RESEARCH ARTICLE

Treatment of Vemurafenib-Resistant SKMEL-28 Melanoma Cells with Paclitaxel

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Abstract

Vemurafenib has recently been used as drug for treatment of melanomas with BRAFV600E mutation. Unfortunately, treatment with only vemurafenib has not been sufficiently effective, with recurrence after a short period. In this study, three vemurafenib-resistant BRAFV600E melanoma cell lines, A375P R, A375M R and SKMEL-28 R, were established from the original A375P, A375M and SKMEL-28 cell lines. Examination of the molecular mechanisms showed that the phosphorylation levels of MEK and ERK, which play key roles in the RAS/RAF/MEK/ERK signaling pathway, were reduced in these three cell lines, with increased phosphorylation levels of pAKTs limited to SKMEL-28 R cells. Treatment of SKMEL-28 R cells with 100 nM paclitaxel resulted in increased apoptosis and decreased cellular proliferation, invasion and colony formation via reduction of expression levels of EGFR and pAKTs. Moreover, vemurafenib-induced pAKTs in SKMEL-28 R were decreased by treatment with an AKT inhibitor, MK-2206. Taken together, our results revealed that resistance mechanisms of BRAFV600E-mutation melanoma cells to vemurafenib depended on the cell type. Our results suggested that paclitaxel should be considered as a drug in combination with vemurafenib to treat melanoma cells.

Keywords: Melanoma - vemurafenib - paclitaxel - treatment resistance - BRAFV600E - EGFR - AKT

Introduction

Currently, skin cancer is one of the most common diseases in the world. The forms of skin cancers that occur most frequently are basal cell cancer, squamous cell cancer and melanocyte cell cancer (melanoma). Although melanoma accounts for only about 10% of skin cancer cases, it causes more than 80% of all skin cancer deaths (Rigel, 2010). In Vietnam, according to statistics of the national academy of dermatology, there were not many cases of melanoma about 10 years ago, but the number of cases has been rapidly increasing since 2007. In the United States, according to statistics of the national cancer institute, there were 50,000 and 77,000 new cases of melanoma in 2010 and 2013, respectively and 9480 deaths due to melanoma in the first 6 months of 2013. However, there is still no effective drug for treatment. The difficulty in studying the mechanism of cancer is believed to be the main reason.

The development of melanoma is related to phosphorylation-induced activation of two signaling pathways: PI3K/PTEN/AKT and MAPK (RAS/RAF/MEK/ERK). AKT is the target molecule in the PI3K/PTEN/AKT signaling pathway (Meier et al., 2005). Phosphorylated AKT (pAKT) promotes proliferation, differentiation and development of cancer cells through activation of mTOR and NF-kappaB pathways (Kim et al., 2001; Meier et al., 2005). ERK is the target molecule in the MAPK signaling pathway and is involved in transcriptional regulation of cells (Gray-Schopfer et al., 2007). The MAPK pathway is activated by many growth factors including NF-kappaB, SCF, FGF, HGF and GDNF (Meier et al., 2005). Increased expression of pERK appears in more than 90% of melanoma cases (Cohen et al., 2002). Inhibition of the expression of molecules such as RAF (BRAF) and MEK leads to reduced expression of pERK (McCubrey et al., 2007).

BRAFV600E mutation, in which the aspartic acid residue is replaced by the valine residue, appears very frequently (over 60%) in cases of melanoma (Davies et al., 2002). Therefore, BRAF is considered as one of the target molecules for mechanism research and drug development (Dankort et al., 2009; Joseph et al., 2010). Vemurafenib, an inhibitor of BRAF expression, has been tested for treatment of melanoma both in vitro and in vivo and has shown positive results (Bollag et al., 2010). After many years of research and trials, vemurafenib has recently been certificated by FDA, US (on 17 August 2011) and Health

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DOI: http://dx.doi.org/10.7314/APJCP.2015.16.2.699

Asian Pac J Cancer Prev, 16 (2), 699-705
Cells were subcultured and refreshed with a new medium CO\textsubscript{2} (FBS) and 1% penicillin/streptomycin at 37\textdegree C in 5% RPMI-1640 (supplemented with 10% fetal bovine serum).

