

Original Article

Overcoming Taxol resistance through the inhibition of EGFR-mediated glucose metabolism in oral cancer cells

Li-Min Liang^{1*}, Lin Feng^{1*}, Zhao-Gao Zhang², Bo Wei¹

¹Department of Stomatology, Chinese PLA General Hospital, Beijing 100853, P. R. China; ²Department of Stomatology, Taizhou Central Hospital, Taizhou 318000, P. R. China. *Equal contributors.

Received July 27, 2016; Accepted August 30, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Background: Epidermal growth factor receptor (EGFR) is frequently expressed in many types of cancer including the oral squamous cell carcinoma (OSCC). As its overexpression is frequently associated with poor clinical outcome. Paclitaxel (Taxol) is a mitotic inhibitor used in cancer chemotherapy. However, although more and more patients accumulated resistance to Taxol, the mechanism remains unclear. The purpose of this study was to assess the novel glucose metabolism features in Taxol-resistant cells and whether there is a synergistic effect produced by incorporating Taxol (paclitaxel) with glycolysis inhibitors in the combined treatment of oral squamous cell carcinoma (OSCC). Methods: We studied two strains of cultured oral cancer cells, one with reduced EGFR expression (OECM-1 cells) and another with elevated EGFR expression (SAS cells). Results: Overexpression of EGFR in OECM-1 cells promoted glucose metabolism, while siRNA knockdown of EGFR in SAS cells inhibited glucose metabolism. Moreover, our data showed a strong correlation between glucose metabolism and Taxol resistance in oral cancer cells. Taxol resistant oral cancer cells displayed highly activated glucose metabolism. Finally, we reported a synergistic effect produced by incorporating Taxol (paclitaxel) with glycolysis inhibitors-2DG and 3BrPA in the combined treatment of oral squamous cell carcinoma *in vitro* and *in vivo*. Conclusion: Our data showed overexpression of EGFR contributed to Taxol resistance in oral cancer cells and the Taxol resistant cells obtained elevated EGFR expression levels. This study provides a novel perspective on the overcoming of chemo-resistant patients in clinical treatment.

Keywords: Taxol, resistance, epidermal growth factor receptor, glucose metabolism, oral cancer

Introduction

Epidermal growth factor receptor (EGFR) is a member of the receptor tyrosine kinase family, and overexpression of EGFR is associated with poor prognosis and progression of many human cancers, including oral cancer [1-3]. At the molecular level, stimulation of EGFR induces intrinsic tyrosine kinase activity and cellular signaling that results in cell growth and proliferation. EGFR stimulation is associated with perturbation of E-cadherin-mediated cell adhesion, development of fibroblast-like morphology, and increased cell motility in certain tumors [4, 5]. In oral cancer, it has been reported that an increased EGFR gene copy number is common in and associated with OSCC development in patients with oral premalignant lesions expressing high EGFR [6], suggesting that EGFR inhibitors may prevent oral cancer in patients with oral premalignant lesions having an increased EGFR gene copy number.

Otto Warburg in 1956 proposed that cancer was caused by defects in mitochondria, forcing cells to shift to energy production through glycolysis despite aerobic conditions [7]. This characteristic of cancers is described as the "Warburg Effect". Clinical studies revealed that Glut-1 expression was an independent marker of prognosis in patients with OSCC. They reported patients who had OSCC with a low Glut-1 expression survived significantly longer compared with patients who had OSCC with a high Glut-1 expression, indicating both glucose transport and glucose metabolism determine the glycolytic tumor phenotype, which might be a significant negative biomarker of prognosis and overall survival in patients with OSCC [8].

Recent reports have demonstrated that ErbB2 which belongs to EGFR family promotes cancer cell growth and glycolysis through increased expression of lactate dehydrogenase isoform A in breast cancer cells [9]. Another study also

showed that inhibiting glycolysis sensitizes cancer cells to the chemotherapeutic agent paclitaxel [10]. Moreover, a novel mitochondria-localized ErbB2 promotes glycolysis and suppresses mitochondrial functions by translocation into mitochondrial of breast cancer cells, revealing an important function of regulation of glucose metabolism by EGFR family members [11].

Taxol (paclitaxel) has recently emerged as an important agent in the treatment of human cancers such as oral, breast, ovarian, prostate and non-small cell lung cancers [12, 13]. The resistance of cancer cells to Taxol and other chemotherapeutic agents is known to result in the subsequent recurrence and metastasis of cancer [14, 15]. Many known mechanisms include the alterations of tubulin structure [16, 17], changes in the drug-binding affinity of the microtubules [18] and cell cycle deregulation [19, 20]. However, whether cancer the detailed molecular mechanisms that may contribute to Taxol resistance of cancer cells are still not fully understood.

In this study, we observed overexpression of EGFR in oral cancer cells contributed to Taxol resistance. Overexpression of EGFR in OECM-1 cells promoted glucose metabolism and oxygen consumption, while siRNA knockdown of EGFR in SAS cells inhibited glucose metabolism and oxygen consumption. Moreover, Taxol resistant oral cancer cells which showed upregulated EGFR expression displayed highly activated glucose metabolism and the combination of glycolysis inhibitors and Taxol had a synergistic effect on the re-sensitization of Taxol resistant cells to Taxol. In addition, our results showed a synergistic effect produced by incorporating Taxol (paclitaxel) with glycolysis inhibitors-2DG and 3BrPA in the combined treatment of oral squamous cell carcinoma *in vitro* and *in vivo*. Finally, we reported a correlation between oral tumor glucose metabolism and Taxol resistance in OSCC patients.

Materials and methods

Cells and culture conditions

Human oral squamous cell carcinoma (OSCC) cell line, OECM-1 and SAS were purchased from ATCC. Briefly, cells are routinely cultured in Dulbecco's modified Eagle's medium (DMEM)

(Gibco BRL, Paisley, UK) containing 10% fetal bovine serum (FBS) (HyClone, Vermont, USA), at 37°C in a humid atmosphere with 5% CO₂.

Antibodies and reagents

Antibodies used in this study were purchased from: EGFR (Cell Signaling: #2239); β -actin (Cell Signaling #4967); GLUT1 (Santa Cruz: sc-7903); LDHA (Cell signaling #2012) and total and cleaved PARP (Cell signaling #9532); Vector containing Wild type ORF clone of Homo sapiens protein EGFR, was purchased from Addgene (Plasmid 11011). Erlotinib was purchased from Roche, Basel, Switzerland; 2-DG and 3-BrPA were purchased from Sigma-Aldrich (St. Louis, MO).

