Sunitinib induced resistance of endothelial cells by up-regulating P-glycoprotein and PI3K/Akt pathway

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Drug resistance is a crucial obstacle to achieve satisfactory chemotherapeutic effects. Numerous studies have shown that the PI3K/Akt signaling pathway plays a significant role in various processes of cellular events and tumor progression, while few studies have focused on the PI3K/Akt signaling pathway in drug resistance of endothelial cells. The present study aims to explore the relationship of PI3K/Akt signaling and cellular resistance to anticancer drugs in human microvessel endothelial cells (HMEC-1). We established stable sunitinib-resiatant human microvessel endothelial cells (HMEC-su) after long-term exposure to sunitinib (a small-molecule tyrosine kinase receptor inhibitor) for 12 months. HMEC-su showed significant alternations of cell morphology and exhibited a 2.32-fold higher IC50 of sunitinib than parental HMEC-1 cells. Expression of P-glycoprotein (P-gp) and breast cancer-resistance protein (ABCG2) which mediates drug efflux, increased significantly in HMEC-su lines compared with HMEC-1 cells by western blots assay. Our study further demonstrates that LY294002 (blocking the PI3K/Akt pathway) enhances the sensibility of HMEC-su to suntinib and inhibits the gene transcription and protein expression of P-gp, ABCG2 in HMEC-su cells. In conclusion, these results indicate that LY294002 could reverse P-gp and ABCG2 mediated-drug resistance to sunitinib in HMEC-su cells by inhibiting PI3K/Akt signaling.

Keywords: Drug resistance. Endothelial cells. P-glycoprotein. Sunitinib. PI3K/Akt pathway.

INTRODUCTION

Drug resistance is a major clinical problem in chemotherapy for cancer treatment. For decades, great efforts have been made to understand the underlying mechanisms which seem to be very complex in the tumor microenvironment. Nowadays, it is known that overexpression of specific trans-membrane proteins, known as ATP-binding cassette (ABC) transporters, plays a determinant role in ATP-dependent efflux of exogenous materials or chemotherapeutics from intracellular compartments (Brayboy *et al.*, 2017). The ABC transporter superfamily contains 7 subfamilies and 48 family members. Over-expression of the ABC transporters in cancer cells has been shown to be responsible for the development of chemo-resistance, such as P-gp (MDR1/ABCB1), ABCG2 (BCRP/MXR/ABCP), and Multidrug Resistance Proteins (MRPs) (De Gooijer *et al.*, 2018; Karthikeyan, Hoti, 2015; Kathawala *et al.*, 2015). Over-expression of these ABC transporters in cancer cells with drug resistance has been repeatedly reported clinically. Tumor angiogenesis is essential for tumor growth as these vessels supply oxygen and nutrients to the tumor cells, and also facilitate tumor metastasis via blood stream. As endothelial cells (ECs) play a crucial role in angiogenesis,

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our study as well as the previous ones provides evidence that endothelial cells acquire drug resistance in tumor microenvironment (Hida *et al.*, 2018; Huang, Christelle, Coelho-Martins, 2013; Zecchin *et al.*, 2017).

Clinic use of tyrosine kinases inhibitors (TKIs) has been adapted clinically in non-small cell lung cancer (NSCLC) (Chen *et al.*, 2018; Na *et al.*, 2017), breast cancer (Shah *et al.*, 2018; Yazdi *et al.*, 2017), and colon cancer (Zhang *et al.*, 2017; O'Neil *et al.*, 2014). Among these TKIs, sunitinib (Sutent TM) is an oral antiangiogenic drug, which targets c-KIT, platelet-derived growth factor receptors α and β (PDGFR α and β), vascular endothelial growth factor receptors (VEGFR) 1, 2, and 3, and other kinases (Li *et al.*, 2018). It has been shown that repetitive use of TKIs in clinical treatment, induces drug resistance due to up-regulation of ABC transporters in cancer cells (Fan *et al.*, 2018; Peng *et al.*, 2012; Katayama *et al.*, 2016). There are some small molecules that specifically block ABC transporters. Examples are verapamil (VRP, blocking P-gp function), and fumitremorgin C (FTC, blocking ABCG2 function). They have been shown to recover the sensitivity of cancer cells to chemotherapeutic drugs significantly; however, their clinical use is limited by safety concerns due to their high toxity and low specificity (Nobili *et al.*, 2006; Breier *et al.*, 2014). Thereby, we need to look for high efficacy novel agents with fewer side effects to overcome drug resistance.

PI3K/Akt signaling pathway regulates cell proliferation, survival, apoptosis, and recently it has been shown to induce drug resistance (Li *et al.*, 2012; Guerrouahen *et al.*, 2014). More interestingly, it has been reported that inhibition of the PI3K/Akt signaling pathway by LY294002, a specific pharmacological inhibitor of PI3K, significantly enhances the cancer cell sensitivity to chemotherapeutic drugs and reverses the ABC transporter proteins-mediated drug resistance (Imai *et al.*, 2012; Liu *et al.*, 2015; Xie *et al.*, 2013). Hence we speculate that the PI3K/Akt signaling pathway could be involved in acquired resistance to TKIs in the endothelial cells.

The aim of the current study was to investigate the role of PI3K/Akt signaling pathway in induction of expression of the ABC transporters in human micro vessel endothelial cells (HMEC-1) after long-term sunitinib treatment. We investigated the overexpression of P-gp gene and protein in the acquisition of resistance to sunitinib and evaluated the efficiency of the use of LY294002 to reverse P-gp-mediate drug resistance by modulating PI3K/Akt pathway in HMEC-1. This study is expected to be helpful in designing novel therapeutic strategies to overcome chemotherapy drug resistance.

