Cancer Letters 361 (2015) 86–96

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

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

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ARTICLE INFO

Article history:
Received 2 February 2015
Received in revised form 19 February 2015
Accepted 19 February 2015

Keywords:
Vemurafenib resistance
Cancer stem cells
Metastatic microenvironment
Biomarkers

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.

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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

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http://dx.doi.org/10.1016/j.canlet.2015.02.041
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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% Hepes (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 Weksler 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% CO_2 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 incubation, 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. 5×10^5 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, BD Pharmingen™, San Jose, CA) or with corresponding isotype controls. After washing, the cells were incubated for 45 minutes at 4 °C with FITC-conjugated secondary antibody (1:50, Jackson Laboratories, Baltimore, MD). Following an additional wash the cells were suspended in 300 µl phosphate-buffered saline (PBSX1) containing 0.1%NaN_3. Antigen expression was determined using Becton Dickinson FACSort and CellQuest software. Baseline staining was obtained by labeling the cells with appropriate isotype 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 cDNA amplification, primers were designed based on the GenBank Nucleotide Database of the NCBI website (Table 1). Amplification reactions were performed with SYBR Green I (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 1
qRT-PCR oligonucleotide primers.

Gene name	Reaction specificity	Accession no.	Sequence
IL1R1	Human	NM_000877.3	S – 5′-GGACATTACTATTGCGT GGTAAG-3′ AS – 5′-TGCTTAAATATGGCTT GTGCAT-3′
JARID1B	Human	NM_006618.3	S – 5′-AGCAGACTGACCGAA GCTCA-3′ AS – 5′-AATTCCATCTCGCTT CCCTC-3′
CYR61	Human	NM_001554.4	S – 5′-CTTAACGAGGACTGCA GCAA-3′ AS – 5′-GTCTGCCCTCTGACT GAGCT-3′
MMP-1	Human	NM_002421.3	S – 5′-GTGCCTGATGTGGCTC AGTT-3′ AS – 5′-ATGGTCCACATCTGCT CTTG-3′
SPINK1	Human	NM_003122.4	S – 5′-CCAAGATATATGACCCT GTCTGT-3′ AS – 5′-TTCTCAGCAAGGCCC AGATT-3′
CEACAM1	Human	NM_001712.4	S – 5′-GTCACCTTGAATGTCAC CTATG-3′ AS – 5′-TGGACGGTAATAGGT GTCTG-3′
ABCG2	Human	NM_004827.2	S – 5′-TGGCTTAGACTCAAGCA CAGC-3′ AS – 5′-TCGTCCCTGCTTAGA CATCC-3′
Nestin	Human	NM_006617.1	S – 5′-AAGATGTCCCTCAGC CTGGA-3′ AS – 5′-GAGGGAAGTCTTGGA GCCAC-3′
Oct4	Human	NM_002701.5	S – 5′-GAAGGAGAAGCTGGAG CAAA-3′ AS – 5′-CATCGGCCTGTGTAT ATCCC-3′
E-cadherin	Human	NM_004360.3	S – 5′-CTCAGAAGACAGAAGAGA GACTG-3′ AS – 5′-GTCAGAGAGAAGACA GAAGACTC-3′
CCL17	Mouse	NM_011332.3	S – 5′-ATCAGGAAGTTGGTG AGCTG-3′ AS – 5′-CAGTCAGAAACACG ATGGCA-3′
CCL22	Mouse	NM_009137.2	S – 5′-CTCGTCCTTCTTGCT GTGGC-3′ AS – 5′-TCTTCCACATTGGCA CCATA-3′
IL-1β	Mouse	NM_008361.3	S – 5′-CAGGCAGGCAGTATC ACTCA-3′ AS – 5′-GAGGATGGGCTCTTC TTCAA-3′
TNF-α	Mouse	NM_013693.2	S – 5′-AGTTCTATGGCCCAG ACCCT-3′ AS – 5′-CACTTGGTGGTTTGC TACGA-3′
RS-9	Human	NM_001013.3	S – 5′-CGGAGACCCTTCGAGA AATCT-3′ AS – 5′-GCCCATACTCGCC GATCA-3′
β2M	Human	NM_004048.2	S – 5′-ATGTAAGCAGCATCAT GGAG-3′ AS – 5′-AAGCAAGCAGAATTTG GAAT-3′
β2M	Mouse	NM_009735.3	S – 5′-CTGGTCTTTCTGGTGC TTGT-3′ AS – 5′-GGCGTGAGTATACTTG AATTTGAG-3′

S, Sense; AS, Anti-sense.

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