Original Article
Targeting STAT3 restores BRAF inhibitor sensitivity through miR-759-3p in human cutaneous melanoma cells

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Abstract: Melanoma treatment with the BRAF V600E inhibitor vemurafenib provides therapeutic benefits, but the common emergence of drug resistance remains a challenge. To define molecular mechanisms of vemurafenib resistance, we generated A375-R, WM35-R cell lines resistant to vemurafenib and found that the p-STAT3 was upregulated in these cells in vitro and in vivo. In particular, activation of the STAT3 pathway was associated with vemurafenib resistance. Inhibition of this pathway with STAT3-specific siRNA (shRNA) sensitized A375-R, WM35-R cells to vemurafenib and induced apoptosis in vitro and in vivo. Moreover, targeting STAT3 induced expression of miR-579-3p and elicited resistance to vemurafenib. However, targeting miR-579-3p with anti- miR-579-3p reversed the resistance to vemurafenib. Together, these results indicate that STAT3-mediated downexpression of miR-579-3p caused resistance to vemurafenib. Our findings suggest novel approaches to overcome resistance to vemurafenib by combining vemurafenib with STAT3 silencing or miR-579-3p overexpression.

Keywords: Melanoma, BRAF V600E inhibitor, signal-transducer-and-activator-of-transcription 3, miR-579-3p

Introduction

Melanoma is the most aggressive form of skin cancer. Patients with metastatic disease have a median survival of less than one year, and outcomes are not improved with chemotherapy or immunotherapy [1]. BRAF somatic mutations that render BRAF constitutively active are observed in 50%-60% of malignant melanomas [2]. Thus, BRAF inhibitors have recently shown promise for the treatment of metastatic melanoma harboring such BRAF mutations [3]. PLX4032 (vemurafenib), a selective RAF inhibitor, showed an unprecedented antitumor response rate in patients with BRAF(V600E) [4] and conferred an overall survival benefit in a pivotal phase 3 study [5]. Unfortunately, most patients rapidly acquire resistance to vemurafenib [6]. In addition, although RAF inhibitor treatment has produced clinical responses in some patients, a subset of tumors is resistant to this agent, indicating the presence of intrinsic resistance or sensitivity to

the RAF inhibitor, highlighting the urgent need for new treatment strategies for BRAF(V600E)-induced melanoma. Besides the BRAF inhibitors, most anticancer drugs have the problem of drug resistance, which limits their effectiveness. Accordingly, understanding the molecular mechanisms of drug resistance is necessary to improve the effectiveness of cancer therapies.

Signal-transducer-and-activator-of-transcription 3 (STAT3), a transcription factor involved in cytokine signaling, participates in the regulation of cell cycle, apoptosis, cell invasion, and angiogenesis [7]. Targeting STAT3 signaling is an attractive therapeutic approach for most types of human cancers with constitutively activated STAT3, including melanoma [8-11]. Recentcell viability studies of B16F10 mouse melanoma cells have shown that the co-delivery of curcumin and STAT3 siRNA significantly inhibited the cancer cell growth compared to either liposomal curcumin or STAT3 siRNA alone

[12]. Bid et al. [13] have reported that dual targeting BRAF$^{(V600E)}$ signaling and STAT3 signaling may be effective in selumetinib-resistant tumors or may retard or prevent the onset of resistance in an in vivo model of childhood astrocytoma. A recent study has reported that STAT3 signaling is induced in BRAF-inhibitor-resistant cells, which made STAT3 phosphorylation increase in BRAF-resistant melanoma cells [14]. Liu et al. [15] found that activation of Stat3 induced resistance to vemurafenib in melanoma cells. In addition, Stat3 silencing inhibited the growth of melanoma cells with acquired resistance to vemurafenib. Furthermore, treatment with the Stat3 inhibitor, WP1066, resulted in growth inhibition in both vemurafenib-sensitive and -resistant melanoma cells. We therefore suggest that Stat3 inhibitor could enhance the sensitivity of the RAF inhibitor to melanoma.

MicroRNAs (miRNAs) are small, noncoding RNAs that modulate gene expression by mRNA silencing or degradation, which usually have pleiotropic effects because of their ability to target simultaneously multiple mRNAs. Changes in miRNAs expression levels are known to play a key role in various human cancers. Recently, microRNAs have been reported to play an important role in inducing resistance to anti-cancer drugs, and specific microRNA alterations occur selectively in cancer cells, rendering these cells resistant to various chemotherapeutic agents [16, 17]. Studies previously showed that expression levels of miR-579-3p decrease from nevi to stage III/IV melanoma samples and even further in cell lines resistant to BRAF/MEK inhibitors. And miR-579-3p ectopic expression impairs the establishment of drug resistance in human melanoma cells [18]. In this paper we identify a mechanism of drug resistance in BRAF mutated melanoma centered around a poorly characterized miRNA, miR-579-3p.

Materials and methods

Chemicals and reagents

Antibodies against pSTAT (Y705), BRAF, STAT3 and actin were obtained from Santa Cruz Biotechnology. Vemurafenib was obtained from Selleck Chemicals. TaqMan probes for miR-579-3p and RNU48 were purchased from Applied Biosystems.