MATERIAL AND METHODS

Reagents

The rabbit monoclonal anti-human P-gp (ab-170904) and ABCG2 (ab-108312) antibodies were purchased from Abcam (Cambridge, United Kingdom). Rabbit antibodies against human Akt (C67E7) and p-Akt (Ser473) were purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). P-gp inhibitor VRP (V106), ABCG2 inhibitor FTC (F9054) and LY294002 (L9908) were from Sigma-Aldrich (St. Louis, MO, USA). Sunitinib (S126061) was from Shanghai Aladdin Bio-Chem Technology Co (Shanghai, RPC).

In vitro assay

Cell culture

Human microvascular endothelial cell (HMEC-1) lines (C0546, Shanghai Guan Dao Biological Engineering Co, Ltd, Shanghai, China) were cultured in RPMI 1640 medium supplemented with 100 µg/mL streptomycin, 100 units/mL penicillin, and 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. To induce the sunitinib resistance in the cells, increasing doses of sunitinib (at concentration of 0.01 µM to 8 µM) were added into the culture media during a period of more than 12 months to obtain a stable sunitinib resistant cell lines (HMEC-su). No mutagenic agents were used in establishment of these HMEC-su resistance cell lines. All of the cells were digested with trypsin-EDTA once or twice a week during the whole experiment. Drug resistant cells were maintained in the culture media containing sunitinib at the concentration of 8 µM.

MTT cell proliferation assay

MTT assay was performed to evaluate cell viability as previously described (Huang, Christelle, Coelho-Martins 2013; Huang *et al.,* 2014). Cells were seeded in 96-well plates (7×10^3 cells/well) after 24 h of inoculation in a medium with 10 % fetal bovine serum concentration. Cells were then treated for 72 h with different concentrations of Sunitinib (5 μM to 20 μM) with unaltered serum concentration or the same concentration of DMSO as control. Then plates were washed for 3 times to remove detached cells before use. After adding 20 μL of MTT reagents at concentration of 5 mg/mL dissolved into 200 μL of phosphate-buffered saline (PBS) per well, plates were incubated at 37 °C for 4 h. Formazan salt crystals were dissolved by dimethylsulfoxide and added into the wells. The absorbance value of each well was determined at 490 nm using a microplate reader (ELX800; GE, USA). Half maximal inhibitory concentration (IC_{50}) was defined as the concentration of drug producing 50% inhibition of cell growth. The resistance index (RI) was calculated as the ratio of IC_{50} values between the resistant and parental cells.

Blocking effect assay

We used P-gp inhibitor VRP at 100 μM, ABCG2 inhibitor FTC at 5 μM, or PI3K/Akt blocker LY294002 at 10 μM to treat the cells for 24 h or 48 h, and then the cell viability was assessed by the MTT assay. The reversal fold value (RF) was used to determine their efficiency to reverse the drug resistance. It was obtained by calculating the ratio of IC_{50} of the cells treated by sunitinib (at 5-20**μM)** alone versus IC_{50} treated by sunitinib (at 5-20**μM)** plus a given inhibitor. All analyses were performed in triplicates and the data were presented as means ± standard deviation (SD).

Western blotting

Western blot assay was performed to detect the protein expression of P-gp and ABCG2 in parental cells,

HMEC-1, and resistance cells HMEC-su. The cultured cells in the 6-well plates were washed with PBS twice and protein was extracted using proper amount of lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). The same amount of protein (30 **μg/lane) was added to each lane and** separated by 8 or 10% SDS-PAGE gel (Beyotime Institute of Biotechnology) and transferred to polyvinylidine difluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked by 5% skimmed dry milk in TBS containing 0.2% Tween-20 at room temperature for 1 h, and then incubated overnight at 4°C with 1:1000 diluted anti-human P-gp, ABCG2, and Akt,p-Akt antibodies, or with the antibodies against β-actin (1:10000), Tubulin (1:10000). After washed for three times in TBS/Tween buffer, the membranes were incubated with labelled goat anti-rabbit or goat anti-mouse IgG (1:10,000; Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd., Beijing) for 1 h at room temperature. The blots were visualized using an enhanced chemiluminescence reagent kit (EMD Millipore). Digital images of the blots were photographed using ChemiDoc XRS+ system and analyzed with Image Lab™ Software, Version 5.2 (BioRad, Hercules, CA, USA).

Quantitative real time PCR (qRT-PCR)

After treatment by 100 μM VRP or 5 μM FTC for 48 h, HMEC-1 and HMEC-su cells were harvested and total RNA was extracted using an TransZol Up Plus RNA Kit (ER501, Transgen, Beijing, China), and cDNA was synthesized using an *easyScrip* One-Step gDNA Removal and cDNA Synthesis SuperMix (AE311, Transgen). The mRNA levels were measured using a *TransStart* Tip Green aPCR Super Mix (AQ141, Transgen) and a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The primers were synthesized by Sangon Biotech (Shanghai, China) and are listed in the Supplementary Table I. The same amount of cDNA (1 μg/sample) was added to each tube in the experiment. The mRNA levels were quantified using the $2^{-\Delta\Delta Ct}$ method.