Establishment of vemurafenib-resistant melanoma cell lines

Three primary human malignant melanoma cell lines (A375P, A375M and SKMEL-28) carrying BRAF\textsuperscript{V600E} mutation were used. A375M and A375P cells kind gifts by Dr. Dorothy C Bennett of St george’s hospital medical school, UK and SK-Mel28 cells were kindly provided by the Riken Bioresource center cell bank. These cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37\textdegree C in 5% CO\textsubscript{2}.

Establishment of vemurafenib-resistant melanoma cell lines

The three cell lines were cultured consecutively in RPMI-1640 (supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37\textdegree C in 5% CO\textsubscript{2}) with or without vemurafenib at 5 \textmu M for 9 months. Cells were subcultured and refreshed with a new medium every 3 days. Cells that survived after this period were vemurafenib-resistant cells.

Crystal violet assay

A crystal violet assay was performed by the method described by Yajima (2012) to primarily assess the viability of cells. Briefly, 3x10\textsuperscript{4} cells were plated in six-well plates and cultured in the medium with or without vemurafenib for 72 hrs. The viable adherent cells were fixed with 10% formalin for 1 hour and stained with 0.1% crystal violet for 30 minutes after washing two times with PBS. The stained cells were solubilized with 0.1% SDS and the absorbance of the solution was measured at 595 nm by a spectrophotometer.

Invasion assay

Cell invasion ability was evaluated by an invasion assay according to the method reported by Thang (2011). Briefly, 2x10\textsuperscript{5} cells in 300 ml of culture medium with 0.5% FBS were applied to a matrigel-coated upper chamber of 8 mm in diameter (8 mm in pore size). Then the upper chambers were placed in 24-well culture plates containing 600 ml conditioned medium with 0.5% FBS to trigger invasion activity and were incubated for 12 hours. Invading cells were stained with hematoxylin/eosin and counted under a microscope.

 Colony formation assay

A colony formation assay was performed to assess the development of tumor in vitro. Anchorage-independent growth was evaluated by the colony formation assay according to the method reported by Thang (2011). After preincubating in the medium for 24 hours, 2.5x10\textsuperscript{4} cells were mixed with 2 ml of 0.36% soft agar in RPMI medium, poured onto slightly solid 0.72% hard agar in RPMI medium and then cultured for 3 weeks. Colonies exceeding 50 \textmu m in diameter were counted and presented as an activity of anchorage-independent growth.

Immunoblotting

Immunoblotting was performed to investigate the protein expression of molecules using the method described by Kato (2010). Briefly, cells were washed twice with ice-cold PBS and lysed in 0.3 ml of lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 12.5 mM b-glycerophosphate, 1.5 mM MgCl\textsubscript{2}, 2 mM EDTA, 10 mM Na\textsubscript{3}PO\textsubscript{4}, 1 mM phenylmethylsulfonyl fluoride, 20 mM aprotinin, 0.5% Triton X-100). Whole cell lysates were resolved on SDS-PAGE and transferred to Hybond-P membranes (GE Health Sciences). The membranes were immunoblotted with various antibodies and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG (Calbiochem) using an enhanced Chemiluminescence (ECL) Western Blotting System (GE Health Sciences) or ECL advance (GE Health Sciences). Rabbit polyclonal antibodies against phosphorylated threonine 202 in ERK1 and phosphorylated tyrosine 204 in ERK2 (Cell Signaling), phosphorylated MEK1/2 (Cell Signaling) and EGFR (Thermo); rabbit monoclonal antibodies against Akt...
and phosphorylated Akt (Cell Signaling); and mouse monoclonal antibodies against phosphorylated tyrosine 1173 in EGFR (Thermo), MEK1/2 (Cell Signaling), ERK1/2 (Cell Signaling) and alpha-TUBULIN (SIGMA) were used as first antibodies.

