Original Article

Apigenin suppresses GLUT-1 and p-AKT expression to enhance the chemosensitivity to cisplatin of laryngeal carcinoma Hep-2 cells: an in vitro study

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Abstract: Glucose transporter-1 (GLUT-1) and PI3K/Akt are known to be closely involved in resistance to chemo-therapy. Co-targeted therapy reducing GLUT-1 expression and PI3K/Akt pathway activity may overcome the chemoresistance of human cancers. Apigenin may inhibit the expression of GLUT-1 and the PI3K/Akt pathway. We hypothesized that over-expression of GLUT-1 and p-Akt was associated with the resistance to cisplatin of laryngeal carcinoma Hep-2 cells. We explored whether apigenin inhibited GLUT-1 and p-Akt, resulting in sensitization of laryngeal carcinoma Hep-2 cells to cisplatin. Real-time RT-PCR and Western blotting confirmed the presence of GLUT-1 mRNA, and GLUT-1 and p-Akt proteins in Hep-2 cells. We found that resistance or insensitivity of Hep-2 cells to cisplatin might be associated with such expression. Apigenin markedly enhanced the cisplatin-induced suppression of Hep-2 cell growth. This effect was concentration- and time-dependent. Thus apigenin may significantly reduce the levels of GLUT-1 mRNA, and GLUT-1 and p-Akt proteins, in cisplatin-treated Hep-2 cells, in a concentration- and time-dependent manner. To conclude, overexpression of GLUT-1 mRNA may be associated with the resistance to cisplatin of laryngeal carcinoma Hep-2 cells. Apigenin may enhance the sensitivity to cisplatin of laryngeal carcinoma cells via inhibition of GLUT-1 and p-Akt expression.

Keywords: Glucose transporter-1, pi3k/akt, laryngeal carcinoma, apigenin, cisplatin, chemosensitivity

Introduction

Laryngeal carcinoma is one of the most common head and neck cancers. Current therapeutic strategies for the early stages of laryngeal carcinoma include various types of larynx-conserving surgery, or radiotherapy. For advanced laryngeal carcinoma, combined therapies are often used, including concurrent chemo-radiotherapy or total laryngectomy, with possible adjuvant therapy [1]. However, the survival rate has not improved over the last few decades [2] because of resistance to chemo-radiotherapy and late metastasis [3, 4]. Thus, overcoming the resistance to chemo-radiotherapy of laryngeal carcinoma is a challenge in cancer therapy.

Laryngeal carcinoma, like other malignant tumors, is hypoxic in nature and exhibits increased glucose uptake and metabolism [5-7]. Glucose transporter-1 (GLUT-1) has been identified as a hypoxic marker [8] and plays a significant role in malignant glucose metabolism; GLUT-1 may contribute to increased FDG uptake [9]. Our previous studies revealed that GLUT-1 might play a role in the mechanism of radiore-sistance of laryngeal carcinoma, and we explored methods of improving the radiosensitivity of laryngeal carcinoma [4, 10]. Recently, the relationship between GLUT-1 expression and resistance to chemotherapy of some human cancer cell lines has gradually received more attention [11-14]. However, no report has yet explored the possible correlation between GLUT-1 expression and resistance to chemotherapy of laryngeal carcinoma.

Abnormal expression of GLUT-1 is associated with the actions of multiple signal transduction pathways, including the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling
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pathway, which plays an important role in the regulation of GLUT-1 expression. Several studies have confirmed that the PI3K/Akt pathway and GLUT-1 affect glucose metabolism [15, 16]. Akt, also called PKB or Rac, is an important downstream serine-threonine regulatory kinase. A variety of molecules can activate Akt; these include insulin, heat shock proteins, and TNFα. Activated Akt plays a central role in PI3K/Akt signal transduction pathways mediating cell growth, survival, and differentiation [17]. Some studies have revealed that PI3K/Akt pathway activity is closely associated with resistance to chemotherapy and, when this pathway is inhibited, the sensitivity to chemotherapy is enhanced [18-20]. Thus, co-targeted therapy that reduces GLUT-1 expression and PI3K/Akt pathway activity may overcome the chemoresistance of human cancers [21, 22].