OSCC patient samples

All primary Human OSCC patient specimens were obtained from patients undergoing surgery for oral tumor during 2009 to 2012 at the Oral Medical Research Center, Chinese PLA General Hospital, Beijing, P. R. China and stored in liquid nitrogen until analysis. All patients provided written informed consent. The study was approved by the Ethics Committee of the Oral Medical Research Center, Chinese PLA General Hospital, Beijing, P. R. China.

Western blotting

Cells were harvested and lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton, 1 mM PMSF and Protease Inhibitor Cocktail (Sigma) for 20 min on ice. Lysates were cleared by centrifugation at 14,000 rpm at 4°C for 10 min. Supernatants were collected and protein concentrations were determined by the Bradford assay (Bio-Rad). The proteins were then separated with a SDS/polyacrylamide gel and transferred to a Nitrocellulose membrane (Bio-Rad). After blocking in PBS with 5% non-fat dry milk for 1 hr, the membranes were incubated overnight at 4-8°C with the primary antibodies in PBS with 5% non-fat dry milk. The following antibodies were utilized: anti-EGFR rabbit antibody (1:1000, Cell Signaling); anti- β actin rabbit antibody (1:1000, Cell Signaling), anti-cleaved PARP and complete PARP Rabbit antibody (1:1000, Cell Signaling), anti-LDHA rabbit antibody (1:1000, Cell Signaling), anti-GLUT1 rabbit monoclonal antibody (1:1000, Cell Signaling).

Relief of Taxol resistance

Membranes were extensively washed with PBS and incubated with horseradish peroxidase conjugated secondary anti-mouse antibody or anti-rabbit antibody (1:2,000, Bio-Rad). After additional washes with PBS, antigen-antibody complexes were visualized with the enhanced chemiluminescence kit (Pierce).

Plasmid DNA transfection

Expression vector containing wild type EGFR were purchased from Addgene.com (Plasmid 11011). Cells were transfected using the Oligofectamine Transfection reagent (Invitrogen) according to the manufacturer's protocol. After 24 h, cells were maintained in regular culture medium containing Puromycin for the selection of stable expression cells.

siRNA experiments

siRNA oligonucleotides for EGFR was purchased from Sigma, with a scrambled siRNA (Sigma) used as a control. The target sequences for EGFR siRNA were CGCAAAGT GTGT-AACGGAATA within exon 13 of the EGFR gene and CTGACTCCGTC CAGTATTGAT within the 5'UTR region of EGFR mRNA. Transfection was performed using the Oligofectamine Transfection reagent (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, whole-cell lysates were prepared for further analysis by Western blot.

cDNA preparation and real time RT-PCR

Total RNA was extracted after homogenization of cells and tissues using RNeasy mini kit (Qiagen Sciences, Maryland MD) and performing DNase digestion (RNase free DNase set, Qiagen, Valencia CA) during the RNA extraction. Total RNA (1 µg) was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City CA). The cDNA reaction was diluted to 1:10 for use as template for real-time RT-PCR.

TaqMan Gene Expression Assays primers and probes specific to EGFR, GLUT1 and LDHA were used for expression analyses and 18S ribosomal primers and probes (Applied Biosystems, Foster City, CA) were used as internal controls. PCR amplifications were performed in a final reaction volume of 10 µl containing, 5.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City,

CA), 0.5 µl of the primers and probes mix and 4.5 µg of the cDNA diluted solution. The cycling conditions were as follows: one cycle of 2 minutes at 50°C, one cycle of 10 minutes at 95°C, 40 cycles of denaturation (15 seconds at 95°C) and annealing/extension (1 minute at 60°C). All reactions were carried out in the Step 1 Plus Real-Time PCR Systems Thermocycler (Applied Biosystems, Foster city, CA). All quantitative PCR reactions were carried out in triplicate and repeated at least twice. The ΔC_t for mRNA expression was calculated relative to the C_t (threshold cycle) of 18S ribosomal RNA. Relative mRNA expression was calculated using the formula $2^{(-\Delta\Delta C_t)}$.

EGFR primers used for Real Time PCR: Forward primer: AGG CAC AAG TAA CAG GCT CAC; Reverse primer: AAG GTC GTA ATT CCT TTG CAC.

GLUT1 primers used for Real Time PCR: Forward primer: AAC TCT TCA GCC AGG GTC CAC; Reverse primer: CAC AGT GAA GAT GAT GAA GAC.

LDHA primers used for Real Time PCR: Forward primer: ATC TTG ACC TAC GTG GCT TGG A; Reverse primer: CCA TAC AGG CAC ACT GGA ATC TC.

Cell viability assay

A total of $5 \times 10^4 \sim 1 \times 10^5$ cells/well were seeded in 6-well plates. Twenty-four hours later, the medium was replaced with fresh medium with or without Taxol and incubated for 24 or 48 h, respectively. Taxol in combination with various concentrations of 2DG or 3BrPA were also used to treat the cells in order to investigate the effect of drug combinations. Cell viability was determined by Typan Blue staining and direct cell counting using hemacytometer.

Glucose uptake assay

Cells were seeded in 12-well plates at 1×10^5 to 3×10^5 cells per well. Culture media was collected at 48 h and stored at -20°C until assayed. Glucose uptake was measured using an Amplex Red Glucose/Glucose Oxidase assay kit (Molecular Probes). Absorbance was measured at 563 nm using a SpectraMax M5 plate reader (Molecular Devices) and the results were normalized to the amount of total protein.

