



Sorafenib sensitizes melanoma cells to vemurafenib through ferroptosis

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Background: *BRAF* gene mutation causes melanoma patients to develop drug resistance after 8–9 months BRAF inhibitors treatment. Therefore, overcoming BRAF inhibitor resistance has important implications for improving patient survival. Sorafenib directly inhibits tumor cell proliferation by blocking the RAF/MEK/ERK-mediated cell signaling pathway. It remains unknown that whether the combination of sorafenib with vemurafenib could sensitize melanoma cells to vemurafenib, and the underlying mechanism needs to be clarified.

Methods: Vemurafenib resistant melanoma cells A375/Vem and SK-Mel-28/Vem were established by exposing to a series of concentration of vemurafenib. Cell viability was measured when A375 and SK-Mel-28 cells treated with vemurafenib or combined with sorafenib. Meanwhile the levels of Iron, GSH, MDA and reactive oxygen species (ROS) were detected. Finally we examined that whether sorafenib sensitizes melanoma cells to vemurafenib through ferroptosis.

Results: We found that sorafenib sensitized melanoma cells to vemurafenib. Sorafenib treatment did not significantly alter the production of ROS and the content of iron, GSH and MDA in vemurafenib resistant cells, but cotreatment of sorafenib and vemurafenib dramatically upregulated ROS production, MDA and iron, but decreased GSH concentration. Interestingly, sorafenib strongly promoted vemurafenib-induced cell death, which was blocked by lipid peroxidation inhibitors ferrostatin-1 but not ZVAD-FMK or necrosulfonamid.

Conclusions: Sorafenib sensitized melanoma cells to vemurafenib by increasing ROS production through ferroptosis. Our study reveals that the combination of sorafenib may provide a novel strategy of vemurafenib resistant melanoma therapy.

Keywords: BRAF inhibitors; sorafenib; ferroptosis; reactive oxygen species (ROS); resistance

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Introduction

Melanoma (MM) is a kind of malignant tumor which originates from melanocytes, and accounts for 3–5% of skin malignant tumors. It has high malignancy and is prone to invasion and metastasis (1), resulting high mortality

rate (2). BRAF kinase is the most important subtype of the Raf family in the MAPK signaling pathway (3). BRAF overexpression or mutation is closely related to the occurrence of melanoma (4). More than 60% melanomas have BRAF^{V600} mutation, which may encodes a constitutively

activated BRAF protein and induces the aberrant mitogen-activated protein kinase (MAPK) signaling (5). Currently, the BRAF inhibitor vemurafenib is approved for melanoma treatment, which inhibits the kinase activity mainly by competitively binding to the ATP binding site (6). Although BRAF inhibitor has a rapid efficacy and good response rate, most melanoma patients develop tumor progression and resistance after 8–9 months of treatments resulting from reactivation of the MAPK pathway and other signaling pathways (7). It seriously affects the survival, prognosis of patients. Therefore, overcoming BRAF inhibitor resistance has important implications for improving patient survival.

Ferroptosis is a recently recognized form of regulated cell death driven by small molecules or conditions that induce lipid-based reactive oxygen species (ROS) accumulation, and this iron-dependent form of cell death is morphologically and genetically distinct from apoptosis, necroptosis, and autophagy (8,9). Ferroptosis is characterized by cell volume shrinkage and increased mitochondrial membranes and is mediated by iron-dependent lipid peroxide accumulation (10). The ferroptosis-inducing compounds, such as erastin and Ras selective lethal 3 (RSL3) could inactivate cellular glutathione (GSH)-dependent antioxidant defenses, leading to the accumulation of toxic lipid ROS (11,12). Glutathione peroxidase 4 (GPX4) is a key antioxidant enzyme that is responsible for removing lipid hydroperoxides within biological membranes (8). Once GPX4 inactivation, GSH will lose ability in removing the local peroxidase reaction, which eventually lead to a lipid ROS accumulation and ferroptosis.

Sorafenib is a novel multi-target anti-tumor drug, which acts on both tumor cells and tumor blood vessels (13). Sorafenib directly inhibits tumor cell proliferation by blocking the RAF/MEK/ERK-mediated cell signaling pathway, and also inhibits VEGF and platelet-derived growth factor (PDGF) receptors to block the formation of tumor neovascularization, indirectly inhibiting the growth of tumor cells (14). In addition, sorafenib-mediated cell death was associated with accumulated ROS (15). Sorafenib combination with triterpenoid oleanolic acid has highly synergistic effects on cell death and suppresses long-term clonogenic survival (16). Sorafenib stimulates significant intracellular ROS production, leading to ferroptosis in hepatocellular carcinoma (17). In melanoma, Tsoi *et al.* proposed that ferroptosis-inducing drugs present an orthogonal therapeutic approach to enhance the efficacy of targeted and immune therapies (18). Notably, It's reported that there is a synergistic effects of vemurafenib and

sorafenib in vemurafenib-resistant A375 cells (19). Thus, we hypothesized that the combination of sorafenib with vemurafenib could sensitize melanoma cells to vemurafenib via ferroptosis.

In this study, we aim to investigate whether sorafenib could sensitize melanoma cells to vemurafenib. We found that sorafenib sensitized melanoma cells to vemurafenib by increasing ROS production through ferroptosis. Our study reveals that sorafenib may be a novel strategy for overcoming vemurafenib resistance in melanoma.