Annexin V apoptosis assay

Annexin V apoptosis assay was performed to investigate the protein expression of molecules using the method described by Schutte (1998). Cells were seeded at 30 to 40% confluence in 6-cm plates. After overnight incubation, medium was aspirated and replaced with medium with or without 100 nM of paclitaxel. After 36 hours, medium was collected. Cells were washed with PBS and trypsinized. PBS wash and trypsinized cells were added to the collected medium in a single tube. Cells were pelleted, washed once with PBS and resuspended in annexin binding buffer (BD Biosciences) at 1×10^6 cells/ml. Cells were stained with propidium iodide (BD Biosciences) and annexin V-FITC according to the manufacturer’s protocol and assayed on a FACSCanto II (BD Biosciences). The percentage of apoptotic cells was measured as the percentage of annexin V-positive cells.

Immunocytochemistry

Immunocytochemistry was performed to investigate the protein expression of molecules using the method described by Fontanini (1995). SKMEL-28® cells were treated with or without 100 nM of paclitaxel for 24 hrs. Cells then were fixed with 1% paraformaldehyde (PFA) and blocked with 5% FBS for 30 minutes. Then the cells were stained with 10 µg/ml anti-human EGFR Alexa Fluor® 594 (red) at room temperature for 3 hours in dark. Nuclei were counterstained with DAPI (blue). The image was captured with a 40X objective.

Statistical analysis

Statistical analysis in this study was performed according to the method described by Yajima (2012). Results from three independent experiments in each group were statistically analyzed by Student’s t-test. The SPSS (version 18) software package (SPSS Japan Inc.) was used for statistical analyses and the significance level was set at p<0.05.

Results

Vemurafenib induced apoptosis via inactivation of the RAS/RAF/MEK/ERK signaling pathway

Firstly, we examined the dose-dependent effect of vemurafenib on cytotoxicity of the three melanoma cell lines carrying BRAFV600E mutation (A375P, A375M and SKMEL-28 cells). Cells were treated with vemurafenib for 48 hrs at different concentrations from 0 µM to 10 µM. In accordance with previous study (Joseph et al., 2010), it was shown that the higher concentration of vemurafenib, the stronger was the toxicity for cells (Figure 1a). Vemurafenib only expressed toxicity to cells at the concentration of 2.5 µM or more (Figure 1a). However, treatment with 10 µM of vemurafenib caused more than 80% and 100% cell death after 48 hrs and 72 hrs, respectively (data not shown). In accordance with previous reports (Halaban et al., 2010; Atefi et al., 2011), treatment with vemurafenib resulted in a big decrease in phosphorylated MEK and ERK (pMEK and pERK), which play central roles in the RAS/RAF/MEK/ERK (MAPK) signaling pathway and a slight decrease in phosphorylated AKT (pAKT) in SKMEL-28 cells in a dose-dependent manner (Figure 1b).

Establishment of three vemurafenib-resistant melanoma cell lines

Three original cell lines, A375M and A375P gifted by Dr. Dorothy C Bennett of St George’s hospital medical school, UK and SKMEL-28 provided by the Riken Bioresource center cell bank, were used to establish vemurafenib-resistant cell lines. A375P, A375M and SKMEL-28 cells were cultured in RPMI medium containing 5 µM of vemurafenib for about 9 months to create three vemurafenib-resistant cell lines named A375P®, A375M® and SKMEL-28® for further experiments. In these three new cell lines, RAS/RAF/MEK/ERK signaling was almost completely inhibited (Figure 3). Although A375P®, A375M® and SKMEL-28® cells all were grew well, their proliferation was slower than that of original cells at day 4 post incubation (Figure 2). Among these three new cell lines, SKMEL-28® grew best with a significant difference in proliferation rate from those of the other two cell lines (Figure 2).