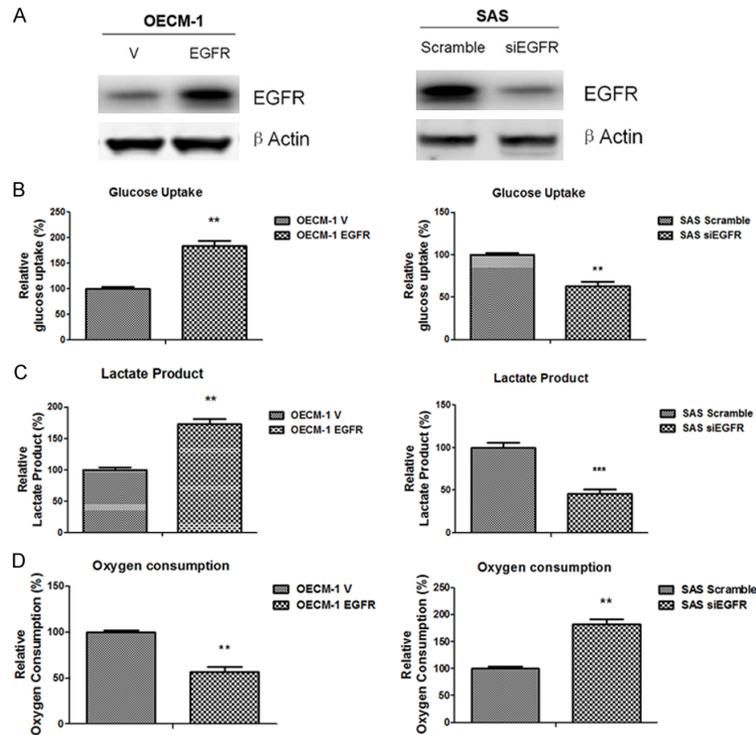


Figure 1. EGFR stimulates glycolysis in human oral squamous cells. (A) Exogenous overexpression of EGFR in OECM1 cells and siRNA knock down of EGFR in SAS cells. β -actin is a loading control. EGFR promotes (B) glucose uptake and (C) lactate production in OECM1 cells (left); SAS cells (right). (D) Oxygen consumption is decreased by overexpression of EGFR in OECM1 cells (upper) and SAS cells (lower). Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **, $P < 0.01$.

Lactate production assay

Lactate production in the medium was detected by using a Lactate assay kit (BioVision) according to a previous report [11].

Oxygen consumption

Oxygen consumption was detected using BD Oxygen Biosensor Systems from BD Bioscience Company. 1×10^6 cells were plated into a 96-well plate provided with the kit. The plates were read for Fluorescence using the following parameters: 485 nm excitation and 630 nm emission. Raw data was analyzed and normalized according to the kit instruction. Each sample was plated in triplicate and each experiment was repeated three times to reach the statistical significance.

Animal experiments

The athymic BALB/c nude mice (5-8 week-old) were housed in the Biological Resource Centre

of Chinese PLA General Hospital, Beijing, P. R. China. Mice were implanted subcutaneously into a mouse mammary fat pad mfp with 1×10^7 OECM-1 TRP cells. Tumor progress was monitored by tumor size measurements at every other day. When the tumor reached a size of greater than 150 mm^3 , the mice were randomly divided into 4 groups (8 mice per group) as follows: PBS-treated control; Taxol alone [10 mg/kg intraperitoneal (i.p.), 2 times/wk for 21 days] and Taxol plus 2DG and 3BrPA (750 mg/kg, i.p., daily for 21 days). Mice mortality rate was recorded daily. All of the experiments involving mouse models were complied with both Chinese laws and the guidelines of the Ethics Committee of Beijing Institutes for Biological Sciences. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

We used the CO_2 chamber for euthanasia of mice, mice were euthanized in their home cage filling full of CO_2 for 3-5 minutes for mice to stop moving or breathing, eyes were fixed and dilated. The adequate steps were taken to avoid unnecessary suffering.

Statistical analysis

The unpaired Student's t-test was used for the data analysis. All data were shown as mean \pm standard error (SE). A statistical difference of $P < 0.05$ was considered significant.

Results

EGFR increases glucose metabolism in OSCC cells

Since it has been reported ErbB2 which belongs to EGFR family which promotes glucose metabolism in human breast cancer cells [9], we started to examine whether the overexpression of EGFR alters glucose metabolism in human oral squamous cell carcinoma (OSCC) cells, we

Relief of Taxol resistance

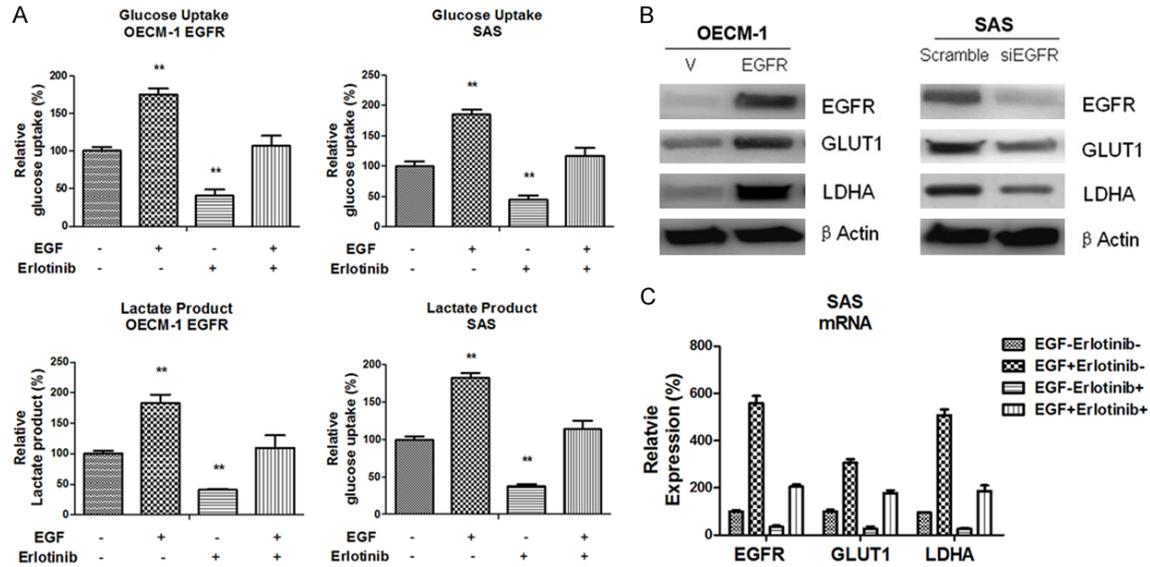


Figure 2. EGFR upregulates the expression of key enzymes in the glucose metabolism pathway in oral cancer cells. A. EGFR inhibitor suppress glucose uptake and lactate product in OECM1 and SAS cells, EGFR ligand stimulates glucose uptake and lactate product. B. EGFR upregulates GLUT1, LDHA protein expression in OECM1 cells (left). Knocking down of EGFR decreases GLUT1 and LDHA protein expression (right). C. mRNA levels of EGFR, GLUT1 and LDHA were decreased by EGFR inhibition, increased by EGF stimulation in SAS cells. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **, $P < 0.01$.

