Upregulation of ABCG2 via the PI3K-Akt pathway contributes to acidic microenvironment-induced cisplatin resistance in A549 and LTEP-a-2 lung cancer cells

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Abstract. Hypoxia always exists in the processes involved in the development of lung cancer and contributes to an acidic microenvironment. Despite that hypoxia in the tumor microenvironment is associated with the formation of chemotherapeutic resistance, the exact role of an acidic microenvironment in the development of hypoxia-induced lung cancer multidrug resistance is still unknown. In the present study, we acidized the medium with 2-(*N*-morpholino)-ethanesulfonic acid (MES monohydrate) to mimic the acidic tumor microenvironment and observed the effects of acidification on lung cancer cell viability, the expression of ATP-binding cassette sub-family G member 2 (ABCG2) and myeloid cell leukemia-1 (Mcl-1), and activation of the PI3K-Akt pathway. Thereafter, we investigated the mechanisms involved in the acidification-induced expression of ABCG2 and Mcl-1, and the potential therapeutic strategy to overcome acidification-associated multidrug resistance formation. We demonstrated that acidification obviously increased the expression of ABCG2 and Mcl-1 via PI3K-Akt-mTOR-S6 pathway activation and contributed to multidrug resistance. Inhibition of PI3K-Akt activity efficiently abolished the effect of acidification on cell viability, indicating that the PI3K-Akt pathway may include potential therapeutic target molecules in acidized microenvironment-associated lung cancer chemotherapeutic resistance.

Introduction

Small cell lung cancer (SCLC), which accounts for ~15% of all lung cancer cases, is the most aggressive metastatic form of lung cancer and does not respond well to surgery or radiotherapy (1). Chemotherapeutic resistance is closely associated with multidrug resistance (MDR). Although a relatively good response can be achieved in the initial stages of lung cancer chemotherapy, chemotherapeutic resistance can develop quickly after initial chemotherapy (2-4). Hence, chemotherapeutic resistance, particularly MDR, is a major obstacle for successful SCLC chemotherapy.

Tumor tissues are composed of tumor cells, fibroblasts and immune cells, which secret pro-inflammatory cytokines such as IL-2, IL-1 and IL-6, and thereby affect the abilities of cell proliferation in the microenvironment (5,6). Hence, hypoxia always exists in the process of tumor tissue development. It was reported that hypoxia-induced acidification may cause this resistance by decreasing cellular uptake along with a lowered cytotoxicity due to pH-dependent topoisomerase type II activity (7). Meanwhile, MDR is also characterized by a reversal of the pH gradient across cell membranes leading to an acidification of the outer milieu and an alkalinization of the cytosol that is maintained by the proton pump vacuolar-type ATPase (V-ATPase) (8,9). ATP-binding cassette transporter proteins such as ATP-binding cassette (ABC) transporters P-glycoprotein (P-gp; MDR1), multidrug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP/ABCG2) (10,11), transporting a wide variety of chemical compounds in an ATP-dependent manner, have been found to contribute to MDR formation in a variety of tumors arising from gastric, renal, endometrium, melanoma and soft tissue (12,13). Our previous studies showed that the upregulation of ABCG2 facilitates MDR formation in lung cancer (14); however, the role of ABCG2 in acidification associated MDR formation is still uncertain.

The PI3K/AKT/mTOR pathway is an intracellular signaling pathway important in regulating the cell cycle. Therefore, it is directly related to cellular quiescence, proliferation, cancer and longevity (15). Apart from the key roles of Akt in regulating co-stimulator molecule expression in dendritic cells (16-18), the activation of Akt has been documented to regulate V-ATPase expression and induce MDR in different types of tumor (19-21). Hence, the activation of Akt may be a key regulator in tumor MDR formation. However, to date, the role of PI3K-Akt activation in acidification-induced chemoresistance is still unclear.