TABLE I - Primers used in reverse transcription-quantitative polymerase chain reaction

Gene	Primer sequence
MDR1	Forward: 5'-CCCATCATTGCAATAGCAGG-3'
	Reverse: 5'-GTTCAAACTTCTGCTCCTGA-3'
BCRP	Forward: 5'-CAGGTCTGTTGGTCAATCTCACA-3'
	Reverse: 5'-TCCATATCGTGGAATGCTGAAG-3'
β-actin	Forward: 5'-CCCATCATTGCAATAGCAGG-3'
	Reverse: 5'-GTTCAAACTTCTGCTCCTGA-3'

Data analysis

All data analysis and group comparisons were performed using student t-test and the one way methods of ANOVA with *Post-hoc* correction using GraphPad Prism 6. For all the results, *p*-values < 0.05 was considered as statistically significant. The data were expressed as means ± standard deviation (SD) or standard error (SE). We usually use ANOVA methods with Post-hoc correction to analyze qPCR data and cell proliferation/resistance assay and present SE in the figures. All data presented were obtained from at least three independent experiments.

RESULTS

Establishment of Sunitinib-resistant HMEC-su cell line

Chronic exposure to Sunitinib in the culture medium over 12 months altered the cell morphology (Figure 1A). The parental cells HMEC-1 were spindle-shaped, uniform in size, and with good transparency. However, there were many morphological changes in sunitinib-treated HMEC-su cells, including the increased number of antennae on the surface of individual cells, irregular shapes, and reduced transparency compared with the p arental cells. Morphological changes emerged after 6 months, and became obvious and established at 12 months (Figure 1B). In addition, the growth rate of the sunitinib-treated cells was slower than that of the parental HMEC-1 cells as previously described (Wu *et al.,* 2020).
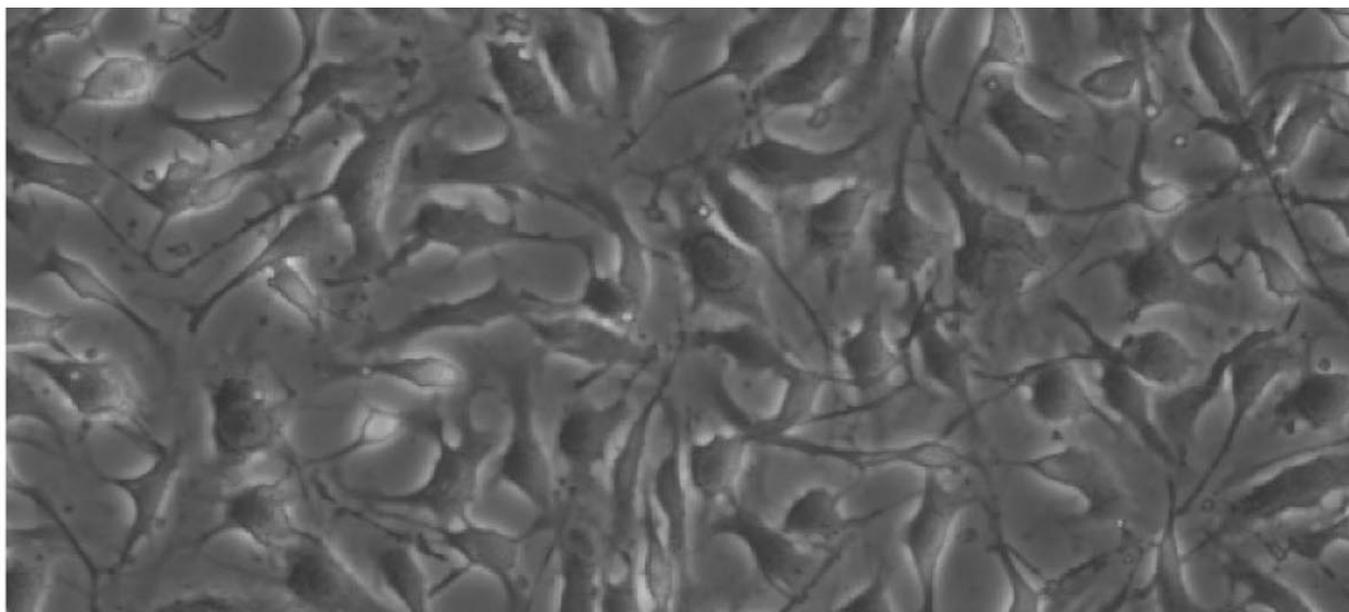
FIGURE 1 - Morphological changes induced by sunitinib. (A) Comparison of cellular morphology between parental cells HMEC-1 and sunitinib-resistant cells HMEC-su at 12 months. (B) Morphological changes of HMEC-su cells compared with HMEC-1 cells at indicated time points. ×200, Arrow indicates vacuole. Scale bar: 200 μm.

Endothelial cells resistances to Sunitinib

The established Su-resistant HMEC-su cell line was maintained in a culture media with sunitinib at a concentration of 8 μM. MTT assay demonstrated that the IC50 value of HMEC-su cells was significantly elevated compared with the IC50 value of HMEC-1 cells from 2 months to 12 months (Figure 2a). After a maintenance for 12 months, the IC50 of the HMEC-su cells was much higher than that that of the HMEC-1 cells (16.910 ± 0.561 μM vs. 7.275 ± 0.306 μM), suggesting that the sensitivity of resistant cells to sunitinib decreased (Table II and Figure 2b).