Cell culture of melanoma cell lines

The human BRAFV600E mutated melanoma cell lines A375, WM35 were maintained in high-glucose RPMI 1640 supplemented with 5% fetal bovine serum. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The vemurafenib resistant A375, WM35 cells were generated by continuous treatment with increasing concentration of PLX4032 up to 10 µM for several months. The culture medium of the resistant cell lines was changed to a PLX4032 free medium 24 h before they were subjected into experiments.

miRNA transfection

Lipofectamine 2000 (Invitrogen, Carlsbad, California) was used for the transfection of anti-miR-579-3p. Forty-eight hours after transfection, cells were treated with PLX4032 for response and apoptosis analysis and real-time PCR.

siRNA transfection

20 nM siRNA (synthesized by biomers.net Germany) against Stat3 (sense: aacuucagacccgucaacaaa-dTdT; antisense: uuuguugacgggucugaag-dTdT) were reversely transfected using the riboxx FECT (riboxx Life Sciences) on 96 well plates. Therefore, the riboxxFECT (1:25) and siRNA were separately diluted using Opti-MEM (Life technologies) and the solutions were gently mixed in a 1:1 ratio. The solution was incubated for 15 min and, subsequently, 50 µl of the solution was transferred to 96 well cavities. Additionally, 2.5×10^3 cells resuspended in 50 µl culture medium were added to each well and incubated for 24 h at 37°C. The transfected cells were subsequently treated with up to 10 µM PLX4032 for 48 h and the viability was measured via the MUH assay.

Construct stable short hairpin RNA (shRNA)-expressing cell lines

Silencing of STAT3 in A375-R and WM35-R cell lines was achieved using lentiviral infection. Lentiviral plasmid vectors containing-control and STAT3 short hairpin RNA (shRNA; Open Biosystems) were co-transfected with packaging vectors and the lentivirus was produced in 293T cells via calcium transfection as the standard methods. STAT3 shRNA-containing vec-

tors were titrated, and A375-R and WM35-R was infected with 25 µL of viral supernatant/per milliliter of medium mixed with polybrene (4 µg/mL medium). Cells were then selected according to their resistance to puromycin (1-3 µg/mL). A stable A375-R and WM35-R cell line was developed thereafter.

Cell viability assay

Cells were seeded in 96 well plates overnight and treated with increasing concentrations of drugs or DMSO (vehicle). The DMSO concentrations were maintained at 0.02% in all wells. After 48 h incubation, cell viability was determined using Cell Titer Blue Cell Viability Assay (Promega, Madison, WI). Median inhibitory concentration (IC_{50}) values were determined using in house software (DIVISA) and plotted in dose-response curves.

FCM analysis of cell apoptosis

The STAT3 siRNA and control siRNA transfected A375-R and WM35-R cells were subsequently treated with up to 10 µM PLX4032 for 48 h. Next, cells were stained with FITC-conjugated Annexin V and propidium iodide (PI) as supplied by an apoptosis detection kit (KeyGEN, China). Cells were analyzed using a FACS Calibur flow cytometer (San Jose, CA, USA). Summit software (FlowJo, USA) was used to determine the number of apoptotic cells.

Western blot

An RIPA buffer was used for total protein extraction. 20 µg of protein was denatured under reducing conditions and separated on 10% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose by voltage gradient transfer. The resulting blots were blocked with 5% (w/v) non-fat dry milk in PBS + 0.1% (v/v) Tween-20. Specific proteins were detected with appropriate antibodies using SignalFireTM Elite ECL Reagent (Cell Signaling Technology). Immunoblotting antibodies were BRAF, STAT3, pSTAT3 and β-actin (I-19, Santa Cruz Biotechnology).

Real-time PCR

Total RNAs were extracted using Trizol reagent (Invitrogen) and reverse-transcribed using the Taqman microRNA Kit (Applied Biosystem). The Taqman MiRNA Assay was used for quantita-

tive PCR. Ct values were employed for quantification of transcripts. MiRNA expression was normalized to the values of RNU24.

Immunofluorescence staining and confocal microscopy

Immunofluorescence was performed using standard techniques. Briefly, cells grown on coverslip in a six-well plate were fixed with 4% paraformaldehyde in PBS. Cells were rinsed with 1 × PBS, permeabilized with PBS and 0.5% Triton X-100 for 5 minutes, and rinsed twice with 1 × PBS. Cells were incubated with 25 µl of anti-pSTAT3 antibody for 30 minutes, followed by rinsing once with 1 × PBS-Triton (0.1%) and twice with 1 × PBS. After incubation with 25 µl of Alexa 499 goat anti-mouse secondary antibody for 30 minutes and repeated rinsing, mounting media containing 4,6-diamidino-2-phenylindole (Sigma-Aldrich) was added to the slides. Cells were visualized with a Zeiss LSM 510 confocal microscope (Oberkochen, Germany).

