Resveratrol Overcomes Cellular Resistance to Vemurafenib Through Dephosphorylation of AKT in *BRAF*-mutated Melanoma Cells

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Abstract. *Background/Aim: The serine/threonine-protein kinase B-Raf (BRAF) V600E mutant (BRAF[V600E]) inhibitor vemurafenib, has improved clinical outcomes for patients with BRAF[V600E] melanoma, but acquired cellular resistance mediated by AKT serine/threonine kinase 1 (AKT) phosphorylation limits its efficacy. We examined the effect of resveratrol on vemurafenib-resistant melanoma cells. Materials and Methods: A vemurafenib-resistant human metastatic melanoma cell line positive for the BRAF V600E mutation was established. The anti-tumorigenic effects of vemurafenib and resveratrol, both alone and in combination, were examined through analysis of cell proliferation and protein expression. Results: The level of phosphorylated AKT (p-AKT) was increased in the primary melanoma cells after treatment with vemurafenib, and the basal level of p-AKT was increased in vemurafenib-resistant melanoma cells. Notably, resveratrol both alone and in combination with vemurafenib effectively suppressed cell proliferation and AKT phosphorylation in both parental and vemurafenib-resistant melanoma cells. Conclusion: Vemurafenib resistance can be reversed by addition of resveratrol in patients undergoing treatment with BRAF inhibitors.*

The oncogenic mutation in serine/threonine-protein kinase B-Raf (*BRAF*), a single-base mutation that substitutes glutamic acid for valine at the amino acid position 600 (V600E) in the

BRAF kinase domain, has been reported in approximately 50% of all patients with melanoma. This mutation leads to the rat sarcoma viral oncogene (RAS)-independent constitutive activation of BRAF and downstream signal transduction in the mitogen-activated protein kinase (MAPK) pathway (1). Vemurafenib selectively targets BRAF and can improve the overall survival rate in patients with *BRAF*-mutant metastatic melanoma (2, 3); however, acquired cellular resistance to BRAF inhibitors is a critical problem during treatment. Indeed, most melanomas resume growth within 5-7 months after the start of therapy (2, 4). Recent studies have implicated the phosphoinositide kinase-3 (PI3K)/AKT signaling pathway in the development of this acquired resistance. A high level of phosphorylated AKT (p-AKT) in cell lines and biopsies appears to predict resistance to vemurafenib (1, 5, 6). Combination therapies that include vemurafenib have emerged as effective therapeutic approaches to treat melanoma until acquired resistance to vemurafenib occurs.

Resveratrol, also known as *trans*-3,5,4'-trihydroxystilbene, is a naturally-occurring polyphenolic compound possessing anticancer capabilities. The growth-inhibitory effects of resveratrol are mediated through several mechanisms, including cyclic AMP (cAMP) and phosphoinositide kinase-3 (PI3K) signaling (7-9). Previously, we demonstrated that mutated oncogenic Kirsten rat sarcoma viral oncogene Homolog (KRAS) inhibits cellular polarity through the increased expression of phosphodiesterase 4B isoform 2 (PDE4B2), which is a cAMP-specific phosphodiesterase family member responsible for the degradation of the second messenger cAMP, and mutated KRAS thereby induces the malignant transformation in colorectal cancer (CRC) cell lines (10, 11). Treatment using a PDE4 inhibitor, such as rolipram or resveratrol, against CRC cells results in the suppression of p-AKT, inducing cellular polarity in 3D culture (10, 12). Similarly, resveratrol suppresses the growth of melanomas through the AKT pathway (13, 14). Furthermore, recent studies have suggested that resveratrol has anti-tumorigenic effects against metastatic melanoma cells,

which usually exhibit multidrug resistance (15, 16). In this study, we aimed to clarify the effects of resveratrol in vemurafenib-resistant melanoma cells.

Materials and Methods

Antibodies and reagents. Vemurafenib was obtained from LC Laboratories (Woburn, MA, USA). Resveratrol (*trans*-3,4',5-trihydroxystilbene) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-p-AKT (D9E) and anti-AKT (pan; C67E7) were obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell culture. Human melanoma cell lines with *BRAF* mutation were established from patient biopsies under the approval of the Fukuoka General Cancer Clinic Institutional Review Board. To generate cell lines with *in vitro* acquired resistance, the parental melanoma cell lines were treated with 10 μM vemurafenib for 1 hour, and then surviving cells were cultured with 1 μM vemurafenib until a vemurafenib-resistant sub-line was established. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 with L-glutamine (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin, streptomycin and amphotericin B (Meiji Seika Pharma Co., Ltd., Tokyo, Japan). All cell lines were mycoplasma free when periodically tested using the Mycoalert assay (Lonza, Rockland, ME, USA).