Methods

Reagents

Vemurafenib (cat No. S1267), sorafenib (cat No. S1040), ferroptosis inhibitor Ferrostatin-1 (Fer-1) (cat no. S7243), caspase inhibitor Z-VAD-FMK (cat No. S7023) and MLKL inhibitor Necrosulfonamide (cat No. S8251) were purchased from Selleck (Shanghai, China).

Cell culture and treatment

Two melanoma cell lines, A375 and SK-Mel-28, were purchased from National Infrastructure of Cell Line Resource (Beijing, China). All cells were cultured in RP1640 medium supplemented with 10% fetal bovine serum under 5% CO₂ and 37 °C conditions for further study.

The resistant MM cells (A375/Vem and SK-Mel-28/Vem) were established in our lab by stepwise exposure to escalating concentrations of Vemurafenib. Briefly, A375 and SK-Mel-28 cells were treated with 0.5–6.0 μM Vemurafenib every 3 days for 6 weeks. By that analogy, the final concentration reached to 6 μM.

For the mechanism investigation, A375 and SK-Mel-28 cells were exposed to ferroptosis inhibitor Ferrostatin-1 (Fer-1) (2 μM dissolve in DMSO), caspase inhibitor Z-VAD-FMK (50 μM dissolve in DMSO) and MLKL inhibitor Necrosulfonamide (1 μM dissolve in DMSO) for 12 hours.

Proliferation measurement

Cell Counting Kit-8 (CCK-8) (Beyotime, Hangzhou, China) was used to determine cell proliferation according to the manufacturer's instructions. Briefly, five-thousand cells were seeded in each 96-well plate and treated with the indicated drugs. After that, CCK-8 reagents (10 μL) were

added to medium. After 1 h incubation, a microplate reader was used to detect the OD value at 570 nm.

Biochemistry detection

Commercial kits (cat No. MAK025, cat No. CS0260, cat No. MAK085, and cat No. MAK143 were purchased from Merck (Shanghai, China) were used to detect the iron, GSH, MDA and ROS concentrations according to the manufacturer' instructions, respectively.

Western blot

After treatment, the cells were collected and used for protein extraction using radio immunoprecipitation assay (RIPA) lysis buffer. Fifty micrograms proteins were separated by 10% SDS/PAG and transferred onto PVDF membranes. The membranes were rinsed with PBS and immunoblotted with primary antibodies (Rabbit mAb anti-NRF2 antibody, cat No. 12721, diluted at 1:1,000, from Cell Signaling Technology; mouse mAb anti-GPX4 antibody, cat No. ab16739, diluted at 1:2,000, from Abcam). Appropriate peroxidase-conjugated secondary antibody was added into the membranes and incubated for 2 h. A Bio-Rad (Hercules, CA) imaging system was used to detect the specific bands.

Statistical analysis

Statistical analyses were processed by using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Each experiment was executed at least three times, and the data are presented as means \pm SD. The difference between the two groups was processed by Student's *t*-test. The differences among multiple groups was evaluated by one-way ANOVA with Bonferroni test. Statistical significance was identified by $P < 0.05$.

Results

The combination of vemurafenib and sorafenib inhibits melanoma cell viability

To investigate whether sorafenib could overcome vemurafenib resistance in melanoma cells, vemurafenib resistant MM cells (A375/Vem and SK-Mel-28/Vem) were established. The morphological changes were obvious. Resistant cells were larger and flatter than their native

counterparts, with abundant cytoplasm and alterations of cytoplasmic membrane shape (*Figure 1A*). A375/Vem and SK-Mel-28/Vem and their parental cells were exposed to a series of concentrations of vemurafenib. The cell viability was significantly decreased in all cells. However, the rates of descent in A375/Vem and SK-Mel-28/Vem cells were significantly lower than that of in their parental cells (*Figure 1B*), implying that A375/Vem and SK-Mel-28/Vem cells obtain a certain degree of resistance. In addition, the IC₅₀ of A375/Vem (4 μ M) and SK-Mel-28/Vem (7 μ M) were significantly higher than in A375 (1 μ M) and SK-Mel-28 (2 μ M) (*Figure 1C*). Moreover, the resistant cells maintained a highly active MAPK pathway in the presence of vemurafenib (*Figure S1*). Then, BRAF-wild-type cells WM35, BRAF-mutation-type cells A375, the resistant cells A375/Vem and SK-Mel-28/Vem cells, were treated with vemurafenib or combined with sorafenib and measured the cell viability. We found that vemurafenib treatment significantly inhibits MM cells viability. This effect was also observed in sorafenib treated cells (*Figures 2,S2*). Importantly, we observed that the combination of vemurafenib with sorafenib showed a synergistic effect in inhibiting cell viability in a dose-dependent manner (*Figures 2,S2*).

