR4 immunostaining. Significance was evaluated using Student’s t-test for each vemurafenib-resistant variant compared to its vemurafenib-sensitive counterpart. All data are means of at least three independent experiments ± SD. (B) qRT-PCR analysis of CCL17 and CCL22 mRNA extracted from brain, lungs and liver of mice inoculated with vemurafenib resistant YDFR.SB3 cells, vemurafenib sensitive YDFR.SB3 cells or healthy mice. The data shown are average values from each group of mice: 5, 5 and 3 respectively. Significance was evaluated using Student’s t-test comparing between each group of mice. *p ≤ 0.05, **p ≤ 0.005. Control – vemurafenib sensitive cells, vemur resist – vemurafenib resistant cells.
Cell migration through collagen layer

Trans-well inserts (6.5 mm diameter polycarbonate membrane with 8.0 μm pores, Corning Costar Corp., New York, NY) were coated with 100 μg/ml collagen type I for 1 hr at 37 °C. After washing the trans-wells with PBSX1 twice, 1 × 10^5 melanoma cells were added to the upper chamber and allowed to transmigrate for 24 hrs after adding serum-free RPMI-1640 medium containing 2 mM CaCl2 and 1 mM MgCl2 to the lower chamber. At the end of the incubation period, cells in the upper chamber were removed using cotton swabs. The bottom side of the trans-well inserts was gently washed in PBSX1 and fixed in ice-cold methanol for 5 minutes, then the cells were stained with Dif-stain Kit (Kaltek, Padova, Italy), according to the manufacturer’s instructions. The number of melanoma cells that migrated through the membrane was determined by counting cells from five independent fields in duplicates using Olympus® IX53 light microscope (Olympus, Center Valley, PA).

Trans-endothelial migration

hCMEC/D3 or hPMEC (5 × 10^4 cells/200 μl) were plated on trans-well inserts, pre-coated with 100 μg/ml collagen type I, for 48 hrs, to form a confluent monolayer. Medium was added only into the upper compartment to prevent the formation of endothelial bilayer [16,17]. 1 × 10^5 melanoma cells expressing mCherry were added to the upper chamber and allowed to transmigrate for 24 hrs after adding serum-free RPMI-1640 medium containing 2 mM CaCl2 and 1 mM MgCl2 to the lower chamber. At the end of the incubation period, cells in the upper chamber were removed using cotton swabs. The bottom side of the transwell inserts was gently washed in PBSX1 and fixed in 4% paraformaldehyde for 15 minutes. Then, the transwell inserts were washed again in PBSX1 and stained with DAPI using Dapi–Fluoromount–GT solution (SouthernBiotech, Birmingham, AL). The number of melanoma cells that migrated through the membrane was determined by counting five independent fields under fluorescence microscopy (Olympus) in duplicates.

Hoechst staining

1 × 10^4 melanoma cells were plated in 96-well plates for 24 hrs in triplicates. Adherent cells were incubated with 5 μg/ml of Hoechst 33342 dye (Sigma-Aldrich) in 100 μl RPMI medium for 60 minutes at 37 °C. After the incubation, cells were rinsed twice with growth medium and PBSX1 was added for measurement in Synergy HT fluorescent multi-well plate reader at wavelength of 360/450 nm [18]. The results were normalized to the amount of cells as obtained from XTT assay (Cell Proliferation Kit, Biological Industries) that was performed simultaneously according to manufacturer’s instructions.

mRNA microarray

mRNA of Vemurafenib-resistant and sensitive melanoma cells was hybridized to GeneChip® Human Transcriptome Array 2.0 (Affymetrix, Santa Clara, CA). Partek Genomics Suite™ software (version 6.5; Partek, St. Louis, MO; http://www.partek.com/pps) was used for microarray analysis. Raw data (CEL files) were normalized at the transcript level using robust multivariate method [19]. Median summarization of transcript expressions was calculated. Gene-level data were then filtered to include only those probe sets that are in the “core” meta-probe list, which represent RefSeq genes and full-length GenBank mRNAs. Fold change (FC) cutoff of ≥1.25 or ≤−1.25 was performed to obtain differentially expressed genes for the various conditions.