TABLE II - Acquired resistance of endothelial cells to sunitinib over 12 months

Agents	HMEC-1 IC$_{50}$ (μM)	HMEC-su IC$_{50}$ (μM)	Resistance Index (RI)
sunitinib	7.275 ± 0.306	16.910 ± 0.561	2.324**

HMEC-1 or HMEC-su were cultured for 72 hours at 37°C in the presence of increasing concentrations of sunitinib and put to MTT assay for detecting cell proliferation. The Resistance index (RI) was defined as the ratio of IC50 value of resistant cells to parental cells. **$P < 0.01$ compared with HMEC-1 cells. Abbreviations: HMEC-1(human microvascular endothelial cells), HMEC-su (sunitinib-resistant HMEC-1 cells), IC50(half-maximal inhibitory concentration).
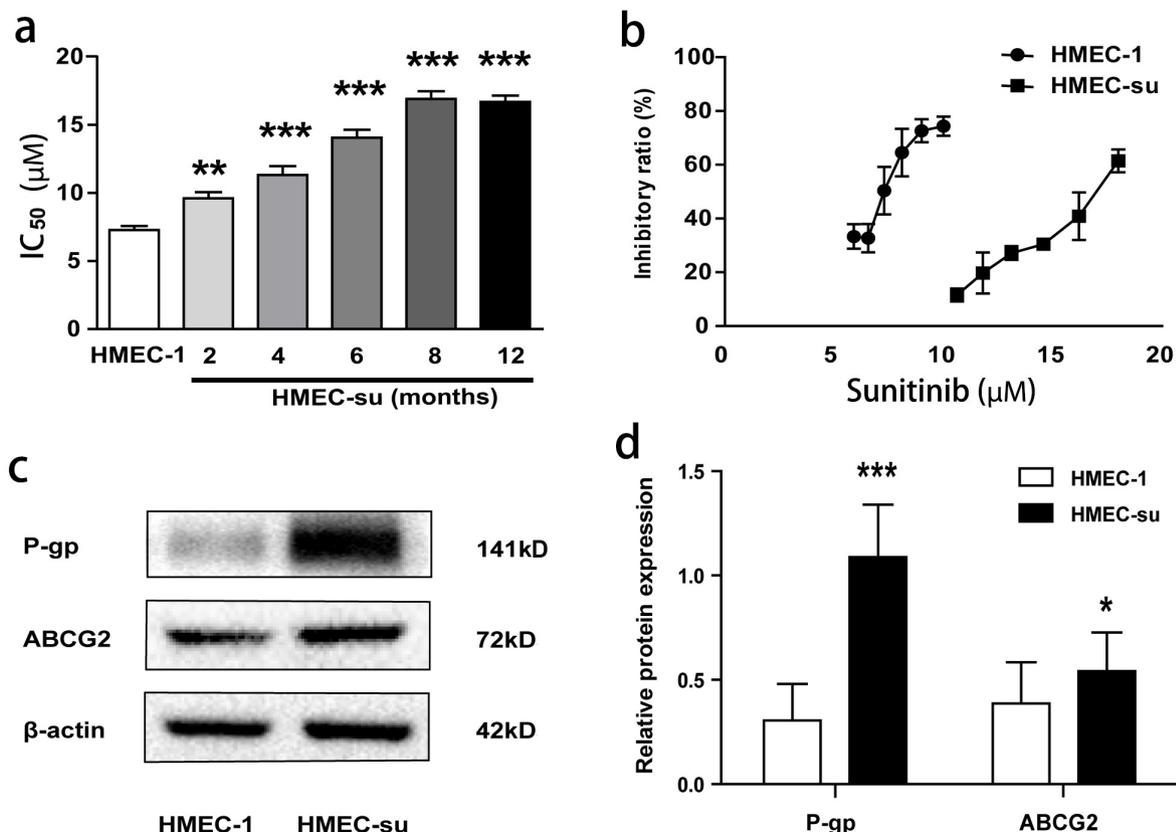
FIGURE 2 - Resistance to sunitinib and MDR protein expression in HMEC-1 cells and HMEC-su cells. (a) IC50 values of HMEC-su at different culture time. (b) Cell proliferation was determined by MTT assay, the inhibitory effects of sunitinib on HMEC-1 and HMEC-su was calculated after exposure to sunitinib at concentration of 5μM to 20 μM for 72 hours. The data shown are the means ± SD of triplicate determinations. **$P < 0.01$ was considered significant while ***$P < 0.001$ was highly significant, HMEC-su cells vs. HMEC-1 cells. (c) Elevated expression of P-gp and ABCG2 in HMEC-su cells in comparison with HMEC-1 cells. (d) Relative expression is shown, in which the intensity of bands for P-gp and ABCG2 was divided by corresponding β-actin band. All data were obtained from three independent western blot experiments. **$P < 0.01$ and *$P< 0.05$ vs. HMEC-1 cells.

Up-regulation of P-gp and ABCG2 in Sunitinib-resistant endothelial cells HMEC-su.