stably transfected wild type EGFR into OECM-1 cell which expressed low level EGFR and the EGFR expression levels were confirmed by immunoblot analysis (Figure 1A). EGFR overexpression was shown in OECM-1 cells, compared to the much lower ErbB2 levels in their corresponding control cells. Glucose uptake, lactate production and oxygen consumption, which are hallmarks of glycolysis, were measured and compared in EGFR-low-expressing and EGFR-high-expressing cells (Figure 1B-D). Overexpression of EGFR in OECM-1 cells showed a significantly higher glucose uptake (Figure 1B) and lactate production (Figure 1C) but lower oxygen consumption rates (Figure 1D) than the OECM-1 control cells, respectively. To verify the above results, we knocked down the expression of EGFR using specific siRNA in SAS cells which originally express high level of EGFR (Figure 1A). Consistently, knocking down of EGFR in SAS cells dramatically decreased the glucose uptake, lactate product but increased oxygen consumption rates (Figure 1B-D). These results strongly suggest an important link between EGFR expression and glycolysis, indicating that EGFR overexpression may promote glycolysis in human oral squamous cell carcinoma.

Promotion of glucose metabolism by EGFR is kinase dependent

As a well-studied oncogene, EGFR signaling pathway is essential to regulate growth, survival, proliferation, and differentiation in mammalian cells. We next to explore whether the kinase activity of EGFR is responsible for the regulation of glucose metabolism of oral cancer cells. Erlotinib has been reported as an EGFR inhibitor by binding in a reversible fashion to the ATP binding site of the receptor and the signal cascades will not be initiated. Our data showed the glucose uptake and lactate products in oral cancer cells with high EGFR expression (OECM-1 and SAS) were inhibited with the treatment of Erlotinib, but promoted by the EGF treatment which stimulated EGFR signaling pathway (Figure 2A).

Glucose metabolic enzymes are upregulated by EGFR in OSCC cells

As we expected, expression levels of major glucose metabolic enzymes were up regulated by overexpression of EGFR in OECM-1 cells and knocking down of EGFR in SAS cells exhibited reversed results (Figure 2B), supporting that EGFR played an essential role in the regulation

Relief of Taxol resistance

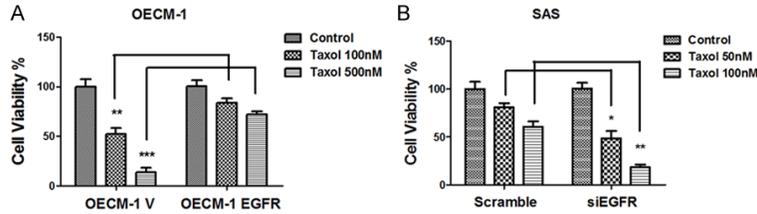


Figure 3. Overexpression of EGFR contributes to Taxol resistance in OECM1 cells. A. OECM V and OECM EGFR cells were treated with Taxol at 100 nM and 500 nM followed by the measurements of cell viability. B. Knockdown of EGFR in SAS cells followed by Taxol treatments at 100 nM and 500 nM, cell viabilities were detected. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **, $P < 0.01$.

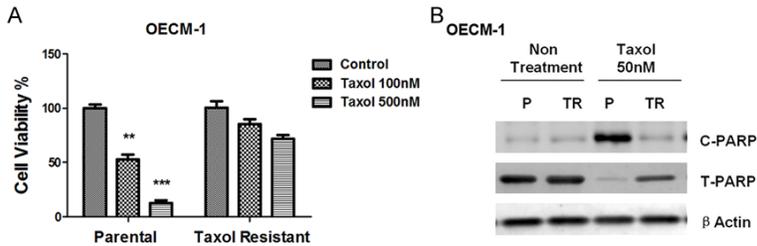


Figure 4. Characterization of Taxol-resistant cells. A. Cell viability analysis was performed to evaluate cytotoxicity of Taxol to OECM parental and Taxol-resistant cells under treatment with indicated concentrations of Taxol for 48 h. B. Taxol-resistant cells and their parental cells were treated without or with 50 nM Taxol for 48 h, then poly (ADP-ribose) polymerase (PARP) and its cleaved protein (c-PARP) were analyzed by Western blotting with specific antibodies, respectively. β -actin was used as a loading control.

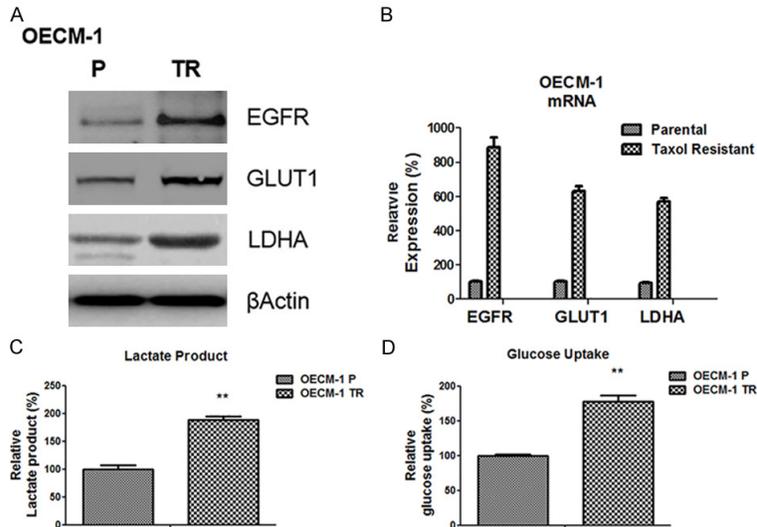


Figure 5. Glycolysis pathway are upregulated in Taxol resistant OECM cells. A. Protein expressions of EGFR, GLUT1 and LDHA in OECM Taxol-resistant cells were upregulated by Western blotting analysis. B. mRNA expression of EGFR, GLUT1 and LDHA were upregulated in OECM Taxol-resistant cells. C. Lactate products of OECM Taxol-resistant cells were increased. D. Glucose uptake of OECM Taxol-resistant cells were increased. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **, $P < 0.01$.

of glucose metabolism. To further strengthen our results, we checked whether inhibition of EGFR by Erlotinib treatments or activation by EGF treatment can alter the mRNA expressions of key enzymes in glucose metabolism, our data showed both GLUT1 and LDHA were up regulated by EFG treatments and down regulated by EGFR inhibitor (**Figure 2C**), indicating a strong role of EGFR pathway involving the regulation of glucose metabolism in oral cancer cells.