The effects of sorafenib on oxidative stress in vemurafenib resistant melanoma cells

We further investigate the mechanism of sorafenib sensitizing MM cells to vemurafenib. Ferroptosis is a new form of programmed cell death characterized with the iron-dependent lipid peroxides, the iron-dependent ROS accumulation triggers ferroptosis mainly by inactivation of GPX4 or GSH deficiency (10). So we treated the vemurafenib resistant cells with either vemurafenib or together with sorafenib (10 μ M) for 48 h, then detected the level of iron and lipid oxidation related molecules. As shown in *Figures 3* and *4*, the concentrations of Iron, GSH, MDA (an end product of lipid peroxidation), and ROS in cells treated with vemurafenib or sorafenib alone have no significant changes compared with the control cells. However, the combination of vemurafenib and sorafenib dramatically increased the concentration of Iron, MDA, and ROS, but reduced GSH in vemurafenib resistant cells compared with the cells treated with vemurafenib and sorafenib alone. In RAF-wild-type cells WM35 and BRAF-mutation-type cells A375, the levels of Iron, GSH, MDA, and ROS were increased, but GSH were reduced

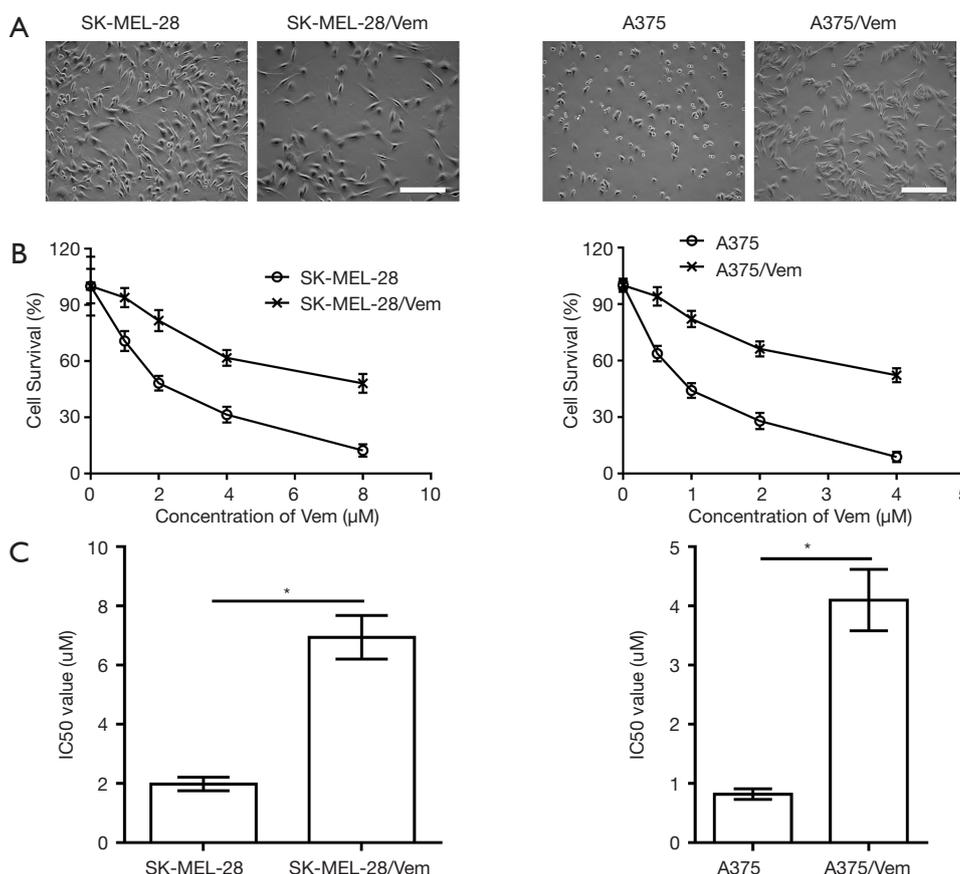


Figure 1 IC50 in vemurafenib-resistant MM cells. Vemurafenib-resistant MM cell lines were established. The cells were treated with a series of concentrations of vemurafenib (Vem). (A) The photographs obtained by light microscope showed the morphology changes of the cells. Bar =200 µm. (B) CCK-8 assay was performed to measure the cell viability in vemurafenib-resistant SK-Mel-28 cells and A375 cells after 48 h-treatment. (C) IC50 was calculated for vemurafenib-resistant SK-Mel-28 cells and A375 cells and their parental cells. MM, melanoma. *, P<0.05. and MLKL inhibitor Necrosulfonamide for 12 hours. CCK-8 assay was performed to measure the cell viability in vemurafenib-resistant A375 cells (A) and SK-Mel-28 cells (B). *, P<0.05.

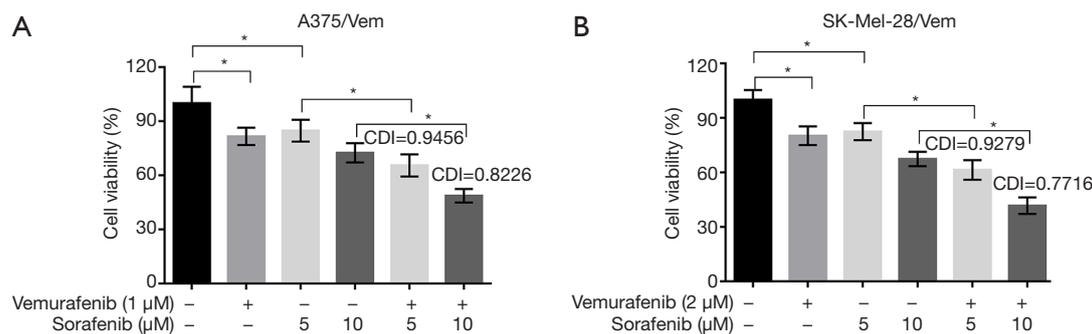


Figure 2 Sorafenib sensitizes MM cells to vemurafenib. The vemurafenib-resistant cells were treated with vemurafenib or sorafenib alone, or both of them for 48 hours. CCK-8 assay was performed to measure the cell viability in vemurafenib-resistant A375 cells (A) and SK-Mel-28 cells (B) after 48 h-treatment. *, P<0.05. The coefficient of drug interaction (CDI) was calculated as follows: $CDI = AB/(A \times B)$. According to the absorbance of each group, AB is the ratio of the combination groups to control group; A or B is the ratio of the single agent group to control group. Thus, CDI value <1, =1 or >1 indicates that the drugs are synergistic, additive or antagonistic, respectively. MM, melanoma.