Our results demonstrated that the protein levels of P-gp and ABCG2 in HMEC-su cells increased approximately 3.61 fold and 1.51 fold, respectively (Figures 2c, d) compared with the parental cells. We also determined the changes of P-gp and ABCG2 mRNA levels by qRT-PCR, and showed the Sunitinib-induced up-regulation of P-gp and ABCG2 in HMEC-su cells (3.28-fold and 1.34-fold, respectively) (Figure S1). However, there was no statistical difference in ABCG2 mRNA level between HMEC-1 and HMEC-su cells. The data indicated that up-regulation of P-gp was in correlation with endothelial cell resistance to sunitinib. Time course study showed that the up-regulation of P-gp and p-Akt protein level could be discerned as early as 6 months after the treatment by sunitinib, and the up-regulation became apparent at 12 month (Figure 3ab).
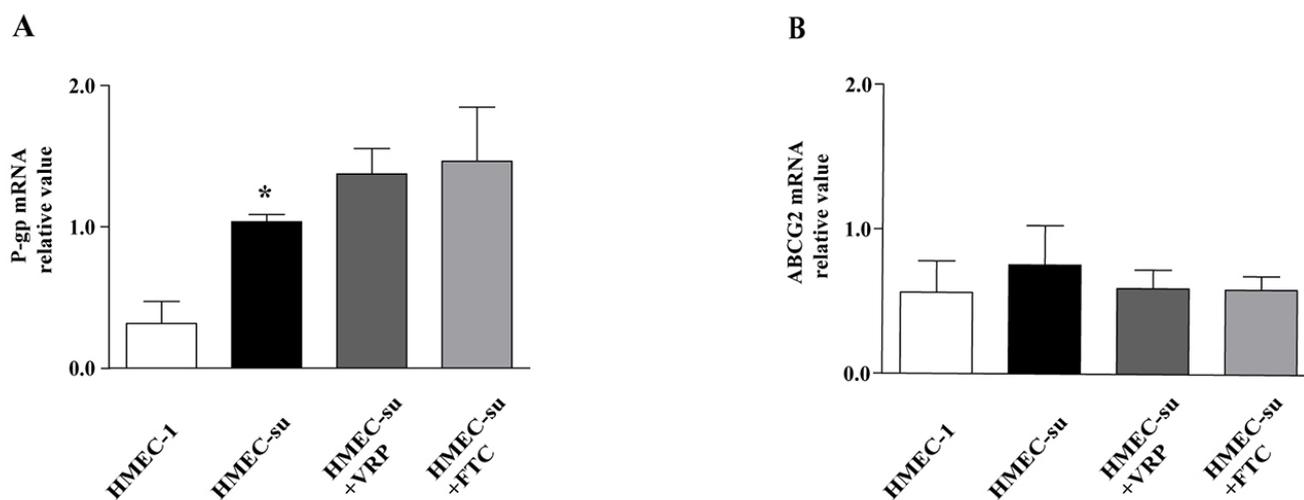
FIGURE S1 - The effect of P-gp and ABCG2 inhibitors on P-gp and ABCG2 gene expression. (A) P-gp and (B) ABCG2 mRNA level in HMEC-1 and HMEC-su cells using qRT-PCR, verapamil (VRP) at 100 µM and ABCG2 inhibitors fumitremorgin c (FTC) at 5 µM were used on both cells for 48h, respectively. $**P < 0.01$ and $*P < 0.05$ vs. HMEC-1 cells.
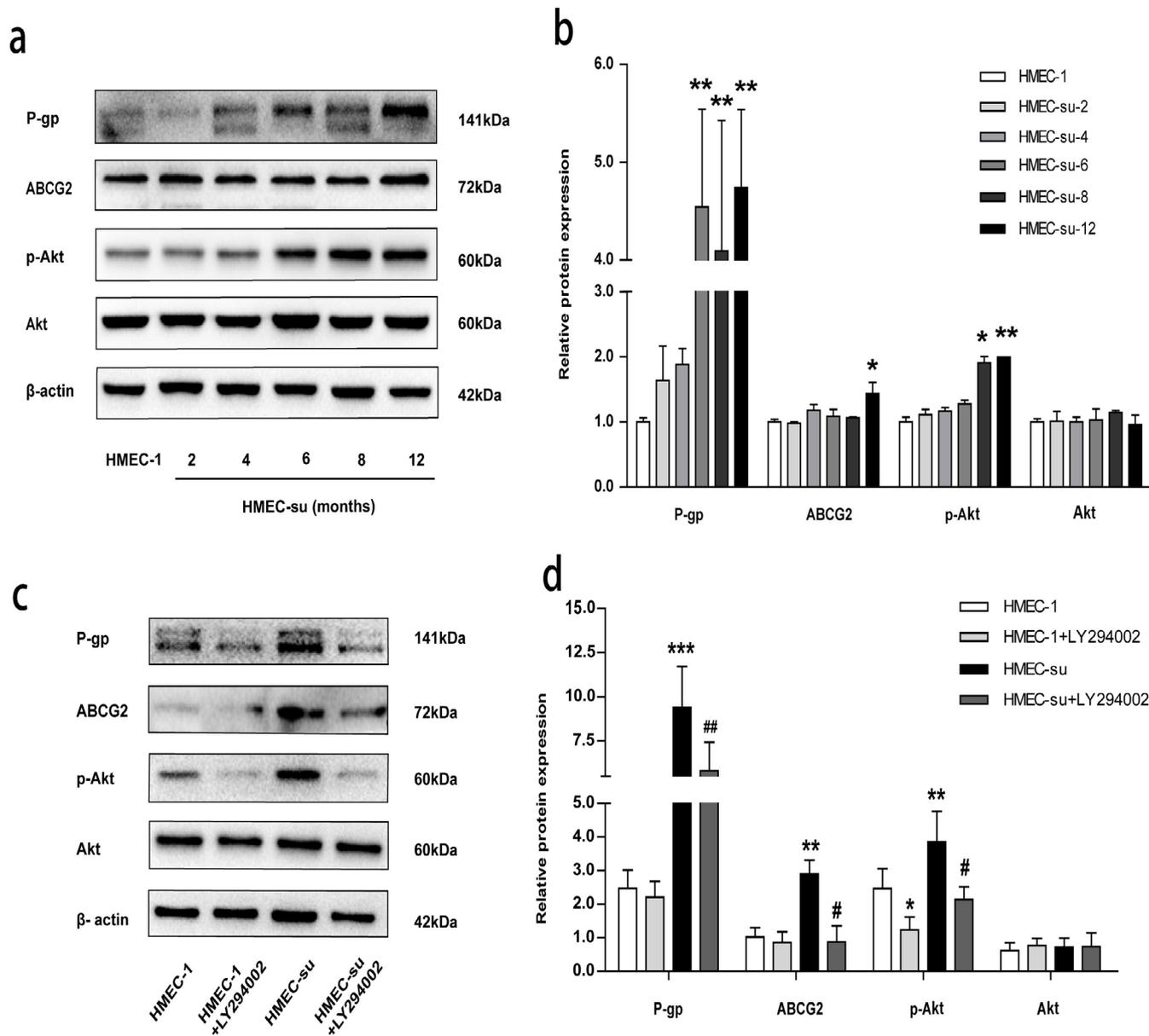
FIGURE 3 - Effect of LY294002 on MDR protein expression and Akt phosphorylation in HMEC-1 and HMEC-su cells. (a) The P-gp and ABCG2, p-Akt and Akt protein expressions in HMEC-su cells and HMEC-1 cells at different months were determined by western blotting and (b) data summary. All data were expressed as mean ± SD of three independent experiments, **$P < 0.01$ and *$P < 0.05$ vs. HMEC-1 cells. (c) LY294002 decreases protein expression of P-gp and ABCG2 in resistance cells HMEC-su at 10 µM after treatment for 24 h, (d) relative expression levels are shown, in which the intensity of bands were divided by corresponding β-actin band. All data are shown as the mean ± SD of three separated experiments, **$P < 0.01$ and *$P < 0.05$ vs. HMEC-1 cells, ##$P < 0.01$ and #$P < 0.05$ vs. HMEC-su cells.