STAT3 knockdown sensitizes melanoma cells towards vemurafenib treatment in vivo

This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health, and the study protocol was approved by the Committee on the Ethics of Animal Experiments of the affiliated hospital of Qingdao University. For the in vivo tumor growth assays, 1×10^6 vemurafenib resistant A375-R and WM35-R cells stably expressing STAT3 shRNA against STAT3 were subcutaneously injected into SCID mice. A375-R and WM35-R cells were as the controls. Treatments were started when tumors reached an average volume of about 0.1 cm³, which was around 7 days after A375-R and WM35-R cells were inoculated. Mice were randomly divided into control and treatment groups (n = 5 animals per group). The treatment group was administered daily injections of vemurafenib (i.p. 25 mg/kg) for 40 days. Tumor size was measured by calipers every 5 days. The tumor volume was calculated with the formula: length × width² × 0.52. In addition, after injections of vemurafenib, mice were injected intraperitoneally (i.p.) with 4 mg of luciferin (Promega) in PBS, and images were recorded by the IVIS Imaging System (Xenogen) 15 min after the injection. The biolu-
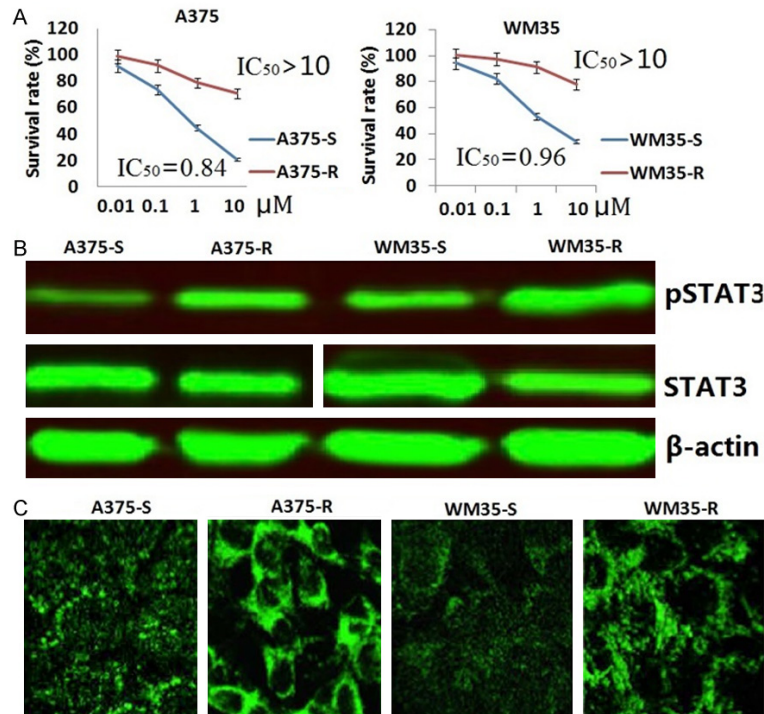
Figure 1. The STAT3 activity confers BRAF inhibitor resistance in BRAF mutant melanoma cells. A. Proliferation of A375-S, WM35-S and the vemurafenib-resistant A375-R and WM35-R cells in the presence of PLX4032 (PLX) for 24 hs. IC_{50} values are means (µM; n = 3) ± standard error of mean. B. Western blot for phosphorylated STAT3 and STAT3 in A375-S, WM35-S, A375-R and WM35-R cells. C. Phosphorylated STAT3 were analyzed by immunofluorescence staining and confocal microscopy.

minescence images were quantified by Living Image software (Xenogen).

Immunohistochemistry

Staining of pSTAT3 on tissue sections by immunohistochemistry (IHC) was carried out using standard techniques. pSTAT3 was detected with 3,3'diaminobenzidine (DAB). Sections were counterstained with Harris hematoxylin.

TUNEL staining

Fresh-frozen sections of tumor tissues from therapy experiments were stained by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL; green; Promega, Madison, WI) and counterstained with Hoechst 1:10,000. An apoptotic body was represented by green fluorescence. To quantify apoptotic cells, TUNEL-positive cells were calculated in 10 random fields at 200 × from five separate slides per group. Average values are presented.

Statistical analysis

For cell culture and animal experiments, data were analyzed using GraphPad Prism software for Windows 7 (version 4; GraphPad Software, San Diego, CA, USA) and the two-tailed Student's *t*-test was used to determine the significances of the results. Results are expressed as mean ± SD. *P* < 0.05 was considered statistically significant.

Results

STAT3 expression is increased in vemurafenib-resistant melanoma cell lines in vitro

To unravel the functional relevance of STAT3 expression on BRAFi resistance development we generated BRAFi resistant melanoma cell lines (A375-R and WM35-R) by continuous treatment with vemurafenib. The in vitro vemurafenib dose-response curves for the parental A375-S, WM35-S and the vemurafenib-resistant A375-R and WM35-R cells are shown in **Figure 1A**. From the survival curves, we computed the respective IC_{50} values in all the cells, and found the IC_{50} values were significantly increased in the vemurafenib-resistant A375-R (IC_{50} > 10 µM), WM35-R (IC_{50} > 10 µM) compared to the parental A375-S (IC_{50} = 0.84 µM), WM35-S cells (IC_{50} = 0.96 µM).