Overexpression of EGFR renders oral cancer cells insensitive to Taxol treatment

As reported before, deregulated signaling through the epidermal growth factor receptor (EGFR) is involved in chemotherapy resistance [3]. We next tried to figure out the roles of EGFR in chemotherapy in oral cancer cells. Our results showed OECM-1 cells with overexpression of EGFR displayed insensitiveness to Taxol treatments (**Figure 3A**). Cell viabilities were significantly increased in OECM-1 EGFR cells compared with control cells with the treatment of Taxol at 100 nM and 500 nM. Consistently, knocking down of EGFR in SAS showed the reverse results with the Taxol treatment at 50 nM and 100 nM (**Figure 3B**), indicating EGFR contributes to Taxol resistance in oral cancer cells.

Taxol-resistant cells originated from oral cancer cells exhibit induced EGFR expression and upregulated glucose metabolism

It has been reported cancer cells with upregulated glucose

Relief of Taxol resistance

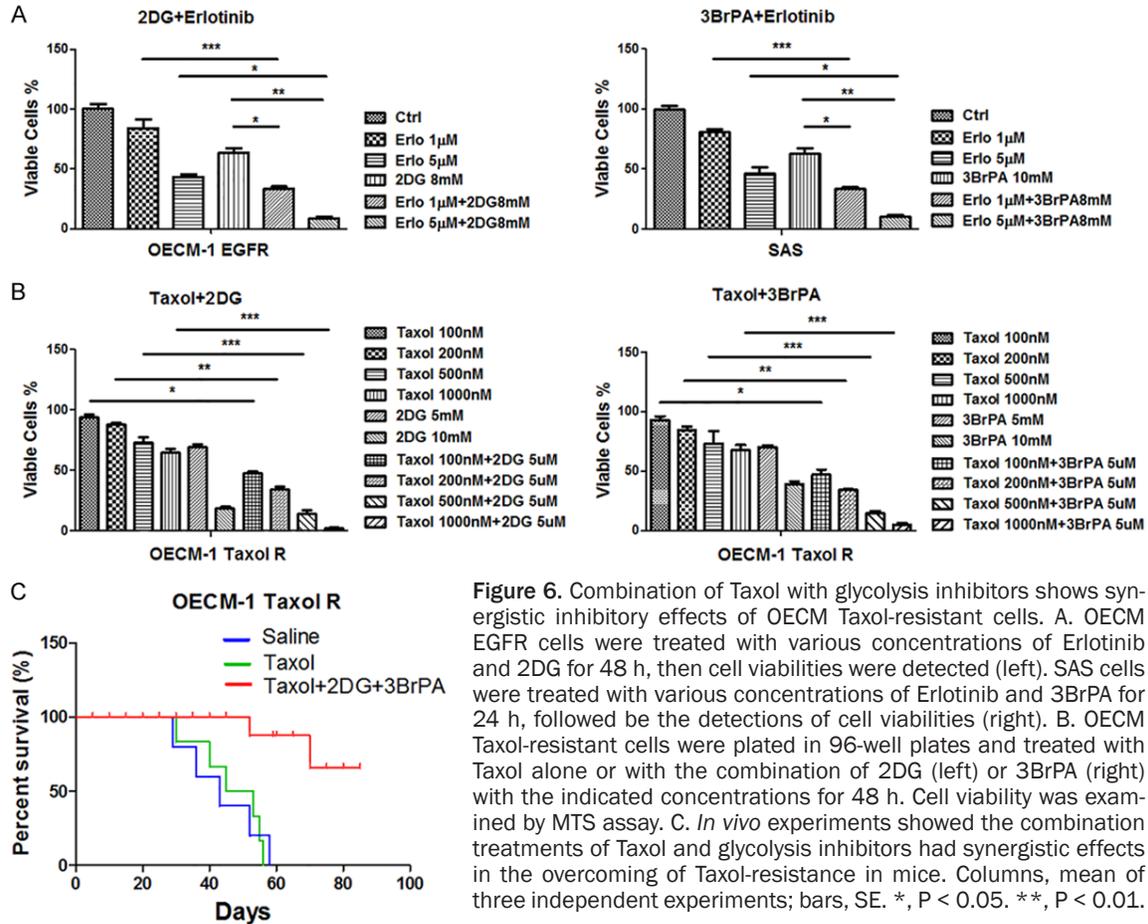


Figure 6. Combination of Taxol with glycolysis inhibitors shows synergistic inhibitory effects of OECM Taxol-resistant cells. A. OECM EGFR cells were treated with various concentrations of Erlotinib and 2DG for 48 h, then cell viabilities were detected (left). SAS cells were treated with various concentrations of Erlotinib and 3BrPA for 24 h, followed by the detections of cell viabilities (right). B. OECM Taxol-resistant cells were plated in 96-well plates and treated with Taxol alone or with the combination of 2DG (left) or 3BrPA (right) with the indicated concentrations for 48 h. Cell viability was examined by MTS assay. C. *In vivo* experiments showed the combination treatments of Taxol and glycolysis inhibitors had synergistic effects in the overcoming of Taxol-resistance in mice. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **, $P < 0.01$.

metabolism show less sensitive to chemotherapy such as Taxol compared with normal cancer cells [10]. We next studied whether EGFR-mediated glucose metabolism had any correlation with Taxol resistance. We established Taxol resistant cell lines using OECM-1 cells by treatment with gradually increasing concentrations of Taxol for the selection of Taxol-resistant cells. After successive Taxol treatments for 3 months, several resistant cell clones were developed from the cells. Taxol-resistant pooled clones (TRP) were used for all subsequent experiments in this study. **Figure 4A** showed OECM-1 TRP cells were resistant to Taxol, only a smaller percentage of apoptotic cells were detected in Taxol-resistant OECM-1 cells, compared to their parental (**Figure 4A**). The protein expression of the cleaved Poly (ADP-ribose) polymerase (c-PARP), an important marker of caspase mediated apoptosis, was also examined by Western blotting after the cells were treated with 50 nM Taxol for 48 h. We found much lower levels of cleaved PARP and correspondingly much higher levels of un-

cleaved PARP in Taxol-resistant OECM-1 cells, compared to parental cells (**Figure 4C**). Taken together, our data showed that OECM-1 TRP cells could tolerate much higher concentrations of Taxol compared to parental cells.