Effect of ABC transporter inhibitors in resistant HMEC-su cells

To assess the effect of functional inhibitors of P-gp and ABCG2, 100 µM VRP and 5 µM FTC were used respectively, to determine the cell sensitivity by MTT assay (Table III). The results showed that blocking P-gp by VRP and ABCG2 by FTC in these cells did not decrease the resistance index of HMEC-su to sunitinib remarkably. Furthermore, qRT-PCR test also indicated

that VRP and FTC slightly decreased the expression of ABCG2; however, had no effect on the gene expression of P-gp in HMEC-su (Figure S1). In addition, treatment with either VRP or FTC alone caused few changes in the protein level of ABCG2 compared to the untreated resistant cells (Figure S2). Therefore, ABC transporter inhibitors VRP and FTC failed to restore drug sensitivity in these cells.

TABLE III - Effects of P-gp and ABCG2 inhibitors in resistance HMEC-su cells

Agents	IC$_{50}$ (µM)		RI	RF
	HMEC-1	HMEC-su		
sunitinib	7.757±0.130	16.910±0.750	2.324**	1.000
+VRP (100 µM)	7.181±0.162	15.120±0.947	2.110*	1.104
+FTC (5 µM)	6.961±0.131	15.595±0.982	2.172*	1.070

MTT assay was used to determine IC50 in the presence or absence of P-gp or ABCG2 inhibitors for 48 h followed by 72 hours of sunitinib treatment. The RI was the ratio of IC50 values between the resistant and parental cell lines. The resistance fold (RF) was calculated as the IC50 of HMEC-su in sunitinib-treatment group to the IC50 of HMEC-su in sunitinib/P-gp inhibitor (VRP: verapamil) or sunitinib/ABCG2 inhibitor (FTC: Fumitremorgin C) group, **P < 0.01, *P< 0.05 vs. HMEC-1 cells.
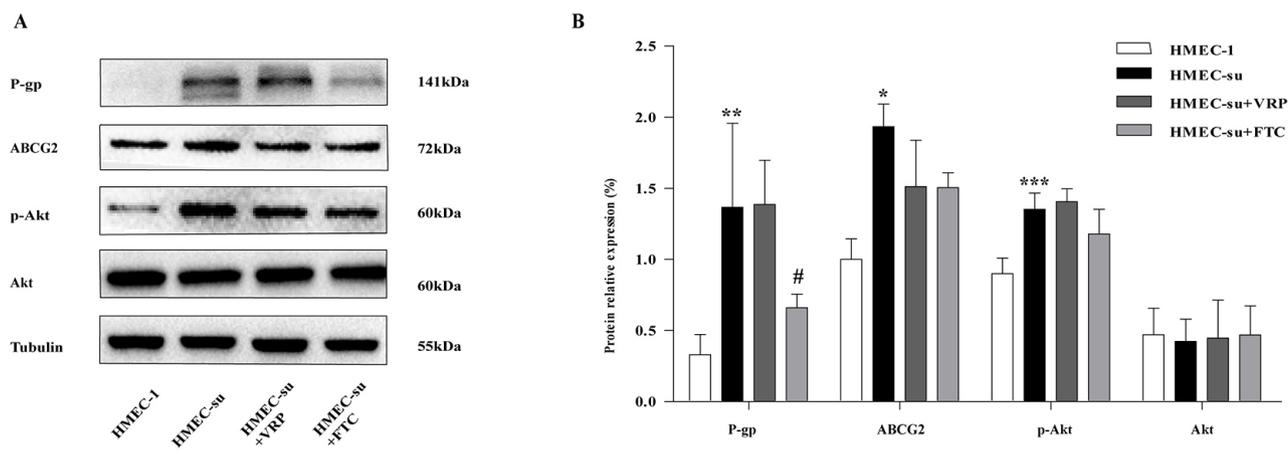
FIGURE S2 - Effect of P-gp and ABCG2 inhibitors on P-gp and ABCG2 proteins expression. Expression of P-gp, ABCG2 and p-Akt, Akt protein in HMEC-1 and HMEC-su cells was determined by Western blotting. Cells were treated with verapamil (VRP) at 100 µM and ABCG2 inhibitors fumitremorgin C (FTC) at 5 µM for 48h. All data were collected from three independent experiments, **P < 0.01 and *P < 0.05 vs. HMEC-1 cells, #P < 0.05 vs. HMEC-su cells.