Western blot analyses of fractionated cell lysates confirmed that the resistance-acquired cell lines A375-R, WM35-R exhibit significantly increased pSTAT3 compared to the parental A375-S and WM35-S cells (**Figure 1B**). To further support that STAT3 was activated in the BRAFi resistant melanoma cell lines, we used immunofluorescence staining and confocal microscopy. As shown in **Figure 1C**, prominent expression of pSTAT3 in vemurafenib resistant melanoma A375-R, WM35-R cells in comparison to the sensitive parental cells (**Fig-**
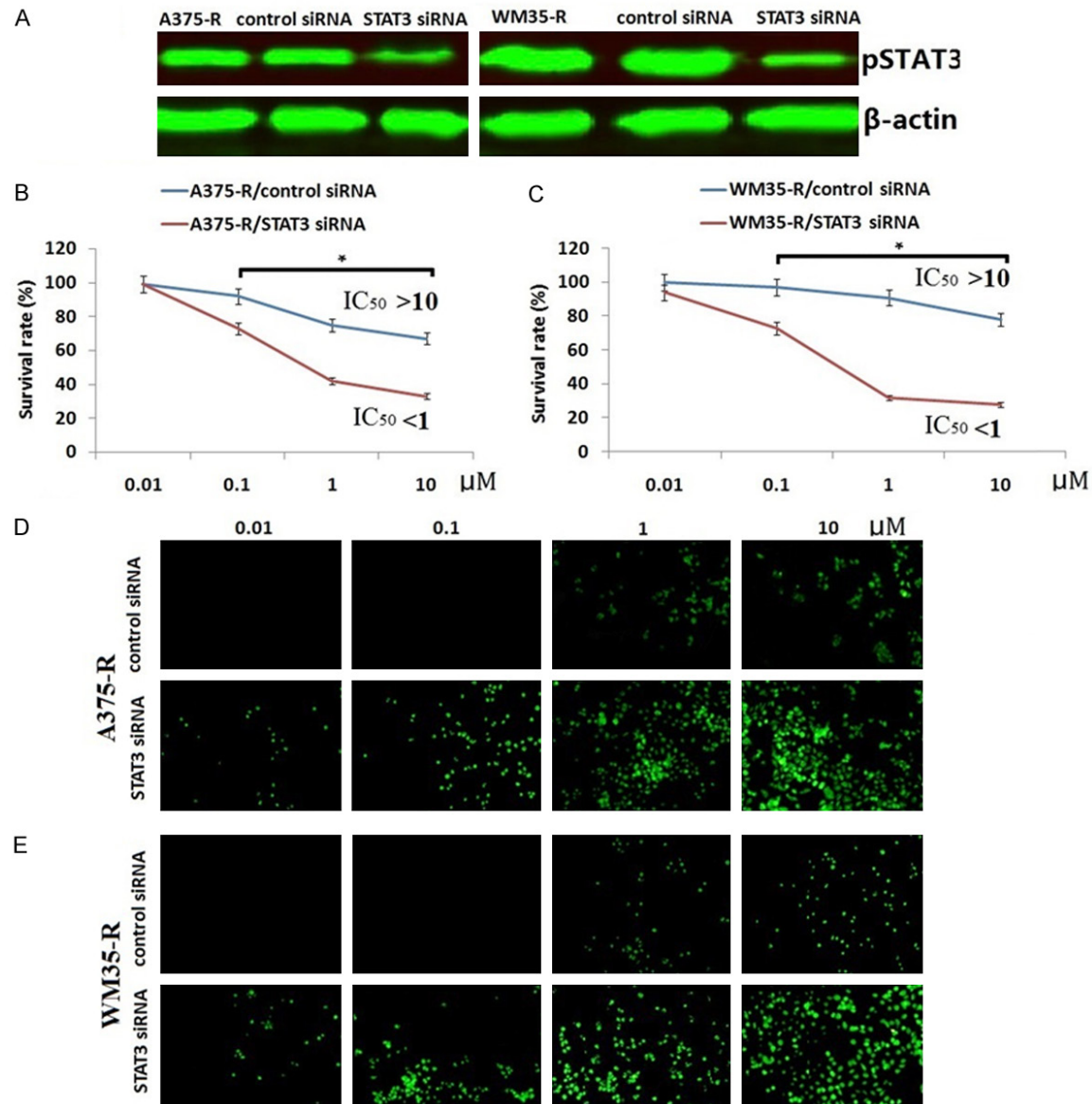
Figure 2. Targeting STAT3 restores the sensitivity of melanoma cells to PLX4032 treatment. A. Western blotting confirmed that STAT3 was inhibited with STAT3-specific siRNA. B. Knockdown of STAT3 sensitized A375-R cells to PLX4032 by cell viability. C. Knockdown of STAT3 sensitized WM35-R cells to PLX4032 by cell viability. D. The TUNEL assay in A375-R cells was performed using the One-Step TUNEL Apoptosis Assay Kit. E. The TUNEL assay in WM35-R cells was performed using the One-Step TUNEL Apoptosis Assay Kit. Images were captured by fluorescence micros-copy. The green color is indicative of TUNEL-positive cells.

ure 1C), indicating that vemurafenib resistant melanoma cells had high STAT3 signaling activity.

Targeting STAT3 regulates sensitivity of melanoma cells to PLX4032 treatment

To further test if the STAT3 pathway mediated BRAF inhibitor resistance, STAT3 was knocked down in resistant cell lines A375-R and WM35-R (**Figure 2A**). The results showed that knockdown of STAT3 in A375-R and WM35-R cells significantly sensitized the cells to PLX4032 by cell viability (**Figure 2B**, **2C**) and apoptosis assay (**Figure 2D, 2E**). Knockdown of STAT3 decreased IC_{50} values from > 10 μM to < 1 μM in both the A375-R and WM35-R cell lines. These results suggested that targeting STAT3 pathway increased PLX4032 sensitivity in melanoma cells.

Targeting STAT3 regulates sensitivity of melanoma cells to PLX4032 treatment through miR-579-3p

To further analyze how targeting STAT3 sensitized the resistant cells to PLX4032 treatment, we first detected the miR-579-3p expression in A375-S, WM35-S, A375-R, WM35-R and harboring the STAT3-specific shRNA A375-R and WM35-R cells by qRT-PCR. The results showed that miR-579-3p expression was significantly downregulated in A375-R and WM35-R cells compared to the A375-S and WM35-S cells (**Figure 3A**, **3B**). However, in the STAT3-specific shRNA A375-R and WM35-R cells, miR-579-3p expression was significantly upregulated compared to the untreated A375-R and WM35-R cells (**Figure 3A**, **3B**).

To further test whether miR-579-3p upregulated by STAT3 silencing plays a role in PLX4032 resistance, A375-R, WM35-R or cells harboring the STAT3-specific shRNA A375-R and WM35-R were transfected with an anti-miR-579-3p expression vector, then treated with PLX4032 and assessed by the TUNEL assay. The results showed that transfection of anti-miR-579-3p in A375-R, WM35-R or cells harboring the STAT3-specific shRNA A375-R and WM35-R decreased miR-579-3p expression (**Figure 3A**, **3B**) and induced resistance to PLX4032 (**Figure 3C**, **3D**).

