A Novel Chemotherapeutic Arene Ruthenium(II) Drug Rawq01 Altered the Effect of microRNA-21 on PTEN/AKT Signaling Pathway in Esophageal Cancer Cells

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Abstract. *Aim: Organometallic arene Ru(II) complexes have long been considered as most promising substitutes for cisplatinum as an anti-tumor drug, with low toxicity towards human normal cells and high selectivity to tumor cells. In this study, we synthesized a novel arene Ru(II) drug named Rawq01. We evaluated its activity in an in vitro model of esophageal cancer (ESCC) and further explored the cellular signaling pathways altered by Rawq01. Materials and Methods: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphe nyltetrazolium bromide (MTT) assay, colony forming assay and apoptosis assays were used to evaluate the antitumor activity of Rawq01. We treated ESCC cells with Rawq01 alone or combined with microRNA-21(miR-21) and LY294002 to explore whether Rawq01 altered the effect of miR-21 on PTEN/AKT signaling pathway in ESCC cell. Results: ESCC cells were sensitive to RAWQ01. We also found that Rawq01 up-regulated the expression of PTEN through mir-21 inhibition and therefore inhibited the PI3K-AKT pathway. Furthermore, when we combined Rawq01 treatment with miR-21 inhibitor or LY294002, the inhibitory effect of Rawq01 was significantly enhanced. Conclusion: The effect of miR-21 on PTEN/AKT signaling pathway is abrogated by the novel arene Ru(II) drug Rawq01. Our data may be useful for the future development of a chemosensitizing strategies through manipulating microRNA expression for tumor treatment.*

Esophageal cancer is the fourth leading cause of cancer deaths in China, where esophageal squamous cell carcinoma (ESCC) is the dominant histological type (1, 2). Although surgical resection can remarkably improve outcomes in a substantial number of patients with esophageal cancer, prognosis is poor. The overall 5-year survival rate of patients is only 5-10% and about 75% of patients die of disease within the first year after diagnosis (3).

Due to the presence of the aromatic π-ligand which eases cell membrane penetration and thus enhances cellular uptake, organometallic arene Ru(II) complexes have long been considered as most promising substitutes for cisplatinum as an anti-tumor drug with low toxicity to normal human cells and high selectivity for tumor cells (4-10). Arene Ru(II) complexes of the type ((η6-arene)Ru(L)(Cl))PF6, where L is typically a chelating diamine ligand (*e.g.* ethylenediamine) exhibit promising anticancer activity *in vitro* and *in vivo* (11, 12). Moreover, the arene Ru(II) complex (η6-*p*-MeC6H4Pri)Ru(P-pta)Cl2, termed RAPTA-C, exhibits low antitumor activity *in vitro* but has an acceptable inhibition of lung metastasis in CBA mice (13, 14). Arene Ru(II) complexes coordinated by chloroquine were also reported to induce apoptosis of human lymphoid cell lines through DNA interactions and display low toxicity to normal mammalian cells (15). Arene ruthenium(II) complexes coordinated by pta and its derivates, such as RAPTA-C and RAPTA-B have also been reported to inhibit metastasis growth in addition to the low toxicity (16, 17). Chatterjee *et al.* also indicated that RAPTA-C induced tumor cell apoptosis and cell-cycle arrest in the G_1/G_0 or G_2/M phase through p53 involvement (18).

The Phosphoinositide 3-kinase(PI3K)/AKT(Protein Kinase B, PKB) signal transduction cascade has been extensively investigated for its roles in oncogenic transformation. PI3K

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participates in multiple cellular processes, including cell growth, transformation, migration and differentiation (19). Following cell activation by growth factors or cytokines, PI3K is recruited to the plasma membrane, where it catalyzes the conversion of membrane phosphoinositide 4,5-biphosphate (PIP2) to generate phosphoinositide 3,4,5-triphosphate (PIP3) (20, 21). The accumulation of PIP3 creates a plasma membrane docking site for AKT, which binds to PIP3 *via* the pleckstrin homology domain and undergoes conformational changes (20,21). Evidence has shown that AKT, a downstream kinase of PI3K, is involved in malignant transformation (22). It is believed that AKT/PI3K promotes cancer cell survival through anti-apoptotic and pro-apopototic mechanisms and accelerates cell-cycle progression by controlling the expression or activity of cyclin D, p27KIP1 and p21CIP1. Furthermore, PI3K activity has been linked to a variety of human tumors including breast cancer, lung cancer, melanomas, and leukemia (23-26). Inhibition of this pathway is a promising approach for novel chemotherapeutic agents. PIP3 generation caused by P13K activation can be counterbalanced by germline phosphatase and tensin homolog (PTEN), a lipid phosphatase and tumor suppressor that de-phosphorylates PIP3 back to PIP2 therefore controlling the activation of AKT (27, 28). Pathogenic PTEN mutations have been frequently observed in various neoplasms, including glioblastoma, melanoma, prostate cancer, and breast cancer. Although few *PTEN* gene mutations have been found in ESCC tissues, the majority of patients with ESCC have significantly reduced or lack PTEN protein expression (28). The mechanism of PTEN protein inactivation is largely unknown at the moment; further research may yield promising results regarding its role in AKT inhibition and the tumor phenotype.