Cell viability assay. Cell viability and proliferation were determined using the Cell Counting Kit-8 assay (Dojindo Laboratories, Kumamoto, Japan), as described previously (17-20). The melanoma cells were treated for 48 h with vemurafenib and resveratrol, either alone or in combination, or with dimethyl sulfoxide (DMSO) as a vehicle control. The 50% inhibitory concentration (IC_{50}) value was determined using the concentration dependence of viability. The combination-index (CI) was calculated using the formula as previously described (21).

Immunoblotting. Cells were lysed in a RIPA buffer [(50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and protease inhibitor cocktail (Roche, Basel, Switzerland)] and were subjected to immunoblotting, as previously described (10, 22). Quantitative analysis of immunoblotting was performed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

Statistical analyses. Data are presented as means±standard deviations. Statistical analyses were performed using the unpaired two-tailed Student's *t*-test. *p*-Values less than 0.05 were considered statistically significant.

Results

In vitro combination studies of vemurafenib with resveratrol. To generate vemurafenib-resistant melanoma cells, we first established the parental melanoma cells from a patient who had the *BRAF*^{V600E} mutation. Then parental melanoma cells were exposed to a high dose of vemurafenib (1 μM) for 4 months. Chemosensitivity in the parental and vemurafenib-resistant melanoma cell lines towards vemurafenib and

resveratrol was reflected in cell growth inhibition, calculated by IC_{50} at 48 h. The IC_{50} values for vemurafenib for the parental and vemurafenib-resistant cells were 0.3 and 6.5 μM, respectively (Figure 1A). To examine the effects of resveratrol, the parental and vemurafenib-resistant cells were treated with resveratrol alone (Figure 1B). The IC_{10}, IC_{30} and IC_{50} values for resveratrol for the parental cells were 4.2, 18.1 and 49 μM, respectively, while, the corresponding values for the vemurafenib-resistant cells were 4.2, 18.3 and 44 μM, respectively. These results suggest that the survival curves for both cells were similar and that the vemurafenib-resistant cells did not exhibit cross-resistance to resveratrol.

Based on these results, resveratrol concentrations were fixed at 4 μM (IC_{10}) or 18 μM (IC_{30}), and we determined the IC_{50} value for the cells treated with vemurafenib and resveratrol in combination. The IC_{50} values for the parental cells treated with vemurafenib and 4 μM (IC_{10}) and 18 μM (IC_{30}) resveratrol were 0.04 and 0.02 μM, respectively (Figure 1C left panel). Similarly, IC_{50} values for the vemurafenib-resistant cells treated with vemurafenib and 4 μM (IC_{10}) resveratrol or 18 μM (IC_{30}) resveratrol were 0.6 and 0.1 μM, respectively (Figure 1C right panel).

The CI was used to analyze and confirm the synergism observed after the treatment of parental and vemurafenib-resistant cells with vemurafenib and resveratrol, providing a quantitative measure of the degree of drug interaction (Figure 1D). A CI of 1 indicates an additive effect between the two agents, whereas a CI of <1 and CI of >1 indicates synergism and antagonism, respectively. The CI for the parental cells treated with the combination of 0.04 μM vemurafenib and 4 μM resveratrol, and with of 0.02 μM vemurafenib and 18 μM resveratrol was 0.20 and 0.31, respectively (Figure 1D, left panel), suggestive of synergistic effects. Similarly, the CI for the vemurafenib-resistant cells treated with the combination of 0.6 μM vemurafenib and 4 μM resveratrol, and with 0.1 μM vemurafenib and 18 μM resveratrol were 0.19 and 0.43, respectively (Figure 1C right panel), also suggestive of synergistic effects. These results together indicate that addition of resveratrol to vemurafenib synergistically improves the cytotoxic effects of vemurafenib for both parental and vemurafenib-resistant melanoma cells.