We next detected the glucose metabolism changes in OECM-1 TRP cells. As we expected, the expressions of EGFR were induced at both protein and mRNA levels in the OECM-1 TRP cells, as well as other metabolism enzymes (**Figure 5A** and **5B**). The glucose uptake and lactate product were also increased in OECM-1 TRP cells compared with their parental cells (**Figure 5C** and **5D**), indicating EGFR mediated glucose metabolism contributed to Taxol resistance in oral cancer cells.

Combination of Taxol with glycolysis inhibitors shows synergistic inhibitory effects in vitro and in vivo

To examine the role of EGFR in mediating Taxol resistance in human oral cancer cells, we fur-

ther investigated the effects of combining Taxol with glycolysis inhibitors on Taxol-resistant breast cancer cells. We first checked whether blocking EGFR signaling pathway together with glucose metabolism inhibitors significantly inhibited the viability of the EGFR high expressing oral cancer cells. Our results showed combination of Erlotinib and 2DG synergistically inhibited cell viability compared with either agent given alone in OECM-1 EGFR cells (**Figure 6A**, left), similar results showed synergistic inhibiting effects on the cell viability with the treatments of Erlotinib and 3BrPA in SAS cells (**Figure 6A**, right). We next investigated the effects of combining Taxol with glycolysis inhibitor 2DG and 3BrPA on Taxol-resistant oral cancer cells. The treatments with the combination of Taxol with glycolysis inhibitors were much more effective in inhibiting cell viability compared with either agent given alone (**Figure 6B**). Taken together, the combination of Taxol with glycolysis inhibitors had a greater capacity to inhibit oral cancer cell derived Taxol-resistant cells compared to either agent given alone.

To further enhance our conclusions, we performed *in vivo* experiment to see whether the combination of glycolysis inhibitors with paclitaxel chemotherapy could increase animal survival rate. Mice were inoculated OECM-1 TRP cells and when the tumor established, mice were then treated without Taxol, Taxol alone and Taxol plus glycolysis inhibitors via intraperitoneal injection, once a week, for eight consecutive weeks. We observed mice that received treatment with Taxol alone as well as none treatment, most of the animals died within 2 months after treatment. Although Taxol alone did not dramatically repress tumor growth with the inoculation of Taxol resistant cells, treatment with combination of Taxol with glycolysis inhibitors achieved a prolonged survival times (**Figure 6C**). In summary, our results indicated that combination of glycolysis inhibitors with Taxol chemotherapy achieved a significantly better outcome in mice survival rates.

EGFR-mediated upregulation of glucose metabolism is correlated with OSCC concurrency

Finally, we investigated whether there is association between oral tumor glucose metabolism and Taxol resistance in OSCC Patients. Data analyzed from Oncomine database showed EGFR, GLUT1 and LDHA were all upreg-

ulated in multiple OSCC patient samples (**Figure 7A**). We further obtained multiple OSCC patient sample which were separated as Taxol-sensitive and Taxol-resistant, from which we detected the expression of EGFR, GLUT1 and LDHA were upregulated in Taxol-resistant patient samples, indicating the EGFR mediated glucose metabolism contribute to the mechanism of Taxol-resistance in human OSCC (**Figure 7B**).

Discussion

Cancer cells are different from non-neoplastic cells in their metabolic properties, with normal cells relying primarily on the process of mitochondrial oxidative phosphorylation, consuming oxygen and glucose to produce energy. In contrast, cancer cells depend mostly upon glycolysis, the anaerobic breakdown of glucose into the energy-storing molecule ATP, even in the presence of available oxygen. As a well-studied oncogene, the expression level of EGFR in cancer tissues is correlated with prognosis. Recently, multiple studies focused on EGFR and glucose metabolism have been reported. Activation of EGFR has been reported to transiently increase glucose transport. They claimed EGFR-mediated increase in SGLT-generated glucose uptake which is required for the survival of the toxically stressed tumor cells [21]. Another paper published recently revealed that EGFR is a stabilizer of an active glucose transporter, SGLT1, empowering cancer cells with the ability to uptake the basic energy substrate, glucose, regardless the level of extracellular glucose, for their survival [22]. In oral cancer, Synchronous mRNA coexpression of ErbB1, ErbB2, ErbB3 and ErbB4 was detected in oral leukoplakia [23]. Another paper described that overexpression of Glut-1 and increased glucose metabolism in tumors are associated with a poor prognosis in patients with oral squamous cell carcinoma [8]. In this study, we described a novel pathway through which EGFR regulates glucose metabolism which contributes to the Taxol resistance in oral cancer cells. Although the detailed mechanisms have not been thoroughly elucidated, we first reported a linkage between glucose metabolism and chemosensitivity in oral cancer. In a clinical study, the immunoeexpression of EGFR and Her-2 in OSCC samples was evaluated and correlated with the salivary levels of these proteins and the clinic pathological fea-