Apigenin is a natural phyto-estrogen flavonoid present in a wide range of fruits, vegetables (especially celery), beans, and tea. In vitro and in vivo studies have demonstrated that apigenin has potential biological effects, including anti-oxidative, anti-inflammatory, and anti-cancer activities [22]. Of these, the anti-tumor effect is the most prominent [22]. Apigenin may inhibit the expression of some biomarkers to enhance the sensitivity to chemotherapy via downregulation of the PI3K/Akt pathway [23-26]. However, only one study has investigated whether apigenin inhibits the expression of GLUT-1 and the PI3K/Akt pathway [23]. Therefore, we further investigated whether apigenin might concurrently inhibit the expression of GLUT-1 and downregulate the PI3K/Akt pathway in human cancers.

In this study, we hypothesized that over-expression of GLUT-1 and p-Akt was associated with resistance to cisplatin of laryngeal carcinoma Hep-2 cells. Next, we explored whether the effect of apigenin on GLUT-1 and p-Akt sensitized laryngeal carcinoma Hep-2 cells to cisplatin.

Materials and methods

Cells, antibodies, and plasmids

The laryngeal carcinoma Hep-2 cell line was purchased from the Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China). Chloroform, isopropyl alcohol, and anhydrous alcohol were purchased from Hangzhou Changzhen Chemical Plant (Hangzhou, China). Agarose was purchased from Biowest (Spain). TRIzol was purchased from Invitrogen (Carlsbad, CA). Reverse transcriptase MMLV and the TAQ enzyme were purchased from Promega (USA). DNA Marker DL2000, the pcDNA3.1 vector, restriction endonucleases HindIII and XbaI, and T4 DNA ligase were purchased from TaKaRa Co. (Japan). Cisplatin, dimethyl sulfoxide (DMSO), Tween 20, and Ponceau S were purchased from Sigma (St. Louis, MO). Apigenin was purchased from Selleckchem (USA). Primary antibodies against GLUT-1 and p-Akt were purchased from Santa Cruz Biotechnology (CA). The secondary antibodies donkey anti-rabbit and donkey antimouse, cell lysis kits, Supersignal West Femto kits, and PMSF, were purchased from Pierce (USA). Primers were synthesized by Invitrogen. The sequence of the entire coding region of GLUT-1 was obtained from GenBank, and primers were designed using the ClustalX and Omega 2.0 Applied Software.

Cell culture

Hep-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin, at 37°C in a 5% CO2 atmosphere. Cells were trypsinized and harvested after reaching 80-90% confluence.

Preparation of apigenin and cisplatin

Apigenin (13.5 mg) was dissolved in 1,000-µL DMSO and used at 50 mM in all experiments. Cisplatin (5 mg) was dissolved in 1,000-µL DMSO and used at 5 µg/µl in all experiments.

Proliferation assays of Hep-2 Cells using the cell counting Kit-8 (CCK-8) system [27]

Cultured Hep-2 cells were trypsinized using 0.25% trypsin. Cell proliferation was measured using the CCK-8 system (Beyotime, Nanjing, China) according to the manufacturer’s instruc-
tions. The 13 experimental groups were as follows: Hep-2 cells, DMSO, Hep-2 cells+DMSO, 10 µM apigenin+Hep-2 cells, 40 µM apigenin+Hep-2 cells, 160 µM apigenin+Hep-2 cells, 2 µg/ml cisplatin+Hep-2 cells, 3 µg/ml cisplatin+Hep-2 cells, 4 µg/ml cisplatin+Hep-2 cells, 5 µg/ml cisplatin+Hep-2 cells, 10 µM apigenin+5 µg/ml cisplatin+Hep-2 cells, 40 µM apigenin+5 µg/ml cisplatin+Hep-2 cells, and 160 µM apigenin+5 µg/ml cisplatin+Hep-2 cells. Cultures proceeded for 24, 48, and 72 h. Briefly, 5 × 10^3 cells from each group were seeded into wells of 96-well culture plates. Cells were cultured in SFM at 37°C. After 1-6 days, 10 µl of CCK-8 reagent were added to each well, and, after 2 h of incubation at 37°C, the absorbance at 450 nm was measured. OD = OD<sub>cell</sub>−OD<sub>blank</sub>. The cell survival rate was calculated as OD/OD<sub>control</sub> × 100%. All assays were carried out in triplicate.