Targeting STAT3 sensitizes melanoma cells towards vemurafenib treatment in vivo

In the next step, we analyzed whether STAT3 is functionally involved in the resistance mechanisms to BRAF inhibitors in vivo. The A375-R or WM35-R cells and resistant A375-R or WM35-R cells harboring the STAT3-specific shRNA were subcutaneously injected into the mice, until a median tumor volume of approximately 100 mm³ was reached. Mice were randomized into four different therapy groups: 1. A375-R or WM35-R cells did not receive any further therapy; 2. A375-R or WM35-R cells received daily injections of vemurafenib (i.p. 25 mg/kg); 3. A375-R or WM35-R cells harboring the STAT3-specific shRNA did not receive any further therapy; 4. A375-R or WM35-R cells harboring the STAT3-specific shRNA received daily injections of vemurafenib (i.p. 25 mg/kg). The therapy group of mice treated with vemurafenib alone or cells harboring the STAT3-specific shRNA

alone exhibited slow tumor growth as detected by caliper measurements compared to the untreated A375-R or WM35-R cells (**Figure 4A**, **4B**). In vivo imaging showed that A375-R or WM35-R cells harboring the STAT3-specific shRNA received daily injections of vemurafenib (i.p. 25 mg/kg) dramatically decreased A375-R or WM35-R tumor size 40 d after treatment (**Figure 4C**, **4D**). Immunohistochemical staining further confirmed that p-STAT3 was significantly inhibited in animal tumor tissue specimens treated with vemurafenib, shRNA STAT3 or both compared with that treated with the vehicle, indicating that STAT3 can be effectively inhibited with the combination treatment in vivo (**Figure 4E**).

To characterize the mechanism by which targeting STAT3 induces BRAF inhibitor resistance to A375-R or WM35-R cells, the effect of STAT3 on miR-579-3p expression was determined by qRT-PCR. The results showed a significant decrease of miR-579-3p levels in A375-R or WM35-R cells that did not receive any further therapy, and a significant increase in vemurafenib or shRNA STAT3 groups alone, and especially increased in the combined groups (**Figure 4E**, **4F**). The TUNEL assay showed that the number of apoptotic cells significantly increased in vemurafenib or shRNA STAT3 groups alone, and especially increased in the combined groups (**Figure 4G**).

Discussion

Acquired resistance to the second generation BRAF inhibitor vemurafenib is limiting the benefits of long-term, targeted therapy for patients with malignant melanoma that harbor the V600E BRAF mutation [24]. Since many resistance mechanisms have been described, most of them causing a hyperactivation of the MAPK- or PI3K/AKT signaling pathways, one potential strategy to overcome BRAFi resistance in melanoma cells would be to target important common signaling nodes [25, 26]. Known factors that cause secondary resistance include the overexpression of receptor tyrosine kinases (RTKs), alternative splicing of BRAF or the occurrence of novel mutations in MEK1 or NRAS [25].

The STAT3 pathway has been shown to be activated in many types of cancer and is associated with cancer transformation, angiogenesis,

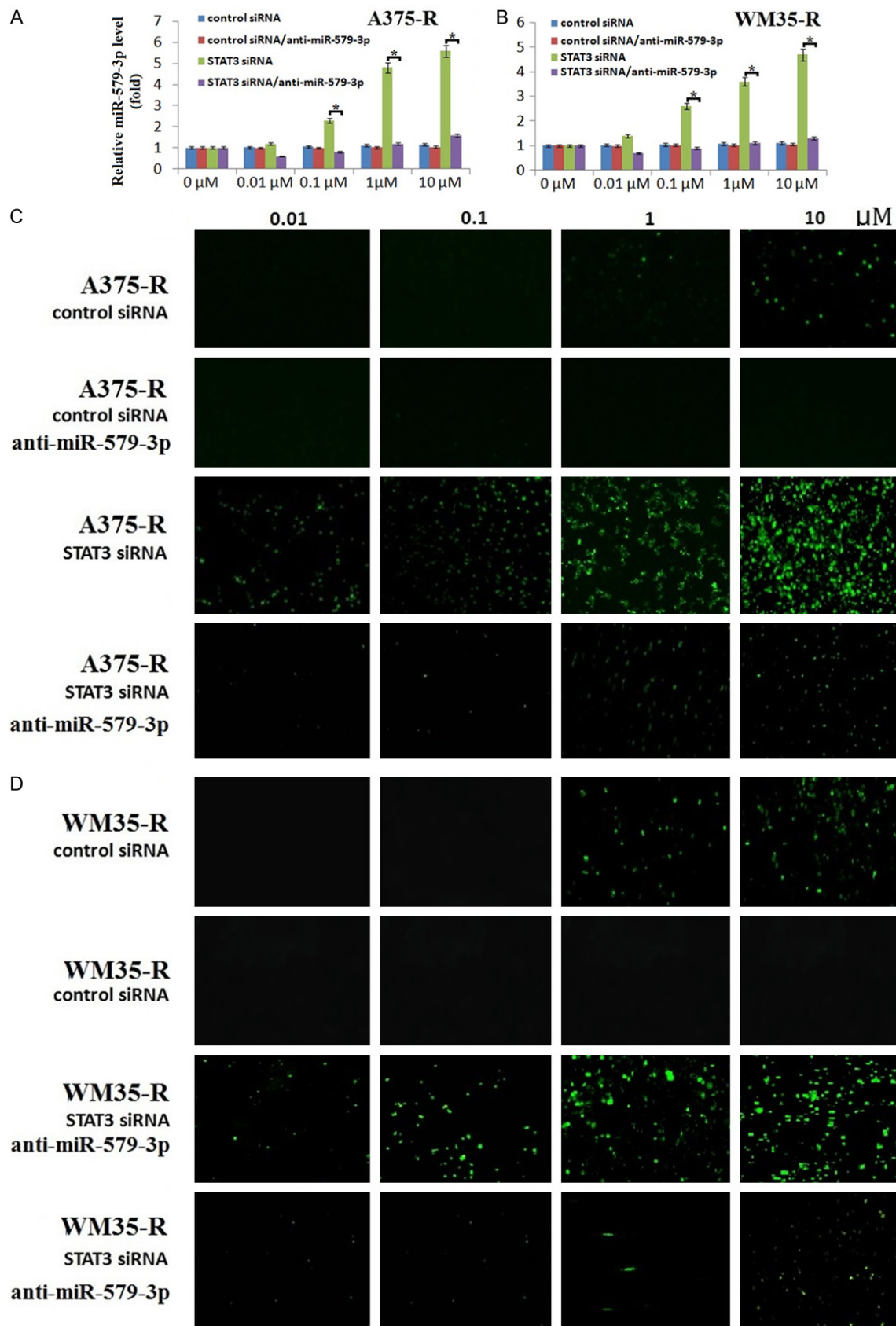# Targeting STAT3 sensitizes cutaneous melanoma cells to BRAF inhibitor