MicroRNAs are small non-coding RNAs that regulate gene expression at the post-transcriptional level either by degradation or translational repression of a target mRNA. Recent studies have shown that microRNAs regulate the expression of several tumor suppressors and oncogenes and contribute to carcinogenesis (29, 30). Specifically, miR-21 stands out as the oncogenic miRNA most often overexpressed in diverse types of malignancy types, and correlated with tumor-suppressor down-regulation (31). To date, miR-21 has been reported to specifically target and down-regulate tumor suppressors such as PTEN (32, 33).

Organometallic arene Ru(II) complexes have long been considered as most promising substitutes for cisplatinum as an anti-tumor drug, with low toxicity to human normal cells and high selectivity to tumor cells. In the present study, we synthesized a novel arene Ru(II) drug named Rawq01 which we evaluated for activity in an *in vitro* model of esophageal cancer cell, and we further characterized the effect of microRNA-21 on PTEN/AKT signaling pathway by this novel organometallic arene Ru(II) complex.

Materials and Methods

Synthesis of Rawq01. The precursor $((\eta^6\text{-}C_6H_6)RuCl_2)_2$ was prepared according to literature (34). 1,10-Phenanthroline-5,6-dione was prepared using similar methods to those reported elsewhere (35). All reagents were used as purchased from commercial suppliers without further purification. The ligand 2-(3-hydroxy-4-methoxyphenyl) imidazo (4,5*f*) (1, 10) phenanthroline (*m*-OH-*p*-OMePIP) was prepared using a similar method as in literature with some modifications and was used after further purification (36). 1,10-Phenanthroline-5,6-dione (1.6 mmol, 347 mg) and 3-hydroxy-4-methoxybenzaldehyde (1.6 mmol, 243.2 mg) in 20 ml of HAc solution containing 2.53 g of NH4Ac solution was refluxed at 110˚C for 4 h. Then 20 ml of water was added and the pH was adjusted to 7.0 at room temperature. The solution was filtered, and dried in vacuum to obtain a yellow precipitate. The product was purified in a silicagel column by using ethanol as eluent, with 62.3% yield, or 341 mg.

Synthesis of $((C_6H_6)Ru(m\text{-}OH\text{-}p\text{-}OMPIP)Cl)Cl\bullet 2H_2O$. (RAWQ01): A mixture of $((\eta^6\text{-}C_6H_6)RuCl_2)_2$ (0.15 mmol, 75 mg) and *m*-OH-*p*-OMPIP (0.3 mmol, 103.2 mg) in dichloromethane (40 ml) was refluxed under argon for 6 h until the solution color changed from brown to yellow (37). A yellow precipitate was obtained under rotary evaporation and purified by re-crystallization in methanol. The yield was 54.5% (103 mg). ESI-MS (in MeOH, *m/z*): 559. 09, $((M\text{-}Cl)^+)$. ^{1}H NMR (DMSO-d_6, δ/ppm) δ: 9.92 (d, *J*=5.2 Hz, 2H), 9.40 (s, 2H), 9.18 (s, 2H), 8.17 (s, 2H), 7.79 (d, *J*=7.6 Hz, 2H), 7.15 (d, *J*=8.5 Hz, 2H), 6.30 (s, 6H).