Gelatin zymography and western blotting analysis

4.5 × 10^4 melanoma cells were plated in 24-well plates for 24 hrs, then growth medium was removed and replaced by 400 μl serum-free RPMI-1640 medium for additional 24 hrs. For Matrix Metalloproteinase-2 (MMP-2) activity detection, conditioned medium was collected and prepared without boiling or reduction, then subjected to SDS polyacrylamide gel electrophoresis containing 0.1% gelatin. After electrophoresis, the gels were washed for 15 minutes 4 times with reaction buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 10 mM CaCl2, 5 μM ZnCl2, 0.02% NaN3) containing 2.5% Triton X-100 and incubated in reaction buffer at 37 °C overnight. Finally, the gels were stained with Coomassie Blue (BioRad, Hercules, CA) and destained in a 20% methanol, 10% acetic acid solution. The results were validated by Western Blot analysis using α-MMP-2 antibody (1:200; R&D systems), as described previously [11]. The obtained MMP-2 activity values were normalized to the amount of viable cells in each well.

![Fig. 3. Vemurafenib resistant cells are enriched for cancer stem cell markers. (A) Flow cytometry analysis of CD271. The FACS diagrams shown are of a representative experiment. The continuous line in the diagram represents negative isotype control; the discontinuous line represents anti-CD271 immunostaining. (B) qRT-PCR analysis of FN1 mRNA expression in human melanoma cells resistant and sensitive to vemurafenib. The obtained values were normalized to hβ2M. (C) Uptake of Hoechst 33342 dye by different melanoma variants. The results were normalized to the amount of cells as obtained from XTT assay. All data are means of at least three independent experiments ± SD. Significance was evaluated using Student’s t-test for each vemurafenib-resistant variant compared to its vemurafenib-sensitive counterpart, *p ≤ 0.05, **p ≤ 0.0005. Control – vemurafenib-sensitive cells, vemur resist – vemurafenib-resistant cells.](image-url)
Statistical analysis

Paired or unpaired Student’s t-test was used to compare in vitro and in vivo results.

Results

Vemurafenib resistance of melanoma cells is associated with an altered gene expression profile

In previous studies we generated variants of human melanoma cells that metastasize to the brain and lungs of xenotransplanted nude mice [11]. In this study we utilized variants that metastasize specifically and spontaneously to brain and lungs of nude mice forming micro-metastasis (YDFR.SB3 and YDFR.CB3CSL3, respectively) in these organs following an orthotopic sub-dermal inoculation. We also used a variant that generates brain macro-metastasis following an intra-cardiac inoculation (YDFR.CB3). All three variants carry a BRAFV600E mutation. Exposing these cells in vitro to several cycles of increasing concentrations of vemurafenib we generated drug-resistant melanoma cells. Using expression microarray analysis we then compared the mRNA expression profile of resistant and sensitive cells. Analyzing the results, we filtered out genes that expressed a change fold of ≥1.25 or ≤−1.25 between the variants, focusing on a cluster of genes that uniformly changed in vemurafenib resistant versus sensitive cells. The most prominent genes that changed in the vemurafenib-resistant cells were those involved in promotion of metastasis, drug resistance, cancer stem cell markers and cell invasion (Table 2). The alteration of the following genes: ABCG2, E-cadherin, IL1R1, JARID1B, MMP1, SPINK1 was validated using qRT-PCR (Fig. 1).

In a previous study we demonstrated that an up-regulated expression of the chemokine receptor CCR4 on human melanoma cells is associated with brain metastasis in nude mice xenografted with human-melanoma cells. It was also shown that the expression of this chemokine receptor is regulated by the brain microenvironment [44,45]. Results of the present study indicated that CCR4 was significantly up-regulated on vemurafenib resistant cells (p < 0.05) (Fig. 2A).