Figure 3. Targeting STAT3 increased sensitivity of melanoma cells to PLX4032 treatment through miR-579-3p up-regulation. A, B. miR-579-3p expression was determined by qRT-PCR in A375-R and WM35-R cells following different treatment. C, D. Cell apoptosis was detected by TUNEL staining in A375-R and WM35-R cells following different treatment. *P < 0.01.
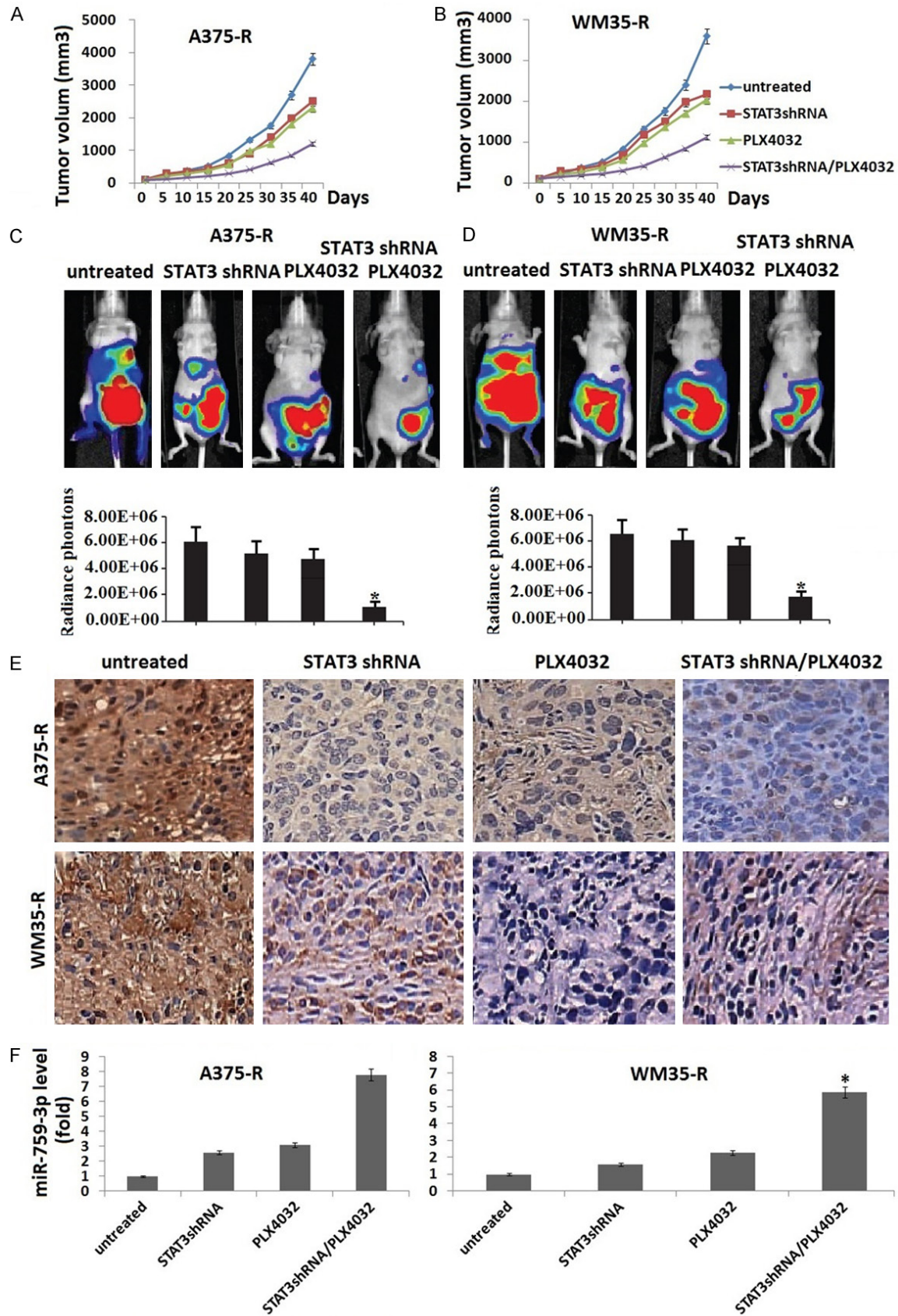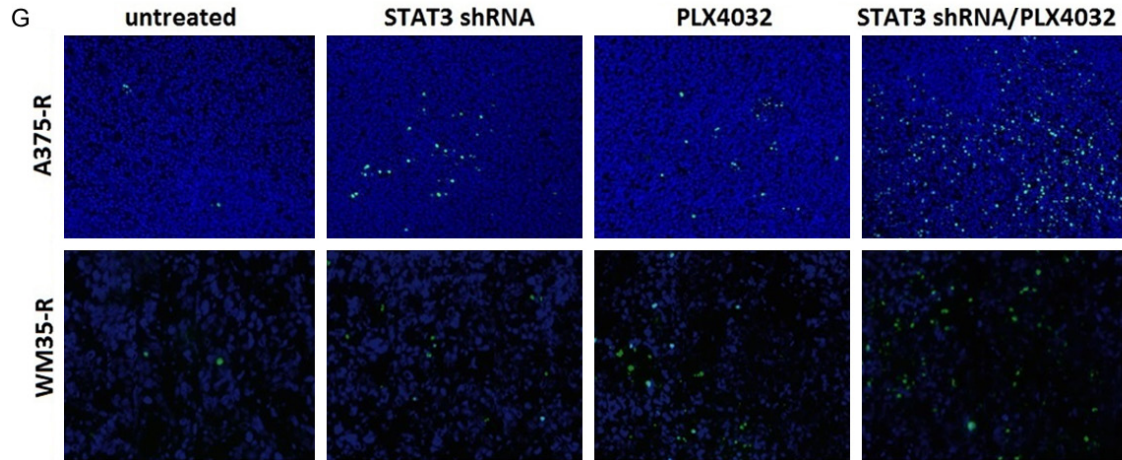
Figure 4. STAT3 inhibit sensitized A375-R or WM35-R cell to vemurafenib in vivo. A, B. Combination with vemurafenib and STAT3 shRNA enhanced antitumor activity in vivo. C, D. Representative in vivo luciferase images of A375-R or WM35-R xenograft nude mice at day 40 after vemurafenib treatment. E. Immunohistochemical staining for p-STAT3 (Y705) with animal tumor tissue specimens; F. miR-579-3p expression was determined by qRT-PCR in animal tumor tissue specimens; G. Cell apoptosis was detected by TUNEL staining in animal tumor tissue specimens.