Detection of GLUT-1 expression in Hep-2 Cells by real-time reverse transcription-polymerase chain reaction (RT-PCR)

Cells of each group described above were homogenized in TRIzol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's protocol. Total RNA concentration was measured by ultraviolet spectrophotometry; and an optical density (OD) 260/280 ratio between 1.8 and 2.0 was deemed to show that the RNA was acceptably pure. Reverse transcription was performed according to the manufacturer's protocol. Briefly, 1 µg of total RNA and Moloney murine leukemia virus (MMLV) reverse transcriptase (Fermentas, Canada) were contained in a 20-µl reaction volume with 0.5 µg/µl of oligo d(T) primer solution, 1 µl of random primer solution (0.2 µg/µl), and 10 µl of DEPC·H<sub>2</sub>O. The reaction mix was first pre-denatured at 65°C for 10 min. After addition of 200 U of MMLV reverse transcriptase, samples were incubated at 42°C for 1 h and annealed at 70°C for 10 min. The above-synthesized cDNAs were used as templates for real-time fluorescence quantitative PCR using the fluorescent dye SYBR Green and the Eppendorf Realplex4 real-time PCR system (Eppendorf Realplex4; Hamburg, Germany). The 20-µl reaction mix consisted of 10 µl of 2 × SYBR Green buffer, 1 µl of template, 1 µl of upstream- and downstream-specific primers, and 8 µl of deionized water. The reaction mix was pre-denatured at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 59°C for 20 s, and 72°C for 20 s. Each sample was run in triplicate. The primers used were as follows: GLUT-1 Forward (F): 5'-CGCAACGAGAGAACC-3'; GLUT-1 Reverse (R): 5'-GTCACCTTTCCCGCATC-3'; GAPDH (control) Forward (F): 5'-TCTTTCTGGCATGCAATGT-3'; and GAPDH Reverse (R): 5'-CAGGAGGACATGATCTTG-3'. The lengths of the PCR products were 123 bp (GLUT-1) and 208 bp (GAPDH).

To distinguish specific and non-specific products and primer dimers, dissociation curve analysis was conducted immediately after amplification by continuous monitoring of the SYBR Green I fluorescence signal at temperatures between 60°C and 95°C. For calculation of differential gene expression levels, the 2^ΔΔCt formula was used.

Analysis of the expression level of GLUT-1 protein by Western blotting

Western blotting was performed as described previously [27]. The levels of the GLUT-1, p-Akt, and GAPDH (control) proteins in each group of Hep-2 cells were assayed using a BAC protein quantitative kit (Wuhan Boster Biological Technology Co. Ltd., Wuhan, China). Briefly, 80-µg amounts of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA). Skim milk solution (2%) was used as a blocking solution (room temperature, 1 h). The membrane was incubated with primary antibodies (anti-GLUT-1, 1:250; -p-Akt, 1:250; -GAPDH, 1:3,000) at room temperature for 3 h, and with secondary antibodies (1:1,000, donkey anti-rabbit; 1:3,000, donkey anti-mouse) at room temperature for 1 h. The proteins were detected using an enhanced chemiluminescence system (Santa Cruz Biotechnology, Santa Cruz, CA) by exposure to X-ray film. Protein levels were analyzed semi-quantitatively using the Kodak Gel Logic Analysis System.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (version 19.0; SPSS Inc., Chicago, IL). A P-value less than 0.05 was deemed to indicate statistical significance.
Results

**Apigenin enhances the sensitivity of Hep-2 cells to cisplatin**

The CCK results showed that the survival rates of Hep-2 cells were significantly reduced with increasing concentrations of apigenin at all timepoints ($P < 0.01$). The survival rates of Hep-2 cells decreased gradually with increasing duration of culture in the presence of 160 µM apigenin ($P < 0.01$, **Figure 1A**).