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In the present study, we modified the pH value of the medium to mimic the tumor acidic microenvironment and investigated the effects of acidification on cell viability, the expression of ATP-binding cassette transporter proteins, and activation of PI3K-Akt. We demonstrated that acidification obviously increased the expression of ABCG2, myeloid cell leukemia-1 (Mcl-1) via PI3K-Akt-mTOR-S6 pathway activation and contributed to MDR. The inhibition of PI3K-Akt activity efficiently abolished the effect of acidification on cell viability, indicating that the PI3K-Akt pathway may include potential therapeutic target molecules in acidized microenvironment-associated lung cancer chemotherapeutic resistance.

Materials and methods

Reagents and antibodies. 2-(*N*-morpholino)ethanesulfonic acid (MES monohydrate) and primers were purchased from Sangon Biotech (Shanghai, China). Cisplatin was purchased from Calbiochem (San Diego, CA, USA). Antibody to ABCG2, antibody to Mcl-1, antibodies to phospho and total kinases were acquired from Cell Signaling Technology (Beverly, MA, USA). Annexin V/propidium iodide (PI) apoptosis detection kit was obtained from KeyGen Biotech (Nanjing, China). RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were acquired from HyClone (Logan, UT, USA). SYBR Premix Ex Taq, TRIzol and PrimeScript reverse transcriptase were obtained from Takara Biotechnology (Dalian, China).

Cell lines. LTEP-a-2 and A549 cells were maintained in our laboratory as previously described (14). Cells were synchronized by serum starvation for at least 6 h before acidification treatment.

Flow cytometric measurements. Cell apoptosis was assayed as previously described (14). Briefly, A549 and LTEP-a-2 cells were pretreated with pH 6.6 for 2 h. Then, the acidized medium was replaced with normal medium for 48 h. After that, the cells were cultured for 24 h in the presence of cisplatin (DDP) (4 μg/ml for A549 cells and 8 μg/ml for LTEP-a-2 cells). The cells were stained with Annexin V-FITC and PI for 20 min at room temperature. Flow cytometry was performed using a FACSCalibur flow cytometer, and the data were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA).

Quantitative PCR. The effects of acidification on MDR-related protein expression were investigated via real-time PCR analyses, as previously described (14). Briefly, whole cellular RNA was extracted, and reverse transcription was performed using PrimeScript reverse transcriptase. To quantify gene amplification, real-time PCR analysis was performed using an ABI 7500 Sequence Detection system in the presence of SYBR-Green (Takara Biotechnology). The cycling parameters were 95˚C for 5 min, followed by 32 cycles of 95˚C for 5 sec, 55˚C for 30 sec and 72˚C for 60 sec, with a final extension at 72˚C for 10 min; a melting curve analysis was subsequently conducted. The relative expression levels (defined as fold-changes) of the target genes were normalized to the folds of the corresponding control cells. The primer sequences outlined in Table I were used in these assays.

Table I. Primer sequences.

Genes	F/R	Sequence
β-actin	F	5'-TCAAGATCATTGCTCCTCCTG-3'
	R	5'-CTGCTTGCTGATCCACATCTG-3'
ABCG2	F	5'-ACTGGCTTAGACTCAAGCACA-3'
	R	5'-ATAGGCCTCACAGTGATAACCA-3'
Mcl-1	F	5'-TGCAGGTGTTGCTGGAGTAG-3'
	R	5'-CCTCTTGCCACTTGCTTTTC-3'

F, forward; R, reverse; ABCG2, ATP-binding cassette sub-family G member 2; Mcl-1, myeloid cell leukemia-1.

Western blot analysis. The cells were treated with pH 6.6 for 2 h and the expression of related proteins was determined via western blot analysis as previously described (16,17). Briefly, proteins were obtained in lysis buffer and loaded onto SDS-PAGE gels for electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking in 5% fat-free milk in Tris-buffered saline and Tween-20 (TBST) for 90 min, the membranes were incubated with primary antibodies at 4˚C overnight. Subsequently, the membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 90 min. After washing 4 times with TBST (for 10 min each), the bound antibodies were visualized using enhanced chemiluminescence (ECL). β-actin was used as a loading control.

Statistical analysis. All experiments were repeated at least three times to confirm the results. The data are presented as the mean ± SEM. Student's t-test and one-way ANOVA with the Newman-Keuls post test were applied. Differences were considered significant at $p<0.05$.

Results

Treatment with acidification increases the chemotherapeutic resistance in A549 and LTEP-a-2 lung cancer cells. MDR formation is the important factor in lung cancer therapeutic failure (22). The tumor microenvironment, which consists of tumor cells, extracellular matrix, immune cells and fibroblasts, is closely involved in MDR formation (23). As the high ability of cell proliferation to blood supply, there was exactly acidized environment in tumor (9). To explore the effect of acidized microenvironment on chemotherapeutic resistance, A549 and LTEP-a-2 cells were firstly treated with MES monohydrate and the value of pH in the medium was determined. As shown in Fig. 1A, the value of pH in the medium of the A549 and LTEP-a-2 cells was controlled by the addition of different volumes of MES monohydrate. The co-relationship index of the standard curve was 0.998 and 0.976 for A549 and LTEP-a-2 cells, respectively, which indicated that the pH value in the medium of cultured cells could be exactly modified. Cell viability observations by crystal violet staining showed that the modification of the pH value from normal to pH 6.6 obviously increased the cell viability in the presence

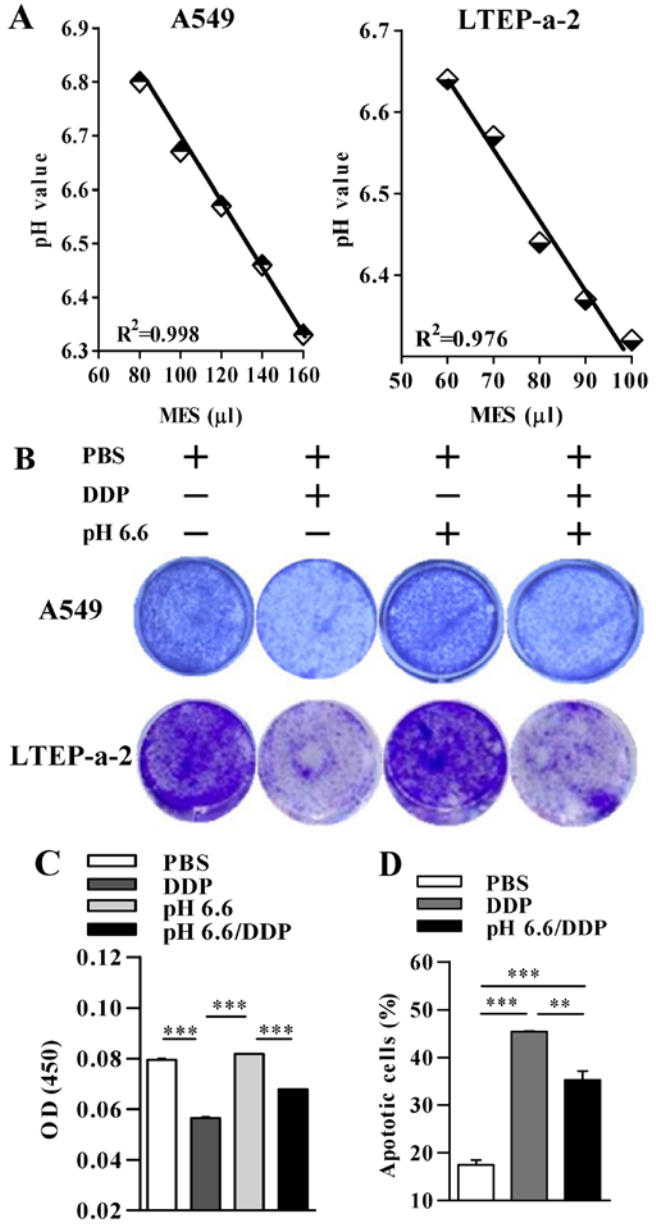

Figure 1. Treatment with acidification increases chemotherapeutic resistance in the A549 and LTEP-a-2 lung cancer cells. To explore the effect of acidification on chemotherapeutic resistance formation, the medium of cultured A549 and LTEP-a-2 cells was treated with different volumes of MES monohydrate, and the value of pH in the medium was determined. (A) The standard curve of A549 and LTEP-a-2 cells was calculated. A549 and LTEP-a-2 cells were firstly treated with MES monohydrate to acidize the medium to pH 6.6 and were maintained for 2 h. Then, the acidic medium was replaced with normal medium and the cells were cultured for 48 h in the presence of cisplatin (DDP) (4 μg/ml for A549 cells and 8 μg/ml for LTEP-a-2 cells). After that, the cells were cultured in pH 7.4 medium and the cell viability was determined by crystal violet staining (B and C) or flow cytometric analyses (D), respectively. The data are presented as the mean ± SEM; **p<0.01, ***p<0.001, one-way ANOVA with Newman-Keuls post test. One representative from 3 independent experiments is shown. DDP, cisplatin.

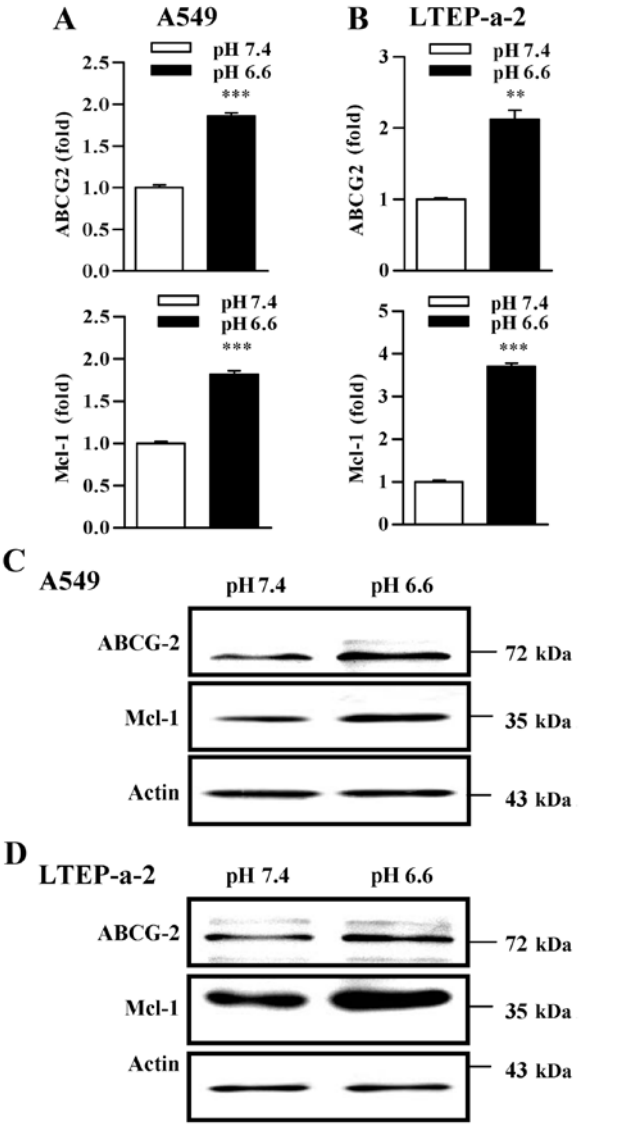

Figure 2. Microenvironment of acidification increases the expression of ABCG2 and Mcl-1 in the A549 and LTEP-a-2 lung cancer cells. To investigate the effect of an acidic microenvironment on chemotherapeutic resistance formation, A549 (A and C) and LTEP-a-2 (B and D) cells were exposed to treatment with pH 6.6 acidification and the whole cellular protein or RNA was extracted. cDNA was reverse transcripted from total RNA and the expression of ABCG2 and anti-apoptotic protein Mcl-1 was determined by RT-qPCR (A and B) and western blot (C and D) analyses, respectively. The data are presented as the mean ± SEM; **p<0.01, ***p<0.001, Student's t-test. One representative from 3 independent experiments is shown.

of cisplatin in both the A549 and LTEP-a-2 cells (Fig. 1B). Analyses of the light absorption value also revealed that a pH 6.6 microenvironment attenuated cisplatin-induced cell death (Fig. 1C). The flow cytometric analyses of cell apoptosis by Annexin V/PI staining demonstrated that whereas the treatment with cisplatin induced ~45% apoptosis in the A549 cells, pH 6.6 acidification of the medium efficiently decreased the percentage of Annexin V/PI-positive cells, which revealed an ~22.2% inhibitory rate (Fig. 1D). All of these results indicate that an acidized microenvironment contributes to MDR formation in lung cancer cells.

Acidification of the microenvironment increases the expression of ABCG2 and Mcl-1 in the A549 and LTEP-a-2 lung cancer cells. Our previous study showed that ABCG2 and anti-apoptotic genes such as Mcl-1 and Bcl-2 could be upregulated and contribute to cisplatin-induced MDR (14). As the treatment with acidic medium increased lung cancer cell viability and attenuated cisplatin-induced apoptosis (Fig. 1),

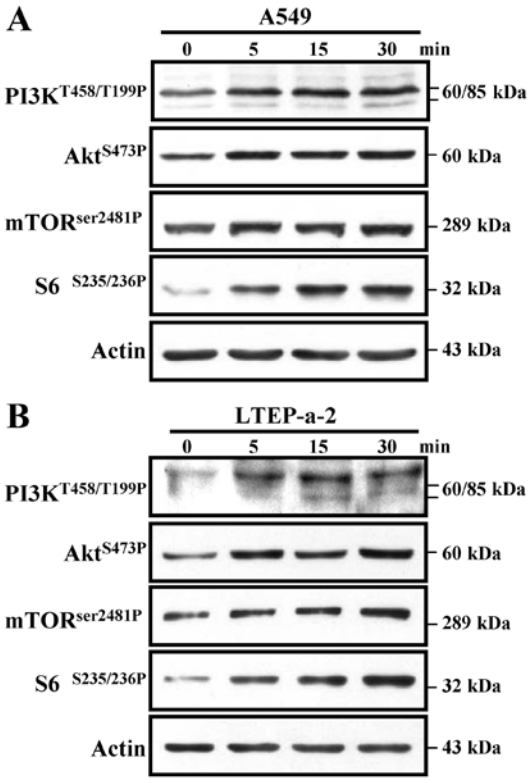

Figure 3. Microenvironment of acidification obviously increases the phosphorylation of PI3K-Akt-mTOR-S6 in A549 and LTEP-a-2 lung cancer cells. To explore the mechanism of acidic microenvironment-increased chemotherapeutic resistance formation, the effect of acidification on kinase phosphorylation was determined. (A) A549 and (B) LTEP-a-2 cells were exposed to pH 6.6 acidification for the indicated periods and the whole cellular protein was extracted. The levels of phosphorylation of PI3K, Akt, mTOR and S6 were determined by western blot analyses. β-actin was used as an internal control. One representative from 3 independent experiments is shown.

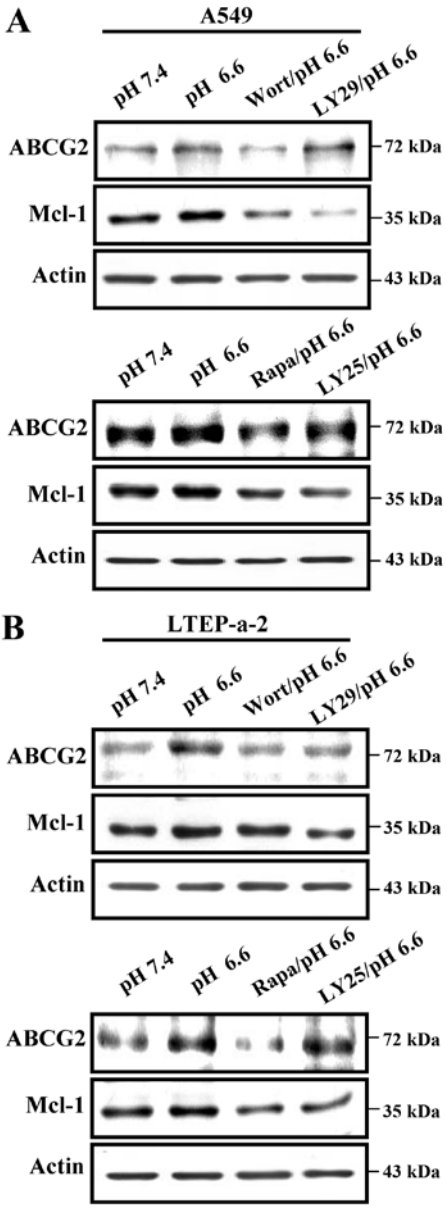

Figure 4. Microenvironment of acidification upregulates the expression of ABCG2 and Mcl-1 via the PI3K-Akt-mTOR-S6 pathway in A549 and LTEP-a-2 lung cancer cells. (A) A549 and (B) LTEP-a-2 cells were pretreated with wortmannin (10 μM), LY294002 (10 μM), rapamycin (10 μM) and LY2584702 (10 μM) prior to pH 6.6 acidification treatment. Then, the whole cellular protein was extracted and the expression of ABCG2 and Mcl-1 was determined by western blot analyses. β-actin was used as an internal control. One representative from 3 independent experiments is shown.

we aimed to ascertain whether ABCG2 and anti-apoptotic proteins are involved in acidification-promoted chemotherapeutic resistance. Toward this end, we modified the pH value of the cultured lung cancer cells and analyzed the effect of acidification on the expression of ABCG2 and Mcl-1. Surprisingly, qPCR analyses showed that the treatment with pH 6.6 acidification not only increased ABCG2 expression, but also augmented Mcl-1 upregulation at the transcription level in both the A549 and LTEP-a-2 lung cancer cells (Fig. 2A and B). Importantly, western blot analyses revealed that upregulation of ABCG2 and Mcl-1 was obviously achieved by the treatment with pH 6.6 acidification (Fig. 2C and D). As ABCG2 and Mcl-1 are the important proteins mediating MDR, the above results indicate that the acidic microenvironment could contribute to lung cancer chemotherapeutic resistance by upregulating the expression of ABCG2 and Mcl-1.

Acidic microenvironment obviously increases the phosphorylation of PI3K-Akt-mTOR-S6 in the A549 and LTEP-a-2 lung cancer cells. Previous studies showed that the phosphorylation of the PI3K-Akt pathway is involved in MDR (20). To explore the effect of acidification on PI3K-Akt kinase phosphorylation, A549 and LTEP-a-2 cells were treated with MES monohydrate to induce pH 6.6 acidification and the phosphorylation of PI3K-Akt was determined by western blot analyses. The results showed that, not only the phosphorylation at T458 or T199 of PI3K, but also the phosphorylation at S473 of Akt could be achieved in both the A549 and LTEP-a-2 cells, which started at 5 min and continue to 30 min after pH 6.6 acidification (Fig. 3A and B). Notably, the activation of Akt downstream kinases mTOR and S6 was also achieved by exposure to pH 6.6 acidification (Fig. 3A and B). All of these results indicate that the PI3K-Akt-mTOR-S6 pathway could be efficiently activated in the acidized microenvironment of lung cancer, and may play a pivotal role in the formation of lung cancer chemotherapeutic resistance.

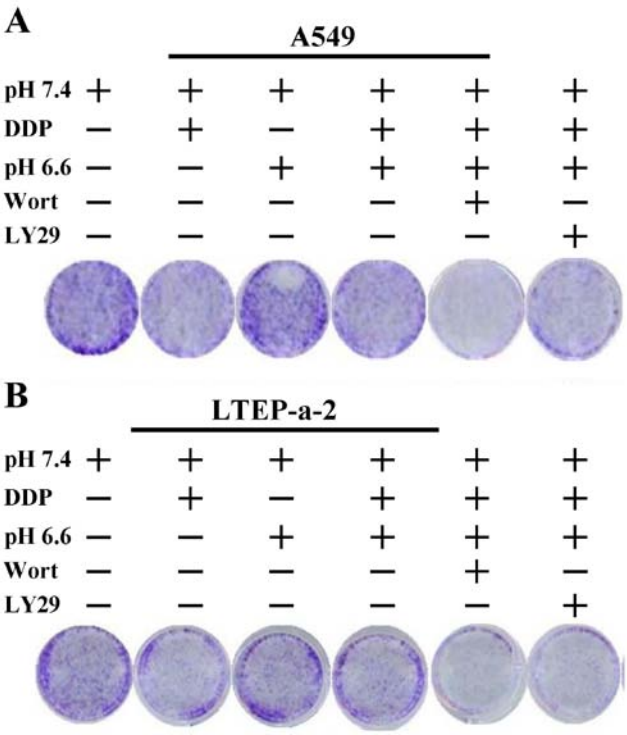

Figure 5. Inhibition of PI3K-Akt-mTOR-S6 activation decreases acidification-promoted chemotherapeutic resistance formation. (A) A549 or (B) LTEP-a-2 cells were pretreated with kinase inhibitors wortmannin (10 μM), LY294002 (10 μM), rapamycin (10 μM) and LY2584702 (10 μM) prior to pH 6.6 acidification. Then, the acidized medium was replaced with normal medium and the cells were cultured for 48 h in the presence of cisplatin (DDP) (4 μg/ml for A549 cells and 8 μg/ml for LTEP-a-2 cells). After that, the cells were cultured in pH 7.4 medium and cell viability was determined by crystal violet staining. One representative from 3 independent experiments is shown.

Microenvironment of acidification upregulates the expression of ABCG2 and Mcl-1 via the PI3K-Akt-mTOR-S6 pathway in A549 and LTEP-a-2 lung cancer cells. Previous studies have shown that activation of PI3K-Akt induced by chemotherapeutic agents facilitates the formation of MDR (20,21). Despite that pH 6.6 acidification efficiently induces the phosphorylation of the PI3K-Akt-mTOR-S6 pathway, the effects of kinase activation on acidification-median upregulation of ABCG2 and Mcl-1 are still unclear. To elucidate this issue, A549 and LTEP-a-2 cells were pretreated with wortmannin, LY294002, rapamycin and LY2584702 to inhibit kinase activation. Then, the expression of ABCG2 and Mcl-1 was determined by western blot analyses. The results showed that not only the inhibition of PI3K and Akt activities but also the deficiencies of mTOR and S6 obviously abolished pH 6.6 acidification-mediated upregulation of the expression of ABCG2 and Mcl-1 in the A549 cells (Fig. 4A). Western blot analyses of LTEP-a-2 cells also revealed the similar phenomena (Fig. 4B). All of these results indicate that the inhibition of the PI3K-Akt-mTOR-S6 pathway may be a potential strategy for overcoming acidic microenvironment-mediated lung cancer chemotherapeutic resistance.

Inhibition of PI3K-Akt-mTOR-S6 activation decreases acidic microenvironment-associated chemotherapeutic resistance in lung cancer cells. Due to the phenomena that acidification increases the activation of PI3K-Akt (Fig. 3), the finding that the inhibition of PI3K-Akt decreased the acidification effect on the expression of ABCG2 and Mcl-1 motivated us to ascertain whether the deficiency of PI3K-Akt activity would be useful to overcome acidification-induced chemotherapeutic resistance. To address this issue, A549 or LTEP-a-2 cells were pretreated with wortmannin or LY294002 and the effect of PI3K-Akt inhibition on acidification-increased cell viability was monitored. Despite that decreased cell viability was achieved following treatment with cisplatin, pH 6.6 acidification obviously increased the survival rate of the cells (Fig. 5A). Importantly, pretreatment with LY294002 and wortmannin efficiently abrogated the effect of acidification on cell viability (Fig. 5A). A similar conclusion could also be derived from the exploration in LTEP-a-2 cells (Fig. 5B). All of these observations indicate that PI3K-Akt may include potential molecules to regulate acidized microenvironment-mediated MDR in lung cancer chemotherapy.

Discussion

In the present study, we investigated the effects of an acidized microenvironment on cell viability, the expression of ATP-binding cassette transporter proteins, and activation of PI3K-Akt. We demonstrated that acidification at pH 6.6 efficiently upregulated the expression of ABCG2 and Mcl-1 via phosphorylation of PI3K-Akt kinases and contributed to multidrug resistance (MDR) in lung cancer. The decreased cell viability was achieved by the inhibition of PI3K-Akt activity, indicating that PI3K and Akt molecules may be potential therapeutic target molecules in acidic microenvironment-associated chemotherapeutic resistance in lung cancer.

IL-6, which is secreted by immune cells, has been demonstrated to be expressed by tumor cells (24-26). An elevated level of IL-6 has a close relationship with poor clinical outcome of advanced lung cancer patients (27-29). Meanwhile, IL-6 reveals anti-apoptotic effects and promotes MDR via the upregulation of ABCG2 (30-32). Our previous studies found that lung cancer cells, which have a high level of IL-6, revealed not only higher MDR, but also a stronger ability for migration (32,33), indicating that IL-6 may be an oncogene in the formation and the development of lung cancer. In the present study, despite that the acidic microenvironment increased the expression of ABCG2 and facilitated MDR formation, the effects of acidification on pro-inflammatory cytokines such as IL-6 and TNF-α are uncertain and need further investigation.

Ataxia-telangiectasia mutated (ATM), which is involved in DNA damage response and cell cycle checkpoints (34), was documented to increase MDR-associated protein expression, and contribute to chemotherapeutic resistance (14,35). Despite that chemotherapeutic agents trigger the phosphorylation of ATM and initiate the activation of TAK1-IKK-NF-κB (36), our previous studies showed that ATM could also be activated by the treatment with IL-6 facilitating the formation of MDR and metastasis in lung cancer (32,33), indicating that ATM plays an important role in lung cancer chemotherapeutic resistance. In the present study, despite that the activation of PI3K-Akt was achieved by the acidic microenvironment, the

exact effects of acidification on PI3K-Akt phosphorylation are still unknown and need further exploration.

Cancer stem cells are different from common cancer cells due to their ability to produce tumors and resist chemoradiation (37). Apart from ABCB1 (38), ABCG2, CD44 and CD133 were recently recognized as lung cancer stem cell markers (39,40). Previous studies have revealed that IL-6 treatment increases ABCG2 expression at both the translational and transcriptional levels (32), and contributes to chemotherapeutic resistance (32), indicating that IL-6 treatment facilitates the ability of lung cancer cells to acquire cancer stem-like phenotypes. Meanwhile, epigallocatechin gallate, a bioactive polyphenol in green tea, was documented to inhibit the stem cell characteristics of glioma stem-like cells by downregulating P-glycoprotein expression and inhibiting the phosphorylation of Akt (41). In the present study, despite that the acidic microenvironment increased the expression of ABCG2 via the PI3K-Akt pathway, the effects of acidification on other molecules of cancer stem-like phenotypes and the cross-interaction among the components of tumor tissues are complicated and require further elucidation.

Taken together, our data provide a new molecular mechanism for acidification-mediated MDR formation in lung cancer, which is mediated by the combined action of increased expression of ABCG2 and Mcl-1 via the PI3K-Akt pathway. This mechanism provides new insights into the molecular mechanisms of chemotherapeutic resistance and may thus open new opportunities for therapeutic intervention in lung cancer therapy.

Acknowledgements

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