Molecular mechanism of resistance to vemurafenib depends on cell type

We next performed immunoblot analysis to examine protein and phosphorylated levels of MEK (pMEK), ERK (pERK), AKT (pAKT). It was shown that pMEK in all of the A375P®, A375M® and SKMEL-28® cells was almost completely inhibited (Figure 3). This result is in accordance well with previous reports (Halaban et al., 2010; Atefi et al., 2011; Lucchetti et al., 2011). While levels of pERK were almost inhibited in A375P® and A375M® cells, its level was still quite high in SKMEL-28® cells (Figure 3a). This result is somehow different from the previous reports (Corcoran et al., 2013; Girotti and Marais, 2013) in which expression levels of pERK in vemurafenib resistant cell lines were quite high. The difference in these patterns might be caused by different exposure time to vemurafenib and the concentration of vemurafenib used for making acquired resistant cells. In previous papers (Vergara et al., 2012; Wang et al., 2014) they let cells exposed to vemurafenib for almost 3-4 months with gradually increasing of concentration of vemurafenib from 0 to 2.5 µM. However in this study, we always let cells exposed to 5 µM of vemurafenib for 9 months to select the resistant cells. The most interesting finding was that while levels of both pThr308-AKT and pSer473-AKT were greatly decreased in A375P® and A375M® cells, the levels were greatly increased in SKMEL-28® cells (Figure 3a-b). This result may explain the higher proliferation rate of SKMEL-28® cells than those of A375P® and A375M® cells. In this study we also indicated that the level of reactivation of pAKT has a correlation with expression level of pERK in the resistant
Vemurafenib on Proliferation of BRAF\textsuperscript{V600E}-Carrying Melanoma Cells.

Figure 1. Dose-Dependent Effect of Vemurafenib on Toxicity of BRAF\textsuperscript{V600E}-Carrying Melanoma Cells. A The three melanoma cell lines A375P, A375M and SKMEL-28 were treated with 0, 0.5, 1.0, 2.5, 5.0 and 10.0 µM of vemurafenib. Living cells were stained with crystal violet. Ratios of living cells between treated and non-treated cells were determined by the crystal violet assay and are shown in a graph. *, ** and ***, Significantly different (p<0.05, p<0.01 and p<0.001) from the control by student’s t-test. b SKMEL-28 cells were treated with 0 (lane 1), 1.0 (lane 2), 2.5 (lane 3) and 5.0 (lane 4) µM vemurafenib for 48 hours. Protein and phosphorylation levels of MEK and p-MEK in SKMEL-28 cells were measured by immunoblotting. TUBULIN was used as an internal control.

Figure 2. Effect of Long-Term Treatment with Vemurafenib on Proliferation of BRAF\textsuperscript{V600E}-Carrying Melanoma Cells. The original A375P, A375M and SKMEL-28 cell lines and vemurafenib-resistant A375P\textsuperscript{R}, A375M\textsuperscript{R} and SKMEL-28\textsuperscript{R} cell lines were cultured and their proliferation rates at day 1, day 2 and day 4 were determined by crystal violet assays. Ratios of proliferation in original melanoma cells and vemurafenib-resistant melanoma cells are shown in the graph. * and **, significantly different (p<0.05 and p<0.01) from the control by student’s t-test.

Paclitaxel reduced cancer characteristics and induced apoptosis of SKMEL-28\textsuperscript{R} cells

We then examined the effects of paclitaxel on cancer characteristics including proliferation, invasion and colony formation of SKMEL-28\textsuperscript{R} cells. Treatment of SKMEL-28\textsuperscript{R} cells with 100 nM paclitaxel for 4 days caused a slight change in morphology of the cells (Figure 4a). Paclitaxel-treated SKMEL-28\textsuperscript{R} cells became larger with formation of multi-nuclei cells. The reason for this might be that paclitaxel reduces the mitotic activity of the cells. Treatment with 100 nM paclitaxel also caused great decreases in proliferation (Figure 4b), invasion (Figure 4c-d) and colony formation (Figure 4e-f) of SKMEL-28\textsuperscript{R} cells. We then examined the effect of paclitaxel on apoptosis of SKMEL-28\textsuperscript{R} by using Annexin V Apoptosis assay (Flow cytometry method). Our result indicated that 100 nM of paclitaxel induced apoptosis of SKMEL-28\textsuperscript{R} about 3.3 folds, from 4.3% in the SKMEL-28\textsuperscript{R} control cells to 14.2% in paclitaxel-treated SKMEL-28\textsuperscript{R} cells (Figure 4g-h). Moreover, in previous studies, they showed that the combination of AKT inhibitors and BRAF inhibitors caused the decrease in melanoma development in cell lines and patients (Rebecca et al., 2012; Lassen et al., 2014). That means our result matches well with these previous studies to suggest that the simultaneous inhibitions of both AKT and BRAF could be beneficial for melanoma patients.