Resveratrol alone and in combination with vemurafenib suppresses AKT phosphorylation in parental and vemurafenib-resistant melanoma cells. Recently, the results of a study indicated that the level of p-AKT levels is increased in vemurafenib-resistant clones compared to those in parental vemurafenib-sensitive cells (1). In the parental melanoma cells, 0.5 μM (IC_{50}) vemurafenib induced AKT phosphorylation, while 18 μM (IC_{30}) resveratrol did not (Figure 2A). Importantly, the combination of vemurafenib and resveratrol suppressed AKT phosphorylation to the basal level in the parental melanoma cells (Figure 2A). The basal
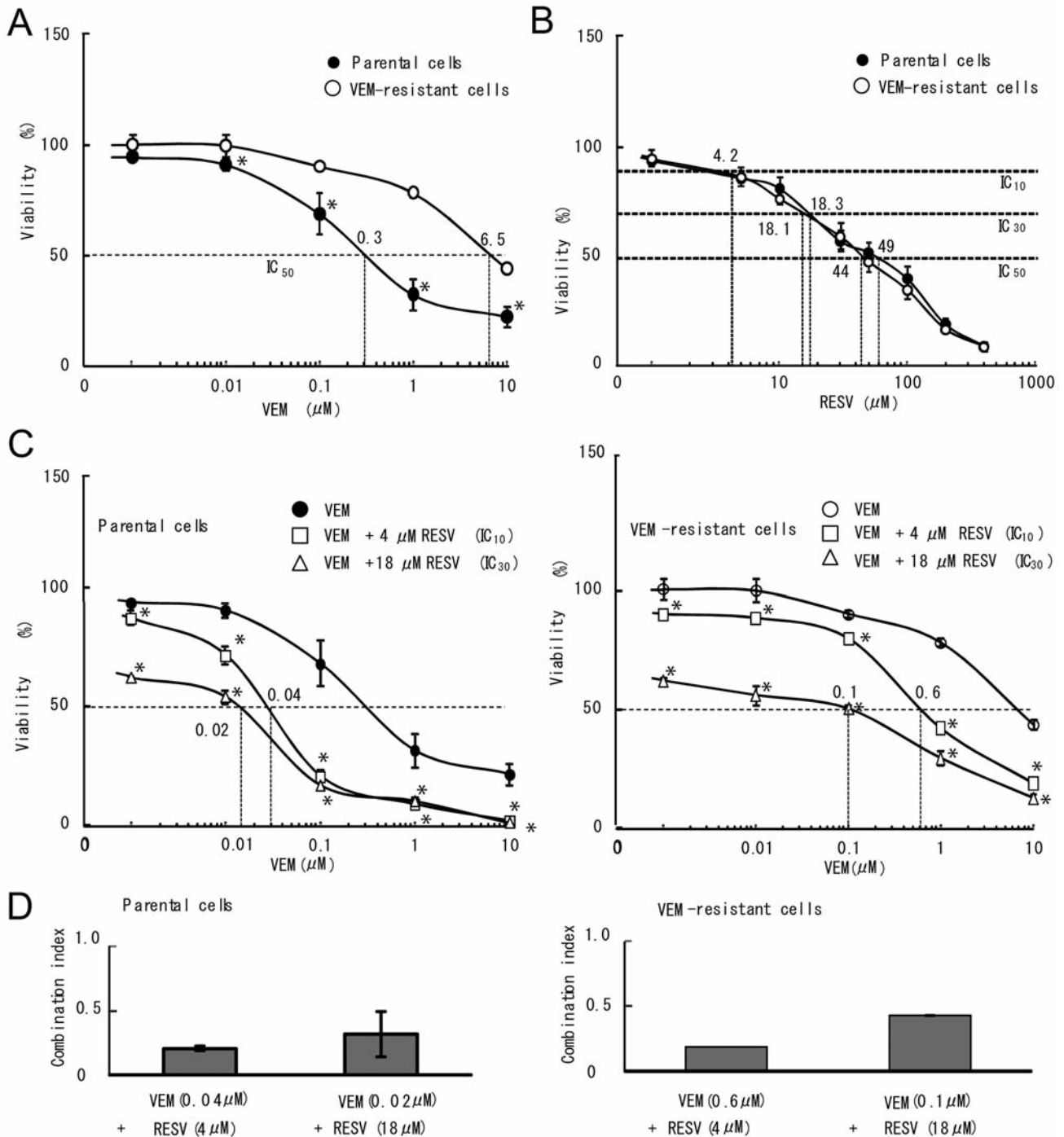
Figure 1. *In vitro combination studies of vemurafenib (VEM) with resveratrol (RESV). Parental and VEM-resistant cells were exposed for 48 h to graded concentrations of VEM (A) or RESV (B) both alone and in combination at a fixed dose of RESV (C). The numbers at the intersection of the horizontal dotted line and the viability curves represent the 10, 30 or 50% inhibitory concentration value (IC_{10}, IC_{30} or IC_{50}, respectively). *Significantly different at $p<0.05$. D: The combination-index for parental and VEM-resistant cells treated with VEM and RESV at different combinations of the dose ratios.*

level of AKT phosphorylation observed in the vemurafenib-resistant melanoma cells treated with the vehicle control (DMSO) increased compared with that in the parental