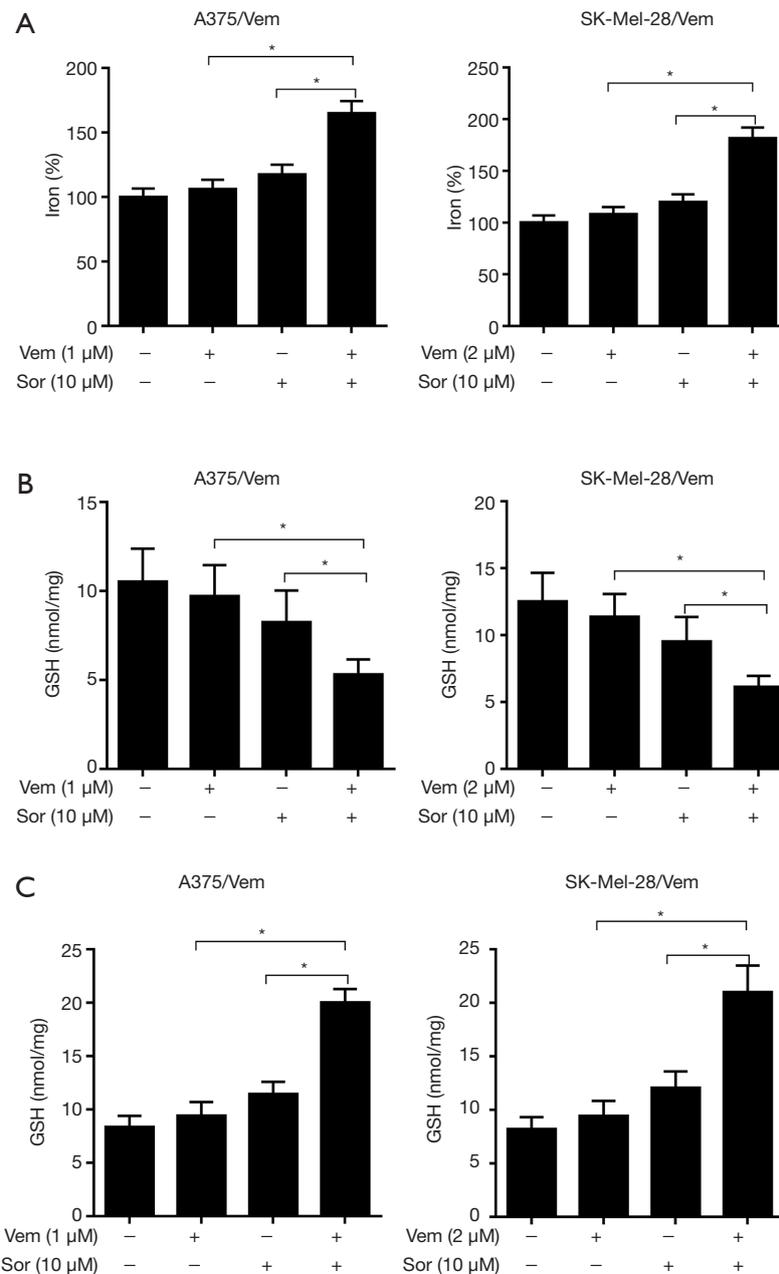


Figure 3 The effects of sorafenib on iron, GSH and MDA in vemurafenib-resistant MM cells. The cells were treated with vemurafenib or sorafenib alone, or both of them for 48 hours. (A) Iron Assay Kit was used to measure the iron concentration. (B) Glutathione Assay Kit was used to measure the GSH concentration. (C) Lipid Peroxidation (MDA) Assay Kit was used to measure the MDA concentration. *, $P < 0.05$.

in cells treated with sorafenib alone or in combination with vemurafenib, and the effects induced by the drug combination were more intense than sorafenib treating alone (Figures S3,S4). These results implied that sorafenib may aggravated oxidative stress in vemurafenib resistant or treated melanoma cells.

Sorafenib sensitizes melanoma cells to vemurafenib through ferroptosis

In addition, we found that the combination of vemurafenib and sorafenib could suppress the antioxidant enzyme GPX4 and nuclear factor (erythroid-derived 2)-like 2