The survival rates of Hep-2 cells were reduced significantly in the presence of various concentrations of cisplatin, compared to the control groups ($P < 0.01$, **Figure 1B**). At 2 and 3 µg/ml cisplatin, the survival rates of Hep-2 cells were significantly reduced with increasing culture duration; however, at 4 and 5 µg/ml cisplatin, the survival rates of Hep-2 cells were not further reduced from 48 to 72 h ($P > 0.05$). At 24 h of exposure, the survival rates of Hep-2 cells were not significantly different when different concentrations of cisplatin were used ($P > 0.05$). At 48 or 72 h of culture, the survival rates of Hep-2 cells were lower in the presence of 3, 4, and 5 µg/ml cisplatin than 2 µg/ml cisplatin ($P < 0.01$, **Figure 1B**); however, the survival rates of Hep-2 cells were higher in the presence of 4 and 5 µg/ml cisplatin than 3 µg/ml cisplatin ($P < 0.05$, **Figure 1B**).

Apigenin markedly enhanced the effect of cisplatin on Hep-2 cells. This effect was apigenin concentration- and time-dependent ($P < 0.01$, **Figure 1C**).

**Expression of GLUT-1 mRNA, and GLUT-1 and p-Akt Proteins, in Laryngeal Carcinoma Hep-2 cells**

The GLUT-1 mRNA and GAPDH mRNA real-time RT-PCR products were of 123 and 208 bp, respectively. Dissociation curve analysis performed at 60-95°C showed only the expected peaks at 87.1°C and 85.1°C for GLUT-1 and GAPDH mRNAs, respectively. Real-time RT-PCR showed that the specific amplified curve for GLUT-1 mRNA and GAPDH. Western blotting confirmed that both GLUT-1 (**Figure 2A**) and p-Akt (**Figure 2B**) were expressed in Hep-2 cells.

**Effects of apigenin and cisplatin on GLUT-1 mRNA and protein levels in Hep-2 cells**

At 24 h, 10 and 40 µM apigenin did not decrease the expression level of GLUT-1 in Hep-2 cells compared to controls ($P > 0.05$); in contrast, 160 µM apigenin did decrease the expression level of GLUT-1 in Hep-2 cells compared to controls ($P = 0.005$). However, the
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The effects of reduction did not differ significantly among 10, 40, and 160 µM apigenin (P > 0.05). At 48 and 72 h, apigenin downregulated the expression of GLUT-1 mRNA in a concentration-dependent manner (P < 0.01, Figure 3A).

At the same apigenin concentration, the GLUT-1 mRNA level was reduced significantly upon prolongation of culture duration (P < 0.01, Figure 3A).

Real-time RT-PCR showed that cisplatin did not always reduce the level of GLUT-1 mRNA at the same culture duration with increasing cisplatin concentration; similar findings were obtained upon prolongation of culture time using an identical apigenin concentration. At 24 h, there was no significant difference in the GLUT-1 mRNA level in the presence of various concentrations of cisplatin. At 48 h, there were no significant differences in GLUT-1 mRNA levels among cultures with 2, 3, 4, and 5 µg/ml cisplatin. At 72 h, there was no significant difference in GLUT-1 mRNA level between cultures with 4 and 5 µg/ml cisplatin (Figure 3B). At 2 and 3 µg/ml cisplatin, there was no significant difference in the GLUT-1 mRNA level upon prolongation of culture duration (24, 48, and 72 h). At 4 and 5 µg/ml cisplatin, there was no significant difference in the GLUT-1 mRNA level between 48- and 72-h cultures.

Apigenin significantly reduced the GLUT-1 mRNA level in cisplatin-treated Hep-2 cells in a concentration- and time-dependent manner (P < 0.01, Figure 3C).

Western blotting revealed that apigenin or cisplatin alone did not significantly reduce the GLUT-1 protein levels (P > 0.05, Figures 2A, 3D). Significant decreases in GLUT-1 protein levels were noted after 48 h of exposure to 40 and 160 µM apigenin of cisplatin-treated Hep-2 cells (P < 0.01, Figure 3D, 3E).