LY294002 enhances the sensitivity of endothelial cells to sunitinib and decreases protein expression of P-gp and ABCG2 in resistant HMEC-su cells

The results of western blotting revealed an increased expression of p-Akt protein in HMEC-su cells compared with parental cells HMEC-1 (Figure 3a, b). To further investigate whether the PI3K/Akt signaling is involved in progression of the endothelial cell resistance to sunitinib, we evaluated the effects of LY294002 (which inhibits the PI3K activity), on HMEC-su cells. MTT assay results clearly showed that LY294002 remarkably restored sensitivity of the HMEC-su cells to Sunitinib, the IC50 values decreased from 16.695 ± 0.466 µM to 10.341 ±

0.664 µM (Table IV). Furthermore, addition of LY294002 significantly decreased the protein expression of p-Akt, P-gp and ABCG2 in HMEC-1 and HMEC-su cell lines (Figure 3c, d), but P-gp and ABCG2 inhibitors did not inhibit expression of the total Akt and p-Akt (Figure S2). Similarly, we also found that LY294002 could suppress the up-regulation of P-gp mRNA level in HMEC-su cells by qRT-PCR assay, as well as ABCG2 mRNA in HMEC-su cells (Figure 4). These results demonstrated that LY294002 can reverse ABC family-mediated drug resistance in endothelial cells to Sunitinib by inhibiting PI3K/Akt signaling.

TABLE IV - LY294002 enhances the sensitivity of endothelial cells to sunitinib

Agents	IC_{50} (µM)		RI	RF
	HMEC-1	HMEC-su		
sunitinib	7.275±0.306	16.695±0.460	2.295***	1.000
+LY294002 (10 µM)	6.002±0. 851	10.341±0.664	1.723**	1.614##

The IC50 value of HMEC-1 and HMEC-su cells after LY294002 treatment for 24 h followed by 72 hours of sunitinib exposure were detected by MTT assay, the RI was the ratio of IC50 values between the resistant and parental cell lines. The resistance fold (RF) was calculated as the IC50 of HMEC-su in sunitinib-treatment group to the IC50 of HMEC-su with LY294002 at 10 µM, ***P < 0.001 and **P< 0.01 vs. HMEC-1 cells. ##P < 0.01 and #P< 0.05 vs. HMEC-su cells.
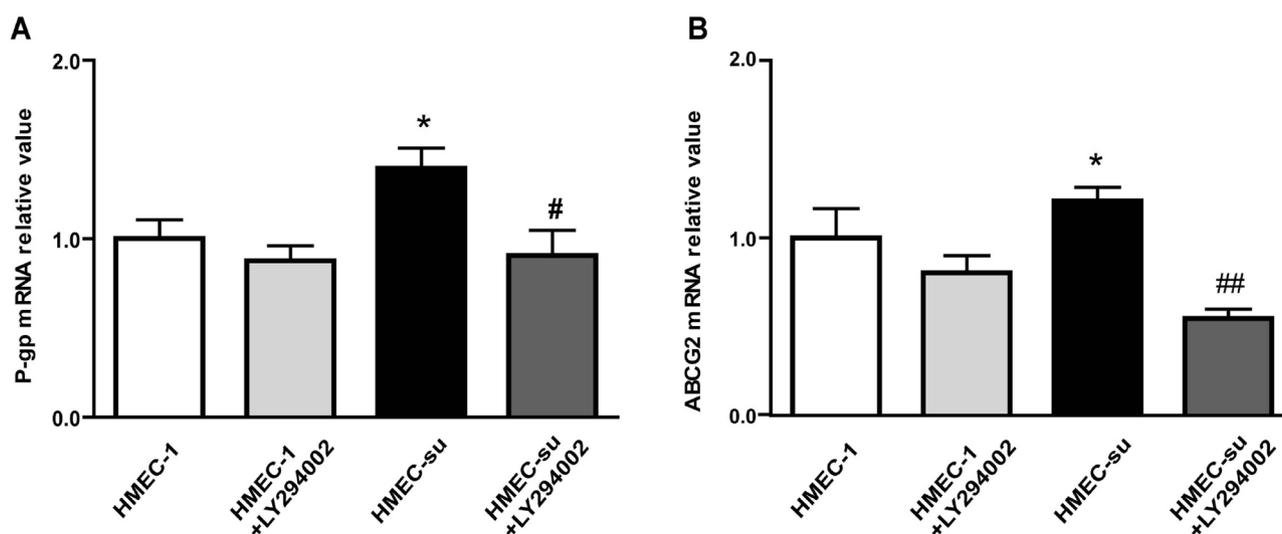
FIGURE 4 - LY294002 down-regulates gene level of (A) P-gp and (B) ABCG2 in resistant HMEC-su cells at 10 µM after 24 h treatments. Data reflect means ± SE values of three independent experiments with one-way ANOVA with Post-hoc correction. **P < 0.01 and *P< 0.05 vs. HMEC-1 cells, ##P< 0.01 and #P< 0.05 vs. HMEC-su cells.