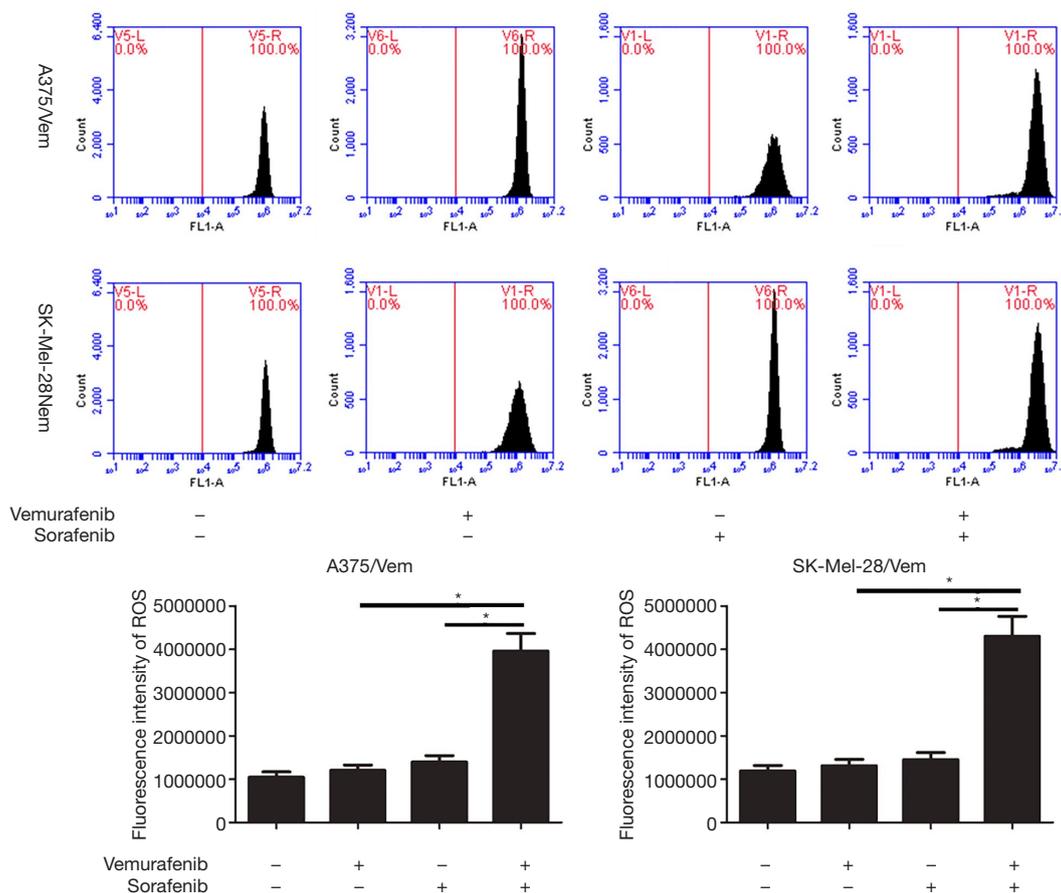


Figure 4 The effects of sorafenib on ROS production in vemurafenib-resistant MM cells. The cells were treated with vemurafenib or sorafenib alone, or both of them for 48 hours. Fluorometric Intracellular ROS Kit was used to measure the ROS concentration. *, P<0.05. ROS, reactive oxygen species; MM, melanoma.

(NRF2) (Figure 5). Vemurafenib resistant cells were treated with vemurafenib or sorafenib, and then exposed to the ferroptosis inhibitor Ferrostatin-1 (Fer-1), caspase inhibitor Z-VAD-FMK and MLKL inhibitor Necrosulfonamide. We found that the caspase inhibitor Z-VAD-FMK and MLKL inhibitor Necrosulfonamide did not significantly alter the inhibitory effects of vemurafenib and sorafenib (Figure 6). However, ferroptosis inhibitor Ferrostatin-1 treatment abolished the inhibitory effects of sorafenib or together with vemurafenib in A375/Vem and SK-Mel-28/Vem cells (Figure 6).

Discussion

MM is an aggressive disease. Molecular targeted therapies largely improved the overall survival of MM (20). BRAF inhibitors Vemurafenib is a drug that targeting to mutations

in the BRAF/MEK/ERK pathway. The application of Vemurafenib results in a good clinical outcome as well as better safety and efficacy. However, BRAF inhibitors resistance brings out new targets in this pathway (21). One of the promising targets is the constitutive activation of the PI3K/AKT pathway that leading to intrinsic BRAF resistance (22). MAPK/ERK and PI3K/AKT pathways can be activated by c-Met (23), leading to drug resistance via upstream or downstream effectors (24).

In normal conditions, the MAPK signaling network was activated by receptor tyrosine kinases through RAS proteins. RAS can regulate BRAF in the MAPK pathway to trigger MEK 1/2 activation, and subsequent activation of ERK 1/2 to induce several pro-growth factors and inhibit pro-apoptotic signals (25). A high percentage of melanomas have a mutated MEK1/2-activated MAPK signaling pathway (26), which leads to the promotion of