invasion, and metastasis and with immune system suppression [27]. Our present results provide additional insight into the complexity of the vemurafenib resistance phenotype of melanoma and advance the basis upon which such resistance may be overcome. The first major finding in this work is that STAT3 was activated in the vemurafenib-resistant melanomas cells *in vitro* and *in vivo*. We clearly show that reduced STAT3 levels by targeting STAT3 can strongly enhance the growth inhibitory and apoptotic effects of vemurafenib in these vemurafenib-resistant cells. This suggests that STAT3 plays a role in a subset of melanoma cells with acquired resistance towards vemurafenib, and knocking down STAT3 inhibits the development of resistance to vemurafenib.

Although targeting STAT3 combined with the BRAF V600E inhibitor vemurafenib provides therapeutic benefits, the underlying mechanisms remain unknown. Small noncoding microRNAs (miRNAs) have been confirmed to regulate the expression of target mRNAs by repressing their translation [28]. A growing body of evidence shows that dysregulation of miRNA expression contributes to the acquisition of drug resistance by cancer cells [29]. Nevertheless, relatively few studies have explored the roles of miRNAs in resistance to BRAF inhibitor therapy, although several studies identified miRNAs that alter some of the oncogenic factors in melanoma cells [30].

miR-579-3p has been found to act not only as an oncosuppressor whose downregulation is linked to the progression of metastatic melanoma, but also as a factor contributing to the development of drug resistance, and its overexpression was able to reduce melanoma cell growth both individually and in combination with the BRAF inhibitor vemurafenib [31]. In the present study, we found that targeting STAT3 upregulated miR-579-3p in vemurafenib-resistant melanomas cells *in vitro* and *in vivo*. *However,* targeting miR-579-3p expression was able to revert drug resistance to a BRAF inhibitor in combination with STAT3 silencing and, more importantly, to reverse the establishment of acquired drug resistance, suggesting that STAT3 regulates miR-579-3p, which affected the sensitivity of melanoma cells to the BRAF inhibitor vemurafenib. In this regard it will be important in the future to investigate in detail the mechanisms responsible for miR-579-3p expression and how these are affected during melanoma progression and the development of drug resistance. miR-579-3p could be regulated by specific transcription factors, whose expression is altered during the development of drug resistance.

In summary, we found that the activation of STAT3 pathways mediates vemurafenib resistance. We further found that STAT3-mediated vemurafenib resistance occurs through the inhibition of miR-579-3p. Our results suggest

that the combination of STAT3 silencing or miR-579-3p overexpression may be productive approaches for melanoma therapy.

Disclosure of conflict of interest

None.

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References

[1] Tsao H, Atkins MB, Sober AJ. Management of cutaneous melanoma. N Engl J Med 2004; 351: 998-1012.

[2] Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S. Mutations of the BRAF gene in human cancer. Nature 2002; 417: 949-954.

[3] Holderfield M, Deuker MM, McMahon M. Targeting RAF kinases for cancer therapy: BRAF-mutated melanoma and beyond. Nat Rev Cancer 2014; 14: 455-467.

[4] Flaherty KT. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med 2010; 363: 809-819.

[5] Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA; BRIM-3 Study Group. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med 2011; 364: 2507-2516.

[6] Nazarian R. Melanomas acquire resistance to B-RAF (V600E) inhibition by RTK or N-RAS upregulation. Nature 2010; 468: 973-977.

[7] Yu H, Jove R. The STATs of cancer--new molecular targets come of age. Nat Rev Cancer 2004; 4: 97-105.

[8] Deng J, Grande F, Neamati N. Small molecule inhibitors of Stat3 signaling pathway. Curr Cancer Drug Targets 2007; 7: 91-107.

[9] Lin L, Deangelis S, Foust E, Fuchs J, Li C, Li PK, Schwartz EB, Lesinski GB, Benson D, Lü J, Hoyt D, Lin J. A novel small molecule inhibits STAT3 phosphorylation and DNAbinding activity and exhibits potent growth suppressive ac-

tivity inhuman cancer cells. Mol Cancer 2017; 9: 217.

[10] Lin L, Hutzen B, Li PK, Ball S, Zuo M, DeAngelis S, Foust E, Sobo M, Friedman L, Bhasin D, Cen L, Li C, Lin J. A novel small molecule, LLL12, inhibits STAT3 phosphorylation and activities and exhibits potent growth-suppressive activity in human cancer cells. Neoplasia 2010; 12: 39-50.