Paclitaxel inhibited vemurafenib-induced EGFR/AKT pathway in SKMEL-28R cells

In previous studies, EGFR had been confirmed as...
Figure 4. Effects of Paclitaxel on Cancer Characteristics of SKMEL-28<sup>a</sup> Cells. SKMEL-28<sup>a</sup> Cells Were Treated With or Without 100 nM Paclitaxel for 48 Hours. a Morphology of control SKMEL-28<sup>a</sup> cells and paclitaxel-treated SKMEL-28<sup>a</sup> cells. b Graph showing proliferation of SKMEL-28<sup>a</sup> cells with or without paclitaxel treatment. c Number of invading SKMEL-28<sup>a</sup> cells treated with 0 or 100 nM paclitaxel in the invasion assay is shown in photographs and d in a graph. e Colonies in SKMEL-28<sup>a</sup> cells with or without treatment with 100 nM paclitaxel in the colony formation assay are shown in photos and f in a graph. g Apoptosis of SKMEL-28<sup>a</sup> cells with or without treatment with 100 nM paclitaxel in the Annexin V Apoptosis assay are shown in photos and h in a graph. * and ** Significantly different (p<0.05, p<0.01 respectively) from the control by Student’s t-test.

Figure 5. Effects of paclitaxel on protein levels of EGFR, AKT and phosphorylation levels of pTyr1173-EGFR, pThr308-AKT and pSer473-AKT in SKMEL-28<sup>a</sup> cell lines. a Protein and phosphorylation levels of EGFR, AKT and p-AKTs were measured by immunoblotting. TUBULIN was used as an internal control. Lane 1: control SKMEL-28<sup>a</sup>; lane 2: SKMEL-28<sup>a</sup>; lane 3: MK-2206-treated SKMEL-28<sup>a</sup>; lane 4: Paclitaxel-treated SKMEL-28<sup>a</sup> and lane 5: MK-2206 and paclitaxel-treated SKMEL-28<sup>a</sup>. b and c Localization and Protein expression levels of EGFR in SKMEL-28<sup>a</sup> with or without paclitaxel treatment were measured by Immunocytochemistry assay.
signaling pathway. We also showed that SKMEL-28 cells resisted to vemurafenib via overexpression of EGFR, pTyr1173-EGFR, pThr308-AKT and pSer473-AKT. These results are in accordance with previous reports (Chen et al., 2012; Gross et al., 2014). Vemurafenib-induced pAKTs were inhibited by MK-2206, an AKT inhibitor or by paclitaxel or by combined MK-2206 and paclitaxel (Figure 5a). Although paclitaxel has been used the treatment of many types of cancers including melanoma, this study is the first study showing that paclitaxel can induce apoptosis and inhibit cancer characteristics of SKMEL-28 cells including proliferation, invasion and colony formation via decreasing of EGFR, pEGFR and pAKT, which were upregulated in SKMEL-28 cells.

Our results suggest that the combination of vemurafenib and paclitaxel should be used to treat induced-pAKT metastatic melanoma tumors, formed by the metastasis of SKMEL-28 cells (Kawada et al., 2004; Peinado et al., 2012), including lymph nodes, lung, liver, brain and bone marrow.

Acknowledgements

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106-NN.02-2013.07. Fee of proof reading for English grammar was funded by Toyoaki Scholarship Foundation. We would like to thank Dr. Nguyen Thi Van Anh for her support and Ms. Bui Thi Van Khanh for her technical assistance.

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