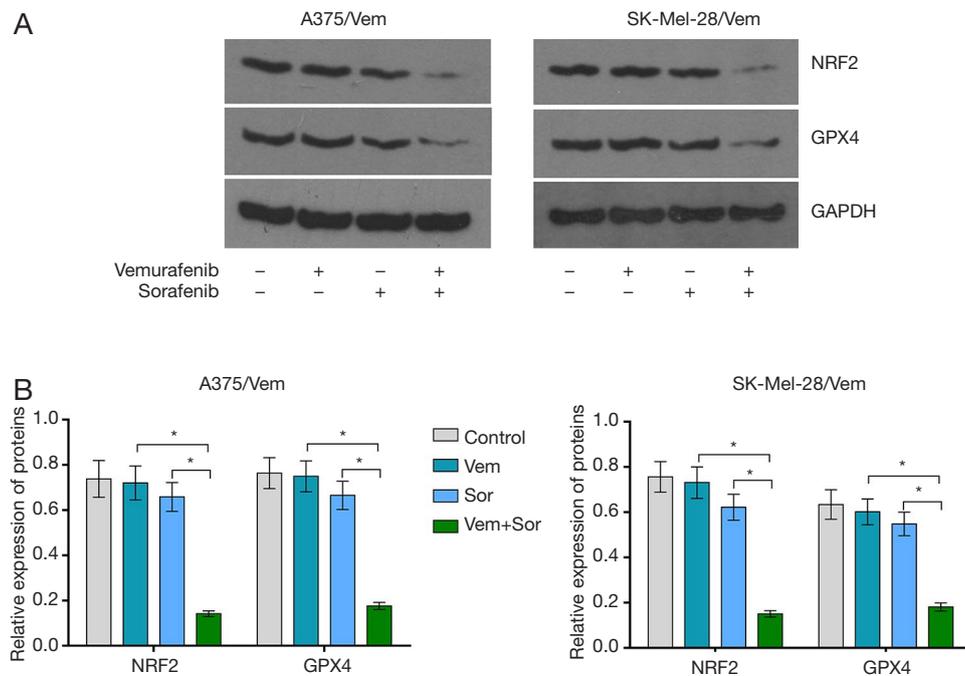


Figure 5 The effects of sorafenib on NRF2 and GPX4 in vemurafenib-resistant MM cells. The cells were treated with vemurafenib or sorafenib alone, or both of them for 48 hours. The untreated cells were used as control. (A) Western blot was performed to analyze the expression of NRF2 and GPX4. (B) The quantification of bands was shown in lower panel. *, $P < 0.05$. MM, melanoma.

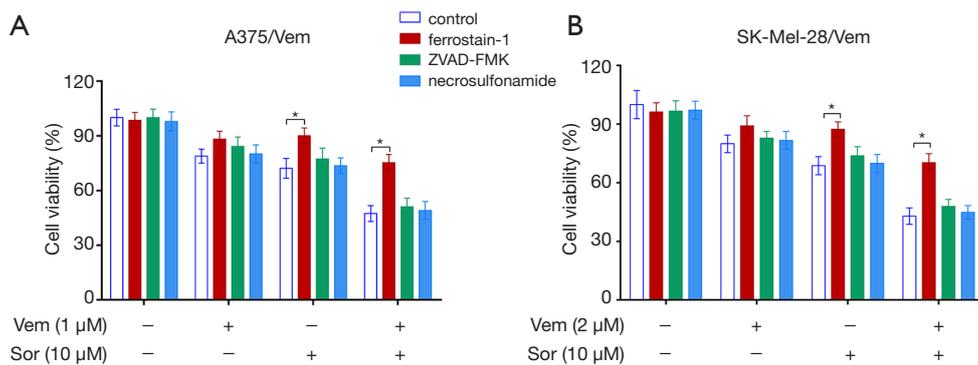


Figure 6 Lipid peroxidation inhibitors ferrostatin-1 reverses Sorafenib-mediated cell death. The cells were treated with vemurafenib or sorafenib alone, or both of them for 48 hours, and then exposed to ferroptosis inhibitor Ferrostatin-1 (Fer-1), caspase inhibitor Z-VAD-FMK and MLKL inhibitor Necrosulfonamide for 12 hours. CCK-8 assay was performed to measure the cell viability in vemurafenib-resistant A375 cells (A) and SK-Mel-28 cells (B). *, $P < 0.05$.

cell proliferation. However, these mutations also result in increased sensitivity to MAPK kinase inhibitors (20). Therefore, targeting MEK1/2 would be a promising candidate for cancer therapy (27). The addition of the MEK inhibitor cobimetinib to vemurafenib was associated with a significant improvement in progression-free survival

among patients with BRAF V600-mutated metastatic melanoma (28).

Sorafenib inhibits tumor growth through disrupting tumor vasculature and targeting the MAPK pathway (29), including VEGFR, and platelet-derived growth factor receptor (PDGFR)- β (1). Sorafenib can efficiently

inhibited the growth of tumors that harboring k-ras or b-raf mutations in a dose-dependent manner, including melanoma (30). In addition, Sorafenib-induced death has also been shown to be associated with oxidative stress (31). Sorafenib could rapidly increase the production of ROS, inducing intracellular glutathione depletion in hepatocellular carcinoma (32). Sorafenib treatment resulted in ROS accumulation and necroptotic cell death activation in Hodgkin's lymphoma (33). It was also demonstrated that melatonin can increase the cytotoxic sensitivity of sorafenib through mitochondria stability and mitophagy in hepatocellular carcinoma cells (15). In this study, we found that sorafenib treatment did not significantly alter the production of ROS and the content of iron, GSH, and MDA in vemurafenib resistant melanoma cells, but cotreatment of sorafenib and vemurafenib dramatically upregulated ROS production, MDA and iron, but decreased GSH concentration. Interestingly, In WM35 and A375 cells, the levels of Iron, GSH, MDA, and ROS were increased, but GSH were reduced in cells treated with sorafenib alone or in combination with vemurafenib. The indistinctive effects on oxidative stress by sorafenib in A375/Vem and SK-Mel-28/Vem cells may be ascribe to the character of multidrug resistance, which contributed to a degree sorafenib resistance in A375/Vem and SK-Mel-28/Vem cells. Anyhow, these results implied that sorafenib may sensitize melanoma to vemurafenib through activation of oxidative stress. Moreover, sorafenib strongly promoted vemurafenib-mediated cell death. And the lipid peroxidation inhibitors ferrostatin-1 blocked these effects. Ferroptosis is characterized by the accumulation of iron-dependent ROS (34), and is can be induced sorafenib in cancer cells (35). MAPK activation and endoplasmic reticulum stress (ERS) upregulation are involved in ferroptosis (36). Ferroptosis is negatively regulated by glutathione peroxidase 4 (GPX4) and nuclear factor erythroid 2-related factor 2 (NRF2), which inhibits ROS accumulation and cellular iron uptake (37). Our recent results showed that cotreatment of sorafenib and vemurafenib suppressed GPX4 and NRF2 expression in vemurafenib resistant melanoma cells, which would increase ROS production and cellular iron uptake (38).