[11] Momtaz S, Niaz K, Maqbool F, Abdollahi M, Rastrelli L, Nabavi SM. STAT3 targeting by polyphenols: novel therapeutic strategy for melanoma. Biofactors 2017; 43: 347-370.

[12] Jose A, Labala S, Ninave KM, Gade SK, Venuganti VVK. Effective skin cancer treatment by topical co-delivery of curcumin and STAT3 siRNA using cationic liposomes. AAPS PharmSciTech 2018; 19: 166-175.

[13] Bid HK, Kibler A, Phelps DA, Manap S, Xiao L, Lin J, Capper D, Oswald D, Geier B, DeWire M, Smith PD, Kurmasheva RT, Mo X, Fernandez S, Houghton PJ. Development, characterization, and reversal of acquired resistance to the MEK1 inhibitor selumetinib (AZD6244) in an in vivo model of childhood astrocytoma. Clin Cancer Res 2013; 19: 6716-6729.

[14] Vultur A, Villanueva J, Krepler C, Rajan G, Chen Q, Xiao M, Li L, Gimotty PA, Wilson M, Hayden J, Keeney F, Nathanson KL, Herlyn M. MEK inhibition affects STAT3 signaling and invasion in human melanoma cell lines. Oncogene 2014; 33: 1850-1861.

[15] Liu F, Cao J, Wu J, Sullivan K, Shen J, Ryu B, Xu Z, Wei W, Cui R. Stat3-targeted therapies overcome the acquired resistance to vemurafenib in melanomas. J Invest Dermatol 2013; 133: 2041-2049.

[16] Abdi J, Jian H, Chang H. Role of micro-RNAs in drug resistance of multiple myeloma. Oncotarget 2016; 7: 60723-60735.

[17] Li X, Shen JK, Hornicek FJ, Xiao T, Duan Z. Noncoding RNA in drug resistant sarcoma. Oncotarget 2017; 8: 69086-69104.

[18] Fattore L, Mancini R, Acunzo M, Romano G, Laganà A, Pisanu ME, Malpicci D, Madonna G, Mallardo D, Capone M, Fulciniti F, Mazzucchelli L, Botti G, Croce CM, Ascierto PA, Ciliberto G. miR-579-3p controls melanoma progression and resistance to target therapy. Proc Natl Acad Sci U S A 2016; 113: E5005-13.

[19] Peart MJ, Smyth GK, van Laar RK, Bowtell DD, Richon VM, Marks PA, Holloway AJ, Johnstone RW. Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. Proc Natl Acad Sci U S A 2005; 102: 3697-702.

[20] Jazirehi AR. Regulation of apoptosis-associated genes by histone deacetylase inhibitors: implications in cancer therapy. Anticancer Drugs 2010; 21: 805-813.

[21] Vo DD, Prins RM, Begley JL, Donahue TR, Morris LF, Bruhn KW, de la Rocha P, Yang MY, Mok S, Garban HJ, Craft N, Economou JS, Marincola FM, Wang E, Ribas A. Enhanced antitumor activity induced by adoptive T-cell transfer and adjunctive use of the histone deacetylase inhibitor LAQ824. Cancer Res 2009; 69: 8693-9.

[22] Carson R, Celtikci B, Fenning C, Javadi A, Crawford N, Carbonell LP, Lawler M, Longley DB, Johnston PG, Van Schaeybroeck S. HDAC inhibition overcomes acute resistance to mek inhibition in BRAF-mutant colorectal cancer by downregulation of c-FLIPL. Clin Cancer Res 2015; 21: 3230-3240.

[23] Lai F, Guo ST, Jin L, Jiang CC, Wang CY, Croft A, Chi MN, Tseng HY, Farrelly M, Atmadibrata B, Norman J, Liu T, Hersey P, Zhang XD. Cotargeting histone deacetylases and oncogenic BRAF synergistically kills human melanoma cells by necrosis independently of RIPK1 and RIPK3. Cell Death Dis 2013; 4: e655.

[24] Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, Kehoe SM, Johannessen CM, Macconaill LE, Hahn WC, Meyerson M, Garraway LA. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. J Clin Oncol 2011; 29: 3085-3096.

[25] Lim SY, Menzies AM, Rizos H. Mechanisms and strategies to overcome resistance to molecularly targeted therapy for melanoma. Cancer 2017; 123: 2118-2129.

[26] Graziani G, Artuso S, De Luca A, Muzi A, Rotili D, Scimeca M, Atzori MG, Ceci C, Mai A, Leonetti C, Levati L, Bonanno E, Tentori L, Caccuri AM. A new water soluble MAPK activator exerts antitumor activity in melanoma cells resistant to the BRAF inhibitor vemurafenib. Biochem Pharmacol 2015; 95: 16-27.

[27] Darnell JE. Validating Stat3 in cancer therapy. Nat Med 2005; 11: 595-596.

[28] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-97.

[29] Ma J, Dong C, Ji C. MicroRNA and drug resistance. Cancer Gene Ther 2010; 17: 523-531.

[30] Pinto R, Strippoli S, De Summa S, Albano A, Azzariti A, Guida G. MicroRNA expression in BRAF-mutated and wildtype metastatic melanoma and its correlation with response duration to BRAF inhibitors. Expert Opin Ther Targets 2015; 19: 1027-1035.

[31] Fattore L, Mancini R, Acunzo M, Romano G, Laganà A, Pisanu ME, Malpicci D, Madonna G, Mallardo D, Capone M, Fulciniti F, Mazzucchelli L, Botti G, Croce CM, Ascierto PA, Ciliberto G. miR-579-3p controls melanoma progression and resistance to target therapy. Proc Natl Acad Sci U S A 2016; 113: E5005-13.