melanoma cells (Figure 2A), suggesting that the long-term exposure of these cells to vemurafenib induces the constitutive phosphorylation of AKT. In the vemurafenib-
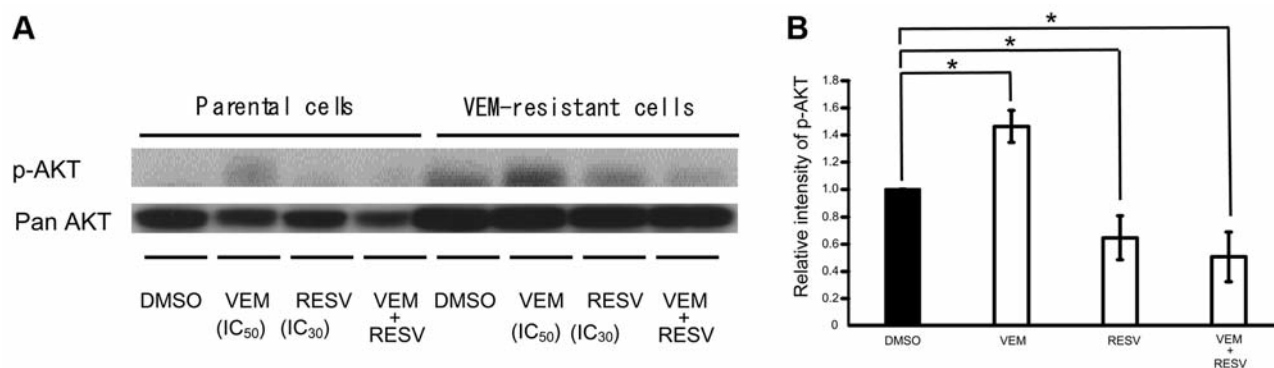
Figure 2. *Resveratrol (RESV) alone and in combination with vemurafenib (VEM) suppresses AKT serine/threonine kinase 1(AKT) phosphorylation in parental and VEM-resistant melanoma cells. A: Western blotting for AKT phosphorylation (p-AKT) and pan AKT in parental melanoma cells and VEM-resistant cells treated with VEM (IC$_{50}$) and RESV (IC$_{30}$), alone and in combination. B: The quantitative analyses of the level of p-AKT in VEM-resistant cells. The white bar represents the relative intensity of the signal for VEM-resistant cells treated with VEM and RESV normalized by the signal for VEM-resistant cells treated with dimethyl sulfoxide (DMSO) alone (black bar). *Significantly different at p<0.05.*

resistant melanoma cells, the level of phosphorylated AKT following treatment with 8.4 μM (IC$_{50}$) vemurafenib increased by 50% compared to that in cells treated with DMSO (Figure 2B). On the other hand, the level of phosphorylated AKT in vemurafenib-resistant melanoma cells treated with 18 μM (IC$_{30}$) resveratrol alone or with vemurafenib and resveratrol in combination were reduced by 35.0% and 49.5%, respectively, compared with cells treated with DMSO (Figure 2B). Taken together, these results suggest that the synergistic effects of vemurafenib and resveratrol in both parental and vemurafenib-resistant melanoma cells are partially exerted through AKT dephosphorylation.

Discussion

In this study, we established vemurafenib-sensitive (parental) and vemurafenib-resistant melanoma cell lines from a patient (Figure 1). Using these cells, we found that vemurafenib-resistant cells did not exhibit cross-resistance to resveratrol. Furthermore, in both parental and vemurafenib-resistant cells, the IC$_{50}$ values for resveratrol and vemurafenib in combination were synergistically lower compared with those for vemurafenib alone (Figure 1). The suppression of AKT phosphorylation observed following treatment with resveratrol alone and in combination with vemurafenib is likely to be the reason for the lower IC$_{50}$ values induced by these treatments in both parental and vemurafenib-resistant melanoma cells (Figure 2).

The activation of the PI3K/AKT signaling pathway contributes to diminished sensitivity to MAPK pathway inhibitor in human melanoma cell lines (23). Here, short-term (48 h) exposure to vemurafenib induced AKT phosphorylation (Figure 2), suggesting that vemurafenib resistance is induced at an early phase. Furthermore, the long-term and constitutive activation of the PI3K/AKT signaling pathway reportedly leads

to multidrug resistance, while inhibition of PI3K/AKT signaling pathway reportedly leads to sensitizing of drug-resistant cells (24). In our study, the level of AKT phosphorylation in the vemurafenib-resistant melanoma cells, established through long-term exposure to vemurafenib, was increased compared to that of parental melanoma cells (Figure 2), suggesting that multidrug resistance is induced in vemurafenib-resistant cells. However, vemurafenib-resistant melanoma cells did not show cross-resistance to resveratrol (Figure 1), and vemurafenib and resveratrol in combination had a synergetic effect in the vemurafenib-resistant melanoma cells (Figure 1). Several recent studies have also clearly demonstrated the synergistic nature of the combination of an inhibitor targeting the PI3K/AKT pathway and a BRAF inhibitor (23, 25-27); however, the use of resveratrol as an AKT inhibitor has not been previously demonstrated in drug-resistant melanoma cells.

The use of vemurafenib and resveratrol in combination before melanoma cells acquire resistance to vemurafenib may result in enhanced melanoma regression and might significantly delay or even prevent the development of vemurafenib-resistant melanoma cells in patients.

Acknowledgements

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