Conclusions

Our study identifies an association between sorafenib and ferroptosis in vemurafenib resistant melanoma cells. Our findings demonstrate that sorafenib treatment may be a novel strategy for overcoming vemurafenib resistance in melanoma.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2020.01.62>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This institutional ethical approval and informed consent were waived.

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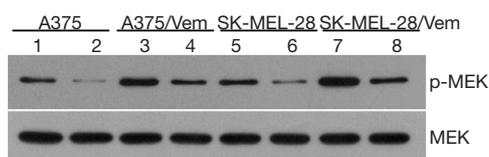


Figure S1 The vemurafenib-resistant cells maintained a highly level of phospho-MEK in the presence of vemurafenib. 1, 3, 5, 7: respectively indicate each cell culturing without vemurafenib; 2, 4, 6, 8: respectively indicate each cell culturing in the presence of vemurafenib, and the drug concentrations were 0.2, 1, 0.4, 2 μM, respectively.

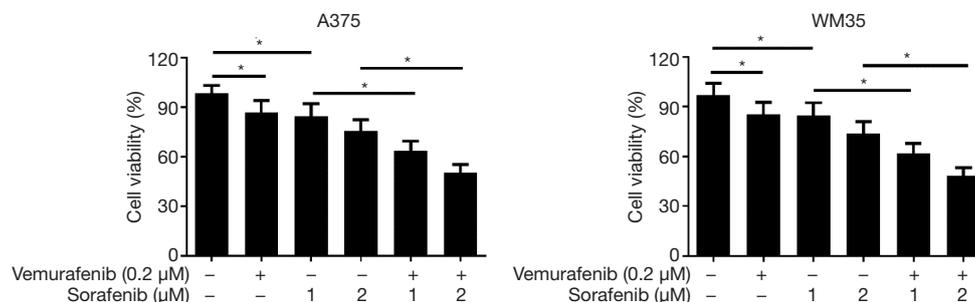


Figure S2 Sorafenib sensitizes A375 and WM35 cells to vemurafenib. The A375 and WM35 cells were treated with vemurafenib or sorafenib alone, or both of them for 48 hours. CCK-8 assay was performed to measure the cell viability after 48 h-treatment. *, P<0.05.