Effects of apigenin and cisplatin on p-Akt protein levels in Hep-2 cells

Western blotting revealed that apigenin or cisplatin alone did not significantly reduce the p-Akt protein level (P > 0.05, Figures 2B, 4A).

Apigenin significantly reduced the p-Akt protein level in cisplatin-treated Hep-2 cells at 48 h of exposure, in an apigenin-concentration-dependent manner (P < 0.01, Figure 4B, 4C). p-Akt protein was barely detected after 72 h of culture in the presence of 40 and 160 µM apigenin (Figure 4B, 4C).

Discussion

GLUT-1 over-expression is a possible mechanism of resistance to chemo-radiotherapy of some human cancers [4, 11-14]. This may be because elevated GLUT-1 expression provides energy to malignant tumors, allowing development of chemo-radioresistance [4]. GLUT-1 causes chemoresistance by increasing cell turnover [28]. GLUT-1 up-regulates the expression levels of the multidrug resistance-1 (MDR-1) [29] and P-glycoprotein (P-gp) genes [30]. To
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In our knowledge, no work on laryngeal carcinoma has addressed a possible correlation between GLUT-1 expression level and resistance to chemotherapy; factors regulating GLUT-1 expression; or the possibility of inhibiting GLUT-1 expression to enhance sensitivity to chemotherapy.

Cisplatin is one of the most common therapeutic agents used to treat head-and-neck cancers [12-14]. However, development of resistance limits the widespread clinical use of cisplatin [12-14]. In this study, the survival rates of Hep-2 cells treated with cisplatin were not further reduced when cisplatin concentrations increased or culture duration was prolonged. In the presence of 4 or 5 µg/ml cisplatin, the survival rate of Hep-2 cells was paradoxically higher than in 3 µg/ml cisplatin. These results suggest that laryngeal carcinoma Hep-2 cells may become resistant or insensitive to cisplatin treatment. Further, we explored the mecha-
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We found that GLUT-1 mRNA and protein were present in laryngeal carcinoma Hep-2 cells. When cisplatin was administrated to Hep-2 cells, the GLUT-1 mRNA levels were not reduced at any timepoint as cisplatin concentrations increased, and we made similar findings upon prolongation of culture time at the same concentrations of apigenin. The effect of cisplatin on the GLUT-1 protein level was similar to that on the GLUT-1 mRNA level. Western blotting revealed that cisplatin alone did not significantly reduce the GLUT-1 protein level. We suggest that the resistance or insensitivity to cisplatin of Hep-2 cells may be associated with enhanced GLUT-1 mRNA and protein levels. We reported similar findings in our previous study of the radioresistance of Hep-2 cells [4]. Therefore, we speculate that GLUT-1 expression plays an important role in the chemo-radioresistance of laryngeal carcinoma Hep-2 cells. Shimanishi et al. also found that proliferation of some oral squamous cell carcinoma cell lines treated with cisplatin was higher under hypoxia than under normoxia; enhanced proliferation was associated with up-regulation of GLUT-1 mRNA and protein levels [14].

Based on the relationship between its over-expression and enhanced chemoresistance to cisplatin, many targeted therapeutic methods aim to inhibit expression of GLUT-1 to enhance sensitivity to chemotherapeutic agents, including cisplatin. Such targeted drugs include small-molecule inhibitors (WZB117, STF-31, CUR+ DOX-loaded micelles, and 2-amino-2-deoxy-D-glucose) [11, 13, 31, 32], and specific molecules targeting GLUT-1 (RNA interference) [12, 14].

Several studies have demonstrated that apigenin enhances the sensitivity of carcinomas to