Relief of Taxol resistance

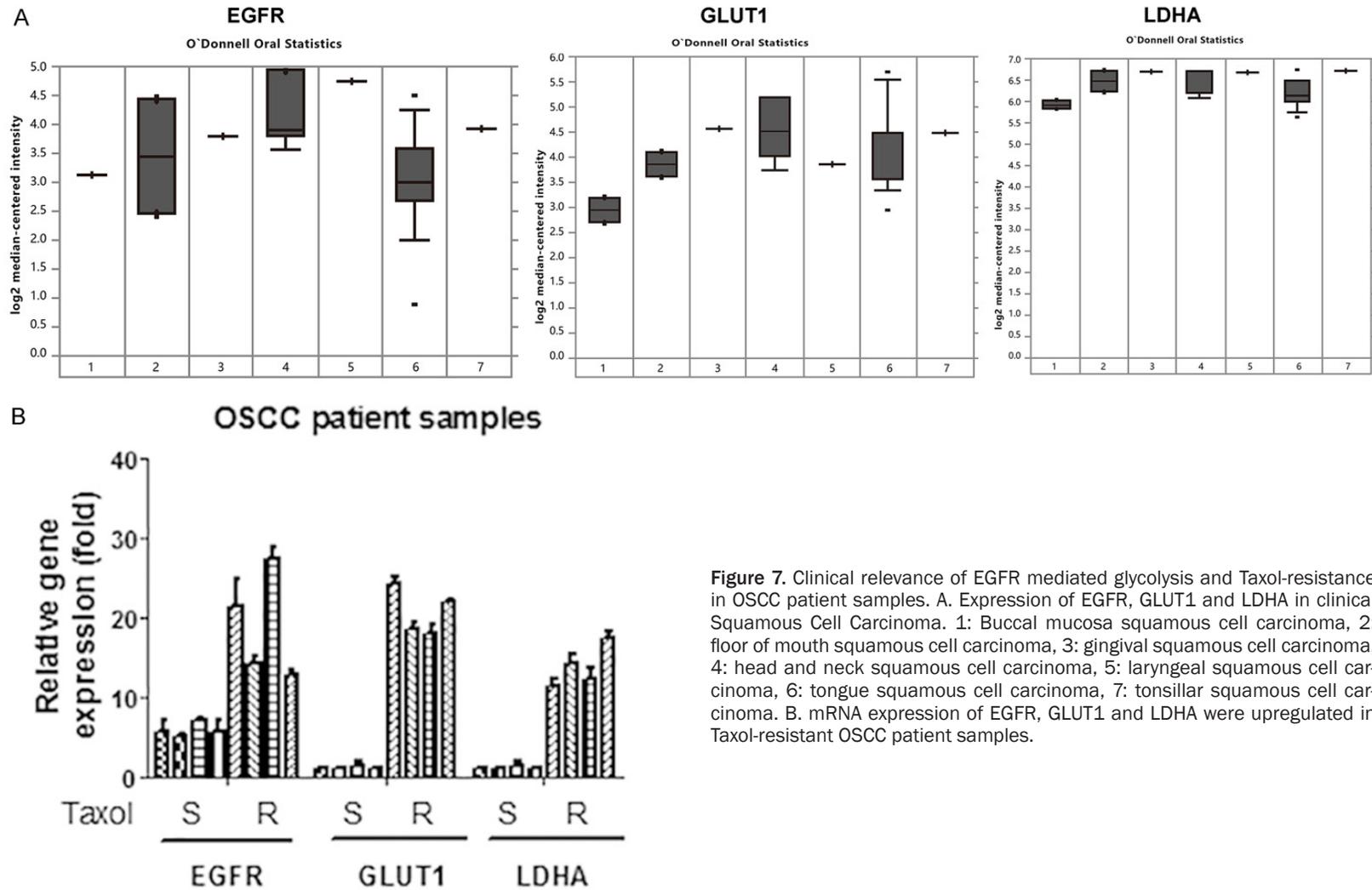


Figure 7. Clinical relevance of EGFR mediated glycolysis and Taxol-resistance in OSCC patient samples. A. Expression of EGFR, GLUT1 and LDHA in clinical Squamous Cell Carcinoma. 1: Buccal mucosa squamous cell carcinoma, 2: floor of mouth squamous cell carcinoma, 3: gingival squamous cell carcinoma, 4: head and neck squamous cell carcinoma, 5: laryngeal squamous cell carcinoma, 6: tongue squamous cell carcinoma, 7: tonsillar squamous cell carcinoma. B. mRNA expression of EGFR, GLUT1 and LDHA were upregulated in Taxol-resistant OSCC patient samples.

tures of the tumors [24]. All the above publications display essential roles of EGFR-mediated glucose metabolism in the tumor progress in OSCC.

Taxol is a widely used chemotherapeutic agent for the treatment of several types of cancers, including oral cancer. However, cancer patients who obtained Taxol resistance may result in the subsequent recurrence and metastasis of cancer, ultimately resulting in death. Although extensive investigations have been done in regards to the resistance of cancer cells to Taxol, currently, the specific mechanisms are still poorly understood. Our data from **Figure 5** showed a strong correlation between glucose metabolism and Taxol resistance in oral cancer cells, Taxol resistant cells come with upregulated EGFR expression and glucose metabolism, which triggered us try to find glycolysis inhibitors to synergistically kill resistant cells with Taxol treatment.

The combination of Taxol with 2-DG and 3-BrPA was found to be more effective in killing Taxol-resistant cells, compared to either Taxol or glycolysis inhibitors treatment alone. The combination therapy reveals a synergistic inhibitory effect by promoting oral cancer cell apoptosis both *in vitro* and *in vivo* (**Figure 6**). Apoptosis is a predominant mechanism by which cancer chemotherapeutic agents kill cells. We report here a novel function via inducing apoptotic cell death, with important implications in the clinical treatment of Taxol-resistant cancers, such as oral cancer.

In summary, to identify the molecules that may contribute to Taxol resistance is important for the management of Taxol resistant oral cancer, in this study, we investigated the role of EGFR-mediated glucose metabolism in the acquired Taxol resistance in human oral cancer cell lines and mice. We observed overexpression of EGFR in oral cancer cells significantly increased glucose metabolism. Interestingly, oral cells with high expression of EGFR are more resistant to Taxol treatment compared with EGFR low expression cells *in vitro*. We identified that compared to Taxol-sensitive cells, Taxol-resistant cells possess an increased expression of EGFR. In addition, compared to Taxol-sensitive cells, Taxol-resistant cells show a higher sensitivity to the glycolysis inhibitor 2DG and 3BrPA. Furthermore, when compared to

single agent therapy, treating cells with the combination of Taxol and glycolysis inhibitors showed a synergistic inhibitory effect on Taxol-resistant oral cancer cells and mice by promoting cellular apoptosis. These results demonstrated that EGFR-mediated glucose metabolism plays an important role in Taxol resistance and potentially it can serve as a therapeutic target for overcoming Taxol resistance in OSCC patients.

Acknowledgements

This study was financially supported by China Postdoctoral Science Foundation Grant (No. 2014M552645) and the National High Technology Research and Development Program (863 program) (No. SS2015AA032102).

Disclosure of conflict of interest

None.