Since metastasis is regulated to a significant part by interactions of tumor cells with their microenvironment [46,47] we hypothesized that the interaction of CCR4 on melanoma cells with the corresponding ligands (CCL17 and CCL22) in the metastatic microenvironment could be functionally involved in metastasis formation [44].

Six weeks after inoculation, mice bearing sub-cutaneous tumors generated by both vemurafenib-sensitive as well as resistant melanoma cells showed an up-regulated CCL17 expression in the lungs compared to lungs of healthy mice (p < 0.05) (Fig. 2B). The brain and liver of mice bearing tumors formed by vemurafenib-resistant cells expressed significantly higher levels of CCL17 than vemurafenib-sensitive cells (p < 0.05). CCL22 expression in the brain of mice bearing tumors generated by vemurafenib-resistant melanoma cells was also higher than that in brains of mice bearing tumors generated by vemurafenib-sensitive cells (p < 0.05).

Vemurafenib-resistant cells are enriched for characteristics of side-population tumor cells

It is widely accepted that cancer stem cells resist various anticancer treatments [6,48]. We therefore analyzed the expression of cancer stem cell markers in vemurafenib sensitive and resistant cells. Figures 1 and 3A,B show that the resistant cells were enriched for cells bearing several stem cell markers such as JARID1B, fibronectin...
Fig. 5. The migration of vemurafenib-resistant melanoma cells through brain and lung vascular endothelial layer is enhanced. (A) Melanoma cell adhesion to brain or lung vascular endothelial cell layer was tested by seeding mCherry expressing melanoma cells on adhered activated endothelial cells for 30 mins. Percentage of the adhered melanoma cells was determined by dividing the fluorescence rate after washing the wells by total fluorescence rate before washing. (B) Melanoma cell migration through brain or lung vascular endothelial cell layer was tested using trans-well inserts coated with collagen type I, on which endothelial cells were plated two days prior to adding mCherry expressing melanoma cells. Representative images are presented. Red staining – mCherry transfected melanoma cells, blue staining – DAPI. All data are means of at least three independent experiments ± SD. Significance was evaluated using Student’s t-test for each vemurafenib-resistant variant compared to its vemurafenib-sensitive counterpart, *p ≤ 0.05, **p ≤ 0.005. Control – vemurafenib-sensitive cells, vemur resist – vemurafenib-resistant cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
and CD271. The resistant cell variants also expressed elevated levels of ATP-binding cassette transporter ABCG2 (Fig. 1). This cellular transporter is directly involved in cancer drug resistance [49]. The chemotherapy extrusion by ABCG2 transporters correlates with the extrusion of the Hoechst 33342 dye. Cancer cells that efficiently exclude this dye are referred to as side-population cells. These cells share characteristics of cancer stem cells [50]. Figure 3C demonstrates that vemurafenib-resistant cells exhibited increased extrusion of the Hoechst 33342 dye.

**The in vitro malignancy phenotype of vemurafenib-resistant melanoma cells**

The 3 vemurafenib-resistant melanoma variants and their original vemurafenib-sensitive variants were tested for several malignancy-linked in vitro functions including MMP secretion, migration through collagen coated membranes, adhesion to brain and lung endothelium and trans-migration through monolayers of these endothelia. Figure 4 indicates that vemurafenib resistant cells express a significant increased MMP activity and a higher transmigration through collagen coated membranes than sensitive cells (p < 0.05). Figure 5A shows that vemurafenib-resistant brain metastasizing melanoma cells (both macro and micro-metastases) adhere significantly better than sensitive cells to lung endothelium (p < 0.05), whereas they adhere significantly less efficiently than vemurafenib-sensitive cells to brain endothelial cells (p < 0.05). Figure 5B demonstrates that vemurafenib-resistant brain metastasizing melanoma cells

### Table 3

<table>
<thead>
<tr>
<th>Adhesion of melanoma cells to endothelia and TEM – summary.</th>
<th>CB3</th>
<th>SB3</th>
<th>CB3CSL3</th>
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<td>Adhesion to brain endothelia</td>
<td></td>
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<td>Adhesion to lung endothelia</td>
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<td>TEM – brain endothelia</td>
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<td>TEM – lung endothelia</td>
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The table summarizes the results of adhesion of melanoma cells to brain and lung vascular endothelial cells and trans-migration of melanoma cells through monolayers of these endothelia (Fig. 4). The results shown are of vemurafenib-resistant cells relative to their non-resistant counterparts. TEM = trans-endothelial migration, NSD = no significant difference.

![Fig. 6](image)

The tumorigenicity of vemurafenib-resistant melanoma cells is increased. (A) The graph shows average tumor volume of mice inoculated sub-cutaneously with YDFR.SB3 vemurafenib-resistant and non-resistant melanoma cells. Each group consisted of 13 mice. Error bars depict standard error around the mean. Representative pictures of mice from each group 4, 5 and 6 weeks after inoculation are shown. Significance was evaluated using Student’s t-test for tumor volume of mice inoculated with vemurafenib resistant cells compared to mice inoculated with their non-resistant equivalents, *p ≤ 0.05. (B) Metastasis formation in the remote organs of mice after orthotopic inoculation with vemurafenib resistant or sensitive melanoma cells. The metastasis presence was determined by qRT-PCR analysis.
transmigrate through both brain and lung vascular endothelial cells much more efficiently than their sensitive counterparts \((p < 0.05)\). Table 3 summarizes these results: The in vitro ability of melanoma cells to adhere to brain and lung endothelia does not predict ability to trans-migrate through these endothelial layers.

The in vivo malignancy phenotype of vemurafenib-resistant melanoma cells

1×10^6 vemurafenib-sensitive or resistant micrometastatic melanoma cells (YDFR.SB3) were inoculated sub-dermally into nude mice. The formation of local tumors was followed up to 6 weeks following inoculation. Figure 6A shows that the tumors formed by vemurafenib-resistant cells were significantly larger than tumors formed by vemurafenib-sensitive cells.

We reported previously that melanoma cells inoculated sub-dermally into nude mice form spontaneous micro-metastasis [11]. The capacity of vemurafenib-sensitive or resistant melanoma cells to form micro-metastasis in the brain and in the lungs was determined by qRT-PCR analysis of human cells in these organs [11]. Figure 6B shows that whereas the vemurafenib-sensitive or resistant melanoma cells did not differ in their ability to form brain micro-metastasis, the capacity of the latter cells to form lung and liver micro-metastasis was increased.

We expected that an up-regulated expression of CCR4 by vemurafenib-resistant melanoma cells and an increased expression of the 2 CCR4 ligands in the brain of mice (as reported in section “Vemurafenib resistance of melanoma cells is associated with an altered gene expression profile”) will lead to an augmentation of the metastatic load in the brain of mice inoculated with these cells [47]. However the results summarized in this section indicated that the load of brain metastasis in mice bearing vemurafenib-resistant cells was not higher than that of mice bearing vemurafenib-sensitive cells. These results, while not discarding the possible involvement of the CCR4-CCL17/CCL22 axis in brain metastasis [47] support the findings that this axis is only one of several determinants of melanoma brain metastasis [11,12].

Discussion

A major finding of this study is that the in vitro induction of vemurafenib-resistance of melanoma cells is associated with an increased malignancy phenotype of these cells. It is not unlikely that selecting for drug resistance selects for tumor and metastasis initiating cells [51,52]. This possibility is supported by the findings that vemurafenib-resistant cells express higher levels of certain stem cell markers and that these cells also express higher levels of ABCG2 functioning as a key molecule in multidrug-resistance of cancer cells by mediating efflux of various compounds, from such cells [53,54]. Another important finding is that vemurafenib-resistant melanoma cells express novel characteristics such as gene products and functions that are not expressed by the drug-sensitive cells. This raises the possibility of attacking resistant cells by specifically targeting these markers.

Figure 7 shows a model summarizing the phenotypic and functional alterations that may accompany vemurafenib-resistant melanoma cells. Vemurafenib-resistant melanoma cells show down-regulation of E-cadherin and up-regulation of molecules involved

Fig. 7. The metastatic cascade is enhanced in vemurafenib-resistant melanoma cells. Vemurafenib-resistant melanoma cells express decreased E-cadherin levels and increased levels of CD271, CCR4, JARID1B, MMP-2 and ABCG2 compared to their non-resistant counterparts. The resistant cells also generate larger primary tumors in nude mice. Moreover, mRNA expression of CCL17 in brain and liver of mice inoculated with vemurafenib-resistant cells was elevated. The in vitro results showed increased trans-endothelial migration through both brain and lung vascular endothelial monolayer by vemurafenib-resistant cells and increased ability to adhere by these cells to lung, but not to brain endothelia. Correspondingly, the capacity of the vemurafenib-resistant melanoma cells to metastasize to the lungs, but not to the brain was increased, comparing to vemurafenib-sensitive melanoma cells.
in promotion of metastasis and genes coding for cancer stem cell markers such as MMP-2, CD271, CCR4, Fibronectin, JARD1B and ABCG2. The resistant cells also generate larger primary tumors in nude mice. The in vitro trans-endothelial migration by vemurafenib-resistant cells through both brain and lung vascular endothelial monolayer was significantly intensified. However, only the ability to adhere to lung, but not to brain endothelia was increased in vemurafenib-resistant cells. Correspondingly, the capacity of the vemurafenib-resistant melanoma cells to metastasize to the lungs, but not to the brain was increased, as compared to vemurafenib-sensitive melanoma cells.

Our results show that an efficient trans-endothelial migration in vitro does not predict metastasis formation in vivo. However, there is a correlation between the ability of melanoma cells to adhere to the endothelium of a specific organ and the capacity to form metastasis within the parenchyma of that organ.

In a recent publication, Paulitschke et al. [55] established a proteome signature of vemurafenib resistant melanoma cells. Some of the results reported above which were obtained with vemurafenib-resistant and sensitive cell variants that originated in the same parent population confirmed their findings. For example our results indicate that vemurafenib-resistant cells express an increased potential for metastasis, higher migratory and adherence functions, down-regulated levels of E-cadherin and higher levels of MMP-2 activity than vemurafenib-sensitive cells.

Numerous attempts are being undertaken to overcome drug resistance [56–59]. Here we suggest that intervening in the interactions between drug-resistant tumor cells and their specific microenvironment may constitute an additional approach to overcome drug resistance. It had been repeatedly shown that tumor-microenvironment interactions are a crucial component in the survival, propagation and progression of cancer cells [46,60,61]. It is therefore not unlikely that the microenvironment of drug-resistant cancer cells could be harnessed to overcome drug resistance. However such attempts have not been sufficiently explored. The observation that vemurafenib-resistant cells reprogram their microenvironment in a different manner from that of drug-sensitive cells suggests that different signaling pathways may operate in interactions between drug-sensitive and drug resistant cancer cells with their respective microenvironments. This may provide the possibility to selectively manipulate and target the signaling pathways of the resistant cells leading to their growth arrest or apoptosis.

Organ specificity of metastasis i.e. the differential biological behavior of cancer cells metastasizing to different organ microenvironments is another point that comes up when analyzing the results of this study. As demonstrated brain and lung metastasizing melanoma cells originating from the same melanoma may express differences in the expression of certain molecules and may interact differentially with microenvironmental components. These results support the notion that metastasis cannot be characterized in generic terms (such as metastatic melanoma or metastatic breast cancer) [62–64]. Characterizing the specific and unique molecular phenotype, biologic behavior, response to treatment etc. of cancer cells that metastasize to different organ sites is essential in the era of personalized medicine [60,65–69].

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Conflict of interest

None.

References