Cell culture and treatments. ESCC lines K150, TE-1, EC-9706, EC-1 and EC-109 was obtained from the department of central laboratory, Shanghai tenth People's Hospital (Shanghai, China). The cell lines were cultured in RPMI 1640 Medium (GIBCO® Invitrogen, CA, USA) supplemented with 5% fetal bovine serum (GIBCO® Invitrogen CA, USA) and penicillin/streptomycin (GIBCO® Invitrogen, CA, USA; 10,000 U of penicillin and 10,000 μg of streptomycin per 1 ml) in a humidified incubator at 37˚C with 5% CO_2. For the transfection experiments, miR-21 inhibitor (5'-UCAACAUCAGUCUGAUAAGCUA-3'), miR-21 mimics (sense 5'-UAGCUUAUCAGACUGAUGUUGA-3', antisense 5'-AACAU CAGUCUGAUAAGCUAU U-3') and a scrambled sequence (5'-CATTAATGTCGGACAACTCAAT-3') were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and each (200 mM) was transfected into ESCC cells by using Lipofectamine TM 2000 (Invitrogen, CA, USA) and OptiMEM I (Invitrogen) according to the manufacturer's instructions. Cultures at 60% confluence were switched to serum-free medium for 16 h, and then treated with the PI3K inhibitor, LY294002 (Santa Cruz Biotechnology, Inc. sc-201426) at the stock concentration(15 μm, in complete cell culture medium). For the Rawq01 treatment alone, freshly-prepared drug was added to cells at the final half maximal inhibitory concentration (IC_{50}) concentration. For the combined treatment, cells were first transfected or treated with LY294002. Twenty-four hours after the treatment, cells were treated by Rawq01 at the IC_{50}.

In vitro drug sensitivity assay. All above cell lines were separately seeded into 96-well plates (2×10^3 viable cells/well) and allowed to attach overnight. After cellular adhesion, freshly-prepared RAWQ01 at final concentration (100 μM, 50 μM, 25 μM and 12.5 μM) were added to corresponding cells. Forty-eight hours after the addition of drugs, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The absorbance at

490 nm (A490) of each well was read on a spectrophotometer. The concentration at which the drug produced 50% inhibition of growth (IC_{50}) was estimated by the relative survival curve. Three independent experiments were performed in quadruplicate.

Colony-forming assays. The five human ESCCs were seeded as single-cell suspension at 100 cells per well in 12-well plates. Twenty-four hours later, cells were exposed to Rawq01 at the IC_{50} concentration. After 14 days, the media were aspirated and plate were washed with phosphate-buffered saline (PBS) and fixed using 4% paraformaldehyde. Plates were subsequently stained with crystal violet solution and gently washed with water. Plates were finally air-dried and photographed. The numbers of total colonies were counted.

Apoptosis assay. Suspended cells were collected by centrifugation (100 ×g for 5 min), and the supernatant decanted. Cells were washed twice with cold PBS and collected by centrifugation (100 ×g for 5 min). Cell apoptosis was detected by using annexin V/PI staining according to the manufacturer's instructions (eBioscience). We used a 400 μl 1×binding buffer to briefly suspend the cells, and adjust the cell to approximately 1×10^6 cells/ml. Five microlitters of V-FITC were added into the suspension, mixed gently and cells were incubated at 4˚C for 15 min in the dark. We finally added 10 μl PI and incubated the mixutre at 4˚C under dark conditions for 5 min. Cells stained with a combination of Annexin V-FITC, propidium iodide (PI), were then analyzed by a flow cytometer.

RNA isolation and (qRT-PCR). For the detection of miR-21 , stem-loop quantitative RT-PCR was performed by using SYBR Premix Ex TaqTM Kit (TaKaRa) according to the manufacturer's instructions. The qRT-PCR primer and thermal cycling conditions for miR-21 and U6 was designed according to Ma *et al*. (32). U6 expression was assayed for normalization, and relative gene expression determinations were made with the comparative delta-delta CT method (38).

Western blotting. The ESCC cells were lysed with RIPA lysis buffer supplemented with protease inhibitors. Proteins were harvested, resolved on an SDS denatured polyacrylamide gel and then transferred onto a nitrocellulose membrane. Antibodies against PTEN, AKT, phosphorylated AKT and endogenous control GAPDH were purchased from Santa Cruz Biotechnology. The antibodies were incubated with the blot overnight at 4˚C. Membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Protein expression was assessed by enhanced chemiluminescence and exposure to chemiluminescent film. Relative protein expression levels were quantified by specific protein/GAPDH ratio, which are presented as mean±standard deviation from three independent experiments.

Statistical analysis. Mean±SEM are shown. Quantitative reverse transcription-PCR assays were, at minimum, performed in triplicate. The data were compared using Student's *t*-test, and a *p*-value of <0.05 was considered statistically significant.