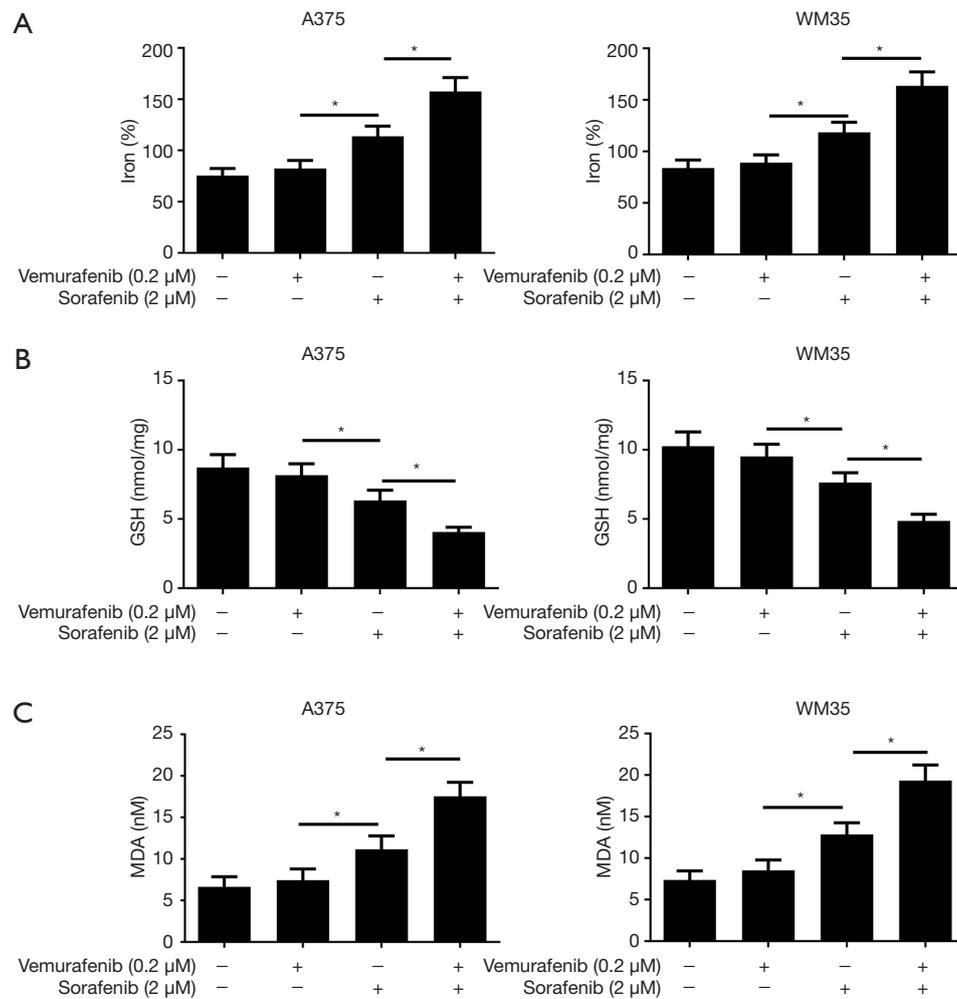


Figure S3 The effects of sorafenib on iron, GSH and MDA in A375 and WM35 cells. The cells were treated with Vemurafenib or sorafenib alone, or both of them for 48 hours. (A) Iron Assay Kit was used to measure the iron concentration. (B) Glutathione Assay Kit was used to measure the GSH concentration. (C) Lipid Peroxidation (MDA) Assay Kit was used to measure the MDA concentration. *, $P < 0.05$.

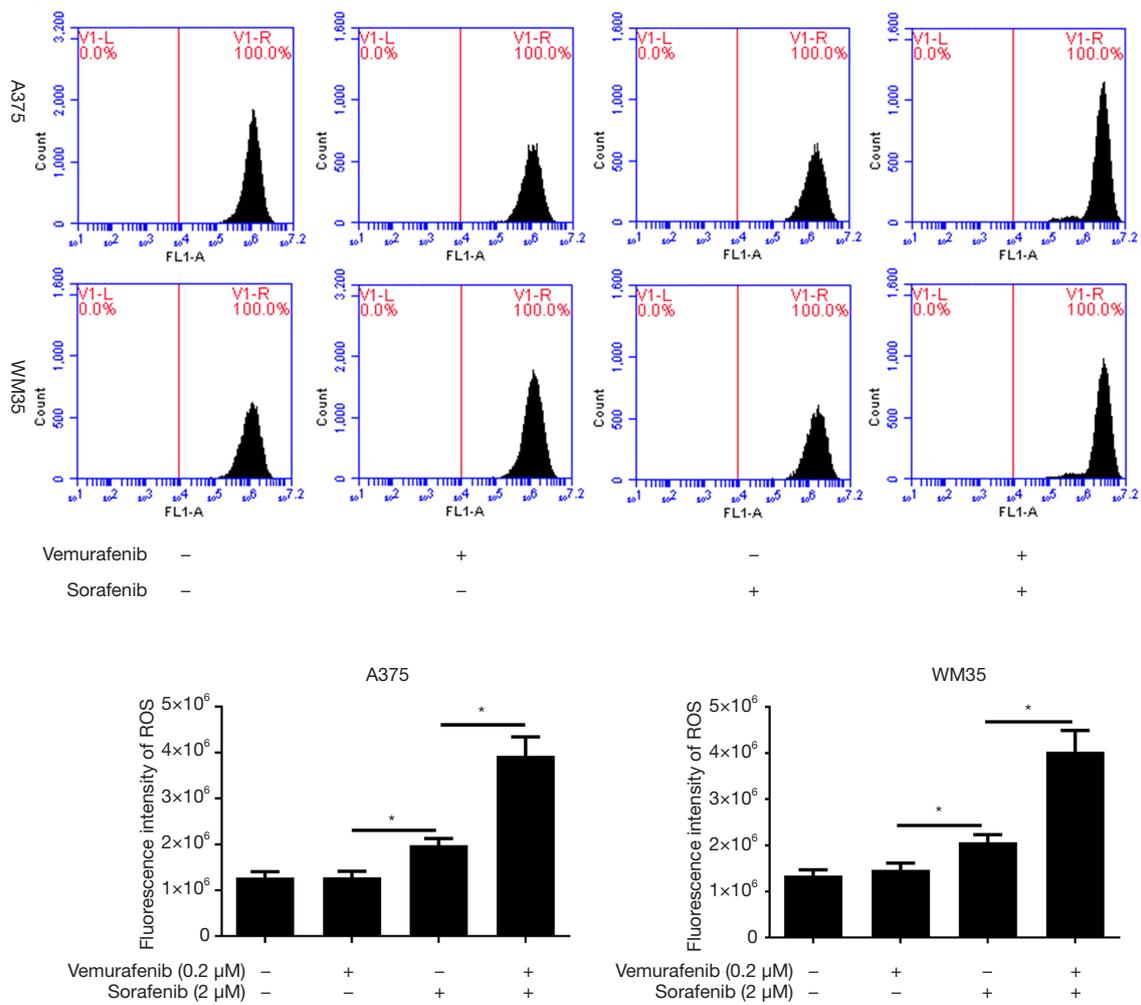


Figure S4 The effects of sorafenib on ROS production in A375 and WM35 cells. The cells were treated with vemurafenib or sorafenib alone, or both of them for 48 hours. Fluorometric Intracellular ROS Kit was used to measure the ROS concentration. *, P<0.05. ROS, reactive oxygen species.