DISCUSSION AND CONCLUSION

Previous studies have shown the tyrosine kinase inhibitor induced drug resistance in tumor cells and endothelial cells *in vitro*, and the resulting development of drug resistance with antiangiogenic therapy in cancer patients (Yazdi *et al.*, 2017; Anreddy *et al.*, 2014; Jayson *et al.*, 2016; Huang *et al.*, 2014). The emerged resistance

reduces their clinical efficacy. In the present study, we first showed the emerging drug resistance in endothelial cells after long-term exposure to Sunitinib *in vitro*. We found that the cell morphology of sunitinib-resistant endothelial cells has undergone significant changes, including an increase in the number of antennas on the cell membranes and irregular cell shapes. We demonstrated that the sunitinib-resistant cells HMEC-su exhibited enhanced gene expression of P-gp and ABCG2, and the up-regulation of these protein levels was demonstrated by western blots. These results provided evidence that resistance to sunitinib in these endothelial cells should be closely related to the over-expression of P-gp and ABCG2 molecules which induce efflux of drug from the cells.

The reversibility of resistance to sunitinib in the HMEC-1 cells was investigated in this study as well. Previous reports have shown the effectiveness of ABC transporter inhibitors in inhibiting drug resistance. For example, verapamil inhibited the increased level of drug efflux in FL/Doxo cells and MK571 and verapamil abolished doxorubicin resistance and act as chemosensitizers for DOX therapy in five different NSCLC-derived cell lines (Wu *et al.*, 2017; Liu *et al.*, 2017). We attempted to use the same strategy to suppress sunitinib resistance in HMEC-1 cells but we found that direct inhibition of the ABCG2 by FTC or P-gp by verapamil was not sufficient to reverse the drug resistance of cells to sunitinib. This could be due to the fact that competitive inhibition of the binding sites of the ABC molecules was not sufficient when these molecules were over-expressed, since verapamil and FTC didn't suppress the over-expression of P-gp and ABCG2 genes in HMEC-su in our study. Our results are consistent with other reports in this regard that inhibitors of ABC transporters including cyclosporin A, verapamil, fumitremorgin C, diethylstilbestrol and MK571 had no effect on multidrug resistance of HMEC-su cells (Huang *et al.*, 2014). In fact, sunitinib and the other TKIs that exhibit substrate-like properties for ABC transporters, also inhibit function of the transporter at lower concentrations by oversaturation of ABC transporters (Anreddy *et al.*, 2014; Elgendy, 2017). Therefore, the inefficiency of ABC transporter blockers in our study has also prompted the search for new agents that could more effectively reverse cancer resistance.

Interestingly, we found that the HMEC-su drug resistant cells exhibited higher PI3K/Akt activity than their parental HMEC-1 cells. This indicated a possible relationship between PI3K/Akt signaling pathway and sunitinib resistance. Therefore LY294002 was used as it blocks the phosphorylation of Akt and PI3K mediated downstream events. More of interest, LY294002 was reported to regulate the cell responses to exogenous substance and anticancer drugs (Xie *et al.*, 2013; Liu *et al.*, 2015; Zhou *et al.*, 2016; Huang *et al.*, 2017; Sui *et al.*, 2014). Our results demonstrated that LY294002 significantly improved the sunitinib sensitivity of HMEC-su cells at 8 µM. We further showed that long-term sunitinib treatment upregulated P-gp 3.61-folds and ABCG2 1.51-folds, however, the addition of LY294002 blocked significantly the gene expressions of P-gp and ABCG2, as well as their protein levels in these HMEC-su cells. As comparison, there was no gene expression change of total Akt in response to LY294002 treatment, showing that this effect was specific for P-gp and ABCG2. Therefore, for the first time, we observed an increase in the chemosensitivity of endothelial cells to sunitinib by blocking the PI3K/Akt pathway, thereby inhibiting the expression of P-gp and ABCG2. These results indicate an alternative way to develop a sensitizer as a new auxiliary agent for sunitinib treatment. Since PI3K/Akt signaling pathway affects cells broadly, the underlying mechanisms that are relevant to the results described above still needs to be explored in further detail. Nevertheless, our findings are encouraging because there is no suitable drug available to reverse the acquired drug resistance to TKIs in clinic. We believe that an understanding of these mechanisms shall be helpful in conceiving the new pharmaceutical strategies and overcoming the resistance to sunitinib and other TKIs in clinic.

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The Lists of Abbreviations

Abbreviations	Expanded Meanings
ABC	ATP binding cassette proteins
ABCG2	Breast cancer-resistance protein
APS	Ammonium persulfate
BSA	Bovine serum albumin
BCA	Bicinchoninic acid
β-actin	Beta-muscle actin
DMSO	Dimethyl sulphoxide
ECL	Enhanced chemiluminescence
ECs	Endothelial cells
FTC	Fmitremorgin C
HMEC-1	Human microvessel endothelial cells
HMEC-su	Sunitinib-resistance human microvessel endothelial cells
MTT	3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
MDR	Multidrug resistance
MRP1/ABCC1	Multidrug resistance-associated proteins
OD	Optical density
P-gp/ABCB1	P-glycoprotein
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene difluoride
PDGFR	Platelet-derived growth factor receptors
Su	Sunitinib
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
TKIs	Tyrosine kinases inhibitors
VECs	Vascular endothelial cells
VRP	Verapamil
VEGFR	Vascular endothelial growth factor receptors