Results

Rawq01 inhibits growth of ESCC cell and induces apoptosis in vitro. To evaluate the antitumor potential of Rawq01 (molecule of structure shown in Figure 1A), we first analyzed their effect on cell proliferation. The inhibitory effects of Rawq01 on viability of ESCC esophageal carcinoma cell lines K150, TE-1, EC-109, EC-9706 and EC-1 were evaluated (Figure 1B) and the IC_{50} values for Rawq01 were 53±6, 52±7, 47±6, 43±6 and 36±6 μM for 48 h treatment, respectively, suggesting that ESCC cells possessed sensitivity to Rawq01.

Furthermore, the colonies of ESCC cells were significantly reduced when treated with Rawq01 at the IC_{50} (Figure 1C). To further analyze the characteristics of the death induced by Rawq01, we performed an apoptosis assay. ESCC cells were stained with annexin V after a 48-h treatment with Rawq01 at the IC_{50}. Compared to control cells, the apoptosis rate in Rawq01-treated cells was close to 10% at 48 h, indicating that some cells had undergone apoptosis (Figure 1D).

Rawq01 down-regulates miR-21 and increases the activity of PTEN in K150 and EC-109 cells. As shown in Figure 2, up-regulation of *PTEN* and down-regulation of *pAKT* is observed in K150 and EC-109 cells when treated with Rawq01, suggesting that Rawq01 triggered the activity of PTEN and thus suppressed hyperactivity of the PI3K-AKT cascade. Interestingly, the other three cell lines did not show any changes in PTEN and pAKT expression, indicating that Rawq01 may inhibit cell growth *via* other mechanisms. Recent studies have showen that miR-21 specifically targets and down-regulates tumor suppressor PTEN (32, 33). Therefore, we hypothesized that Rawq01 up-regulates the expression of PTEN level through miR-21 inhibition. As shown in Figure 2B, we observed down-regulation of miR-21 in Rawq01 treated EC-109 cells. Furthermore, Rawq01 combined with miR-21 inhibitor led to a significant increase of PTEN protein compared to Rawq01 treatment alone, while miR-21 mimics combined with Rawq01 did not lead to a significant change in PTEN protein expression (Figure 2C).

Inhibition of miR-21 expression and AKT pathway increases chemosensitivity to RAWQ01 and apoptosis in ESCC cells. Given that Rawq01 down-regulates miR-21 and inhibits the AKT pathway, we investigated whether inhibiton of miR-21 expression or AKT pathway would enhance the antitumour capability of Rawq01 in ESCC cells. We transfected EC-109 cells with a specific miR-21 inhibitor, or the PI3K inhibitor LY294002. As shown in Figure 3A, cells treated with both miR-21 and Rawq01 exibited slightly reduced PTEN expression and down-regulation of phosphorylated AKT when compared to cells treated with Rawq01 alone. Both miR-21inhibitor and LY294002 could enhance the inhibitory effect of Rawq01 on the AKT pathway. In the proliferation assay, the growth rate was much lower in the cells treated with Rawq01 and miR-21 inhibitor or LY294002 compared with RAWQ01 alone (Figure 3B). Furthermore, we examined the effect of miR-21 knockdown and AKT pathway inhibition on apoptotic cell death. For this purpose,

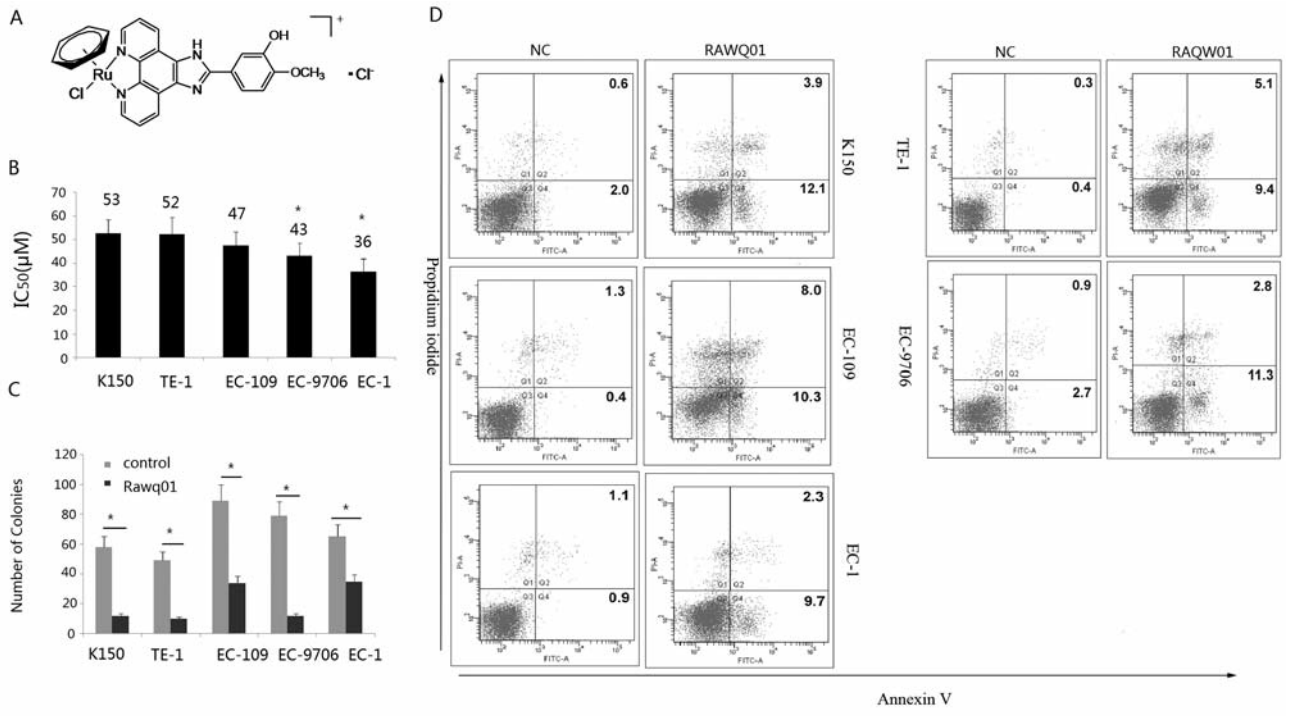

Figure 1. *Arene ruthenium compound RAWQ01 inhibits esophageal carcinoma cell growth and induces apoptosis in vitro. A: Molecular structure of arene Ru(II) compound RAWQ01 B: IC_{50} values represent the concentration required to inhibit cell viability by 50% relative to untreated cells. IC_{50} values for RAWQ01 for five esophageal carcinoma cell lines (K150, TE-1, EC-109, EC-9706 and EC-1). The different cancer cells were treated for 48 h with increasing concentrations of Rawq01, and the inhibitory ratio was determined using the MTT assay as described in Material and Methods. Results are the mean±SD of at least three independent experiments, each performed in triplicate C: Colony-forming assays in vitro. D: After 48 h of the treatment, cells were labeled with Annexin-V and analyzed by flow cytometry.*

we used flow cytometry to determine the percentages of annexin V–positive cells among miR-21 inhibitor-transfected cells, LY294002-treated cells and control cells treated with Rawq01. Knockdown of miR-21 or LY294002 significantly increased the proportion of apoptotic cells after Rawq01 treatment, compared with the negative control (Figure 3C).

Discussion

Recent studies showed that ruthenium (Ru) possesses several favorable chemical properties which indicates that it may be a strong basis for rational anticancer drug design (39, 40). Furthermore, compounds coordinated by large aromatic ligand, such as imidazo (4,5*f*) (1, 10) phenanthroline and its derivates have been extensively reported to bind to DNA molecules in intercalating, groove binding and electronic binding modes, and their utility as candidate of anticancer drugs is anticipated. However, the molecular basis of their effects, especially at the RNA level, is still mostly unknown.

ESCC cells utilize multiple molecular pathways to proliferate and invade tissue during the course of tumor progression. Among several independent cell survival signaling pathways, constitutive up-regulation of PI3K-AKT signaling is particularly important (41, 42). The phosphatase activity of PTEN is crucial in controlling the PI-3K signal transduction pathway and in the activation of the AKT proto-oncogene. When PTEN protein expression is reduced, PI3K/AKT is abnormally activated, followed by disregulation of cellular metabolism, proliferation and differentiation and cells are vulnerable to malignant transformed (43, 44). A survey of 97 patients with ESCC showed that there were statistically significant correlations between nuclear PTEN expression and macroscopic tumor classification, T stage, and American Joint Committee on Cancer stage, indicating that PTEN expression was down-regulated with disease progression (45). Furthermore, Chang *et al*. investigated PTEN expression of patients with ESCC from Liuzhou, an area of high ESCC in northern China. The results showed that PTEN expression is correlated with tumor differentiation, tumor infiltration depth and pTNM staging. The 5-year survival rate in patients with positive expression of PTEN was 82% compared to 39% in patients with negative expression of PTEN (46). Therefore, PTEN may play an important role