Figure 4. The Effects of Apigenin and Cisplatin on p-Akt Protein Levels in Hep-2 Cells. A, B: Western blotting revealed that apigenin or cisplatin alone did not significantly reduce the p-Akt protein level (P > 0.05). C: Apigenin significantly reduced the p-Akt protein level in cisplatin-treated Hep-2 cells at 48 h of exposure, in an apigenin-concentration-dependent manner (P < 0.01). p-Akt protein was barely detected after 72 h of culture in the presence of 40 and 160 µM apigenin. D: The results of Western blotting.
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cisplatin [33-35]. Apigenin, a natural plant flavone, may have chemopreventative and therapeutic effects as an anti-inflammatory, antioxidant, and anti-cancer agent [22]. The anti-cancer mechanism may involve induction of apoptosis, inhibition of angiogenesis, reversal of multidrug resistance, and exertion of an anti-proliferative effect. These increase sensitivity to cisplatin by decreasing the levels of various tumor growth factors and hypoxic markers, and by inhibiting signaling pathways, including those triggered by vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNF-R), and serine/threonine kinase glycogen synthase kinase-3b (GSK-3b) [22, 33-36]. Apigenin may also inhibit expression of GLUT-1 via a mechanism involving PIK/Akt [23]. However, no prior report has shown that apigenin inhibits GLUT-1 expression to chemosen-sitize laryngeal carcinoma cells to cisplatin. In the present study, we found that apigenin suppressed the survival of Hep-2 cells as the apigenin concentration increased, and cell survival decreased gradually upon prolongation of culture time in the presence of a high concentration (160 µM) of apigenin. We also found that apigenin markedly enhanced the ability of cisplatin to suppress Hep-2 cell growth, in a concentration- and time-dependent manner. Further, we found that the apigenin-mediated potentiation of the effect of cisplatin on laryngeal carcinoma Hep-2 cells may be mediated by inhibition of GLUT-1 expression. Although apigenin did not markedly decrease the expression level of GLUT-1 in Hep-2 cells, apigenin alone downregulated the GLUT-1 mRNA level in a concentration-dependent manner, at 48 and 72 h. Apigenin at all tested concentrations also reduced the GLUT-1 mRNA levels upon prolongation of culture duration. Apigenin combined with cisplatin may significantly reduce the GLUT-1 mRNA level. Therefore, we suggest that apigenin induces sensitization of Hep-2 cells to cisplatin by decreasing the expression level of GLUT-1. However, apigenin alone, or in combination with cisplatin, did not significantly reduce the GLUT-1 protein level. This phenomenon was also noted when antisense oligodeoxynucleotides were used to inhibit GLUT-1 expression, which enhances the radiosensitivity of laryngeal carcinoma [4]. Possible explanations are that GLUT-1 mRNA was degraded when the GLUT-1 protein level peaked, or that the GLUT-1 protein level was increasing when the GLUT-1 mRNA level peaked. Also, transcription and translation may be separated temporally and/or spatially [4]. Our results differed from those of Melstrom et al. [23, 34]. These authors found that apigenin concurrently inhibited both GLUT-1 mRNA and protein levels in human pancreatic cancer cells. Thus, the mechanism of action of apigenin should be further investigated.

Melstrom et al. found that apigenin-mediated GLUT-1 down-expression was associated with inhibition of the PI3K/Akt pathway in human pancreatic cancer cells [23]. In the present study, we sought to investigate the p-Akt expression level in laryngeal carcinoma Hep-2 cells and the effect of apigenin on such expression. We found that p-Akt and GLUT-1 proteins were expressed in laryngeal carcinoma Hep-2 cells. Apigenin or cisplatin alone did not significantly reduce the p-Akt protein level. However, apigenin combined with cisplatin markedly decreased the p-Akt expression level in Hep-2 cells, similar to the effect of apigenin on GLUT-1 protein. This finding allows us to speculate that a correlation exists between p-Akt and GLUT-1 expression in laryngeal carcinoma Hep-2 cells. Further work should explore whether apigenin reduces the GLUT-1 expression level to enhance sensitivity to cisplatin via inhibition of the PI3K/Akt pathway in laryngeal carcinoma cells. We plan to investigate the effect of apigenin on GLUT-1 and PI3K/Akt pathway activities in vivo.

In conclusions, GLUT-1 and p-Akt were expressed in Hep-2 cells. Over-expression of GLUT-1 mRNA may be associated with the resistance to cisplatin of laryngeal carcinoma Hep-2 cells. Apigenin may enhance the sensitivity to cisplatin of laryngeal carcinoma via inhibition of GLUT-1 and p-Akt expression.

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Disclosure of conflict of interest

None.

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