Address correspondence to: Li-Min Liang, Department of Stomatology, Chinese PLA General Hospital, 28 Fuxing Road, Haidian District, Beijing 100853, P. R. China. E-mail: liangliminds@163.com

References

- [1] da Cunha Santos G, Shepherd FA, Tsao MS. EGFR mutations and lung cancer. *Annu Rev Pathol* 2011; 6: 49-69.
- [2] Todd R, Wong DT. Epidermal growth factor receptor (EGFR) biology and human oral cancer. *Histol Histopathol* 1999; 14: 491-500.
- [3] Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. *Breast Cancer Res Treat* 2012; 136: 331-345.
- [4] Herbst RS. Review of epidermal growth factor receptor biology. *Int J Radiat Oncol Biol Phys* 2004; 59: 21-26.
- [5] Ono M, Kuwano M. Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. *Clin Cancer Res* 2006; 12: 7242-7251.
- [6] Taoudi Benchekroun M, Saintigny P, Thomas SM, El-Naggar AK, Papadimitrakopoulou V, Ren H, Lang W, Fan YH, Huang J, Feng L, Lee JJ, Kim ES, Hong WK, Johnson FM, Grandis JR, Mao L. Epidermal growth factor receptor expression and gene copy number in the risk of oral cancer. *Cancer Prev Res (Phila)* 2010; 3: 800-809.
- [7] Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the meta-

Relief of Taxol resistance

- bolic requirements of cell proliferation. *Science* 2009; 324: 1029-1033.
- [8] Kunkel M, Reichert TE, Benz P, Lehr HA, Jeong JH, Wieand S, Bartenstein P, Wagner W, Whiteside TL. Overexpression of Glut-1 and increased glucose metabolism in tumors are associated with a poor prognosis in patients with oral squamous cell carcinoma. *Cancer* 2003; 97: 1015-1024.
- [9] Zhao YH, Zhou M, Liu H, Ding Y, Khong HT, Yu D, Fodstad O, Tan M. Upregulation of lactate dehydrogenase A by ErbB2 through heat shock factor 1 promotes breast cancer cell glycolysis and growth. *Oncogene* 2009; 28: 3689-3701.
- [10] Zhou M, Zhao Y, Ding Y, Liu H, Liu Z, Fodstad O, Riker AI, Kamarajugadda S, Lu J, Owen LB, Ledoux SP, Tan M. Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol. *Mol Cancer* 2010; 9: 33.
- [11] Ding Y, Liu Z, Desai S, Zhao Y, Liu H, Pannell LK, Yi H, Wright ER, Owen LB, Dean-Colomb W, Fodstad O, Lu J, LeDoux SP, Wilson GL, Tan M. Receptor tyrosine kinase ErbB2 translocates into mitochondria and regulates cellular metabolism. *Nat Commun* 2012; 3: 1271.
- [12] Henley D, Isbill M, Fernando R, Foster JS, Wimalasena J. Paclitaxel induced apoptosis in breast cancer cells requires cell cycle transit but not Cdc2 activity. *Cancer Chemother Pharmacol* 2007; 59: 235-249.
- [13] Tan M, Yu D. Molecular mechanisms of erbB2-mediated breast cancer chemoresistance. *Adv Exp Med Biol* 2007; 608: 119-129.
- [14] Chen LP, Cai SM, Fan JX, Li ZT. PEBA Regimen (Cisplatin, Etoposide, Bleomycin, and Adriamycin) in the Treatment of Drug-Resistant Choriocarcinoma. *Gynecol Oncol* 2002; 56: 231-234.
- [15] Donnenberg Vera S, Donnenberg Albert D. Multiple Drug Resistance in Cancer Revisited: The Cancer Stem Cell Hypothesis. *J Clin Pharmacol* 2005; 45: 872-877.
- [16] Panda D, Miller HP, Banerjee A, Ludueña RF, Wilson L. Microtubule dynamics in vitro are regulated by the tubulin isotype composition. *Proc Natl Acad Sci U S A* 1994; 91: 11358-11362.
- [17] Kavallaris M, Kuo DY, Burkhart CA, Regl DL, Norris MD, Haber M, Horwitz SB. Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific beta-tubulin isoforms. *J Clin Invest* 1997; 100: 1282-1293.
- [18] Gonçalves A, Braguer D, Kamath K, Martello L, Briand C, Horwitz S, Wilson L, Jordan MA. Resistance to Taxol in lung cancer cells associated with increased microtubule dynamics. *Proc Natl Acad Sci* 2001; 98: 11737-11741.
- [19] Tan M, Jing T, Lan KH, Neal CL, Li P, Lee S, Fang D, Nagata Y, Liu J, Arlinghaus R, Hung MC, Yu D. Phosphorylation on tyrosine-15 of p34(Cdc2) by ErbB2 inhibits p34(Cdc2) activation and is involved in resistance to taxol-induced apoptosis. *Mol Cell* 2002; 9: 993-1004.
- [20] Lu J, Tan M, Huang WC, Li P, Guo H, Tseng LM, Su XH, Yang WT, Treekitkarnmongkol W, Andreeff M, Symmans F, Yu D. Mitotic deregulation by survivin in ErbB2-overexpressing breast cancer cells contributes to Taxol resistance. *Clin Cancer Res* 2009; 15: 1326-1334.
- [21] Huber SM, Misovic M, Mayer C, Rodemann HP, Dittmann K. EGFR-mediated stimulation of sodium/glucose cotransport promotes survival of irradiated human A549 lung adenocarcinoma cells. *Radiother Oncol* 2012; 103: 373-379.
- [22] Weihua Z, Tsan R, Huang WC, Wu Q, Chiu CH, Fidler IJ, Hung MC. Survival of cancer cells is maintained by EGFR independent of its kinase activity. *Cancer Cell* 2008; 13: 385-393.
- [23] Kobayashi H, Kumagai K, Gotoh A, Eguchi T, Yamada H, Hamada Y, Suzuki S, Suzuki R. Upregulation of epidermal growth factor receptor 4 in oral leukoplakia. *Int J Oral Sci* 2013; 5: 14-20.
- [24] Bernardes VF, Gleber-Netto FO, Sousa SF, Silva TA, Aguiar MC. Clinical significance of EGFR, Her-2 and EGF in oral squamous cell carcinoma: a case control study. *J Exp Clin Cancer Res* 2010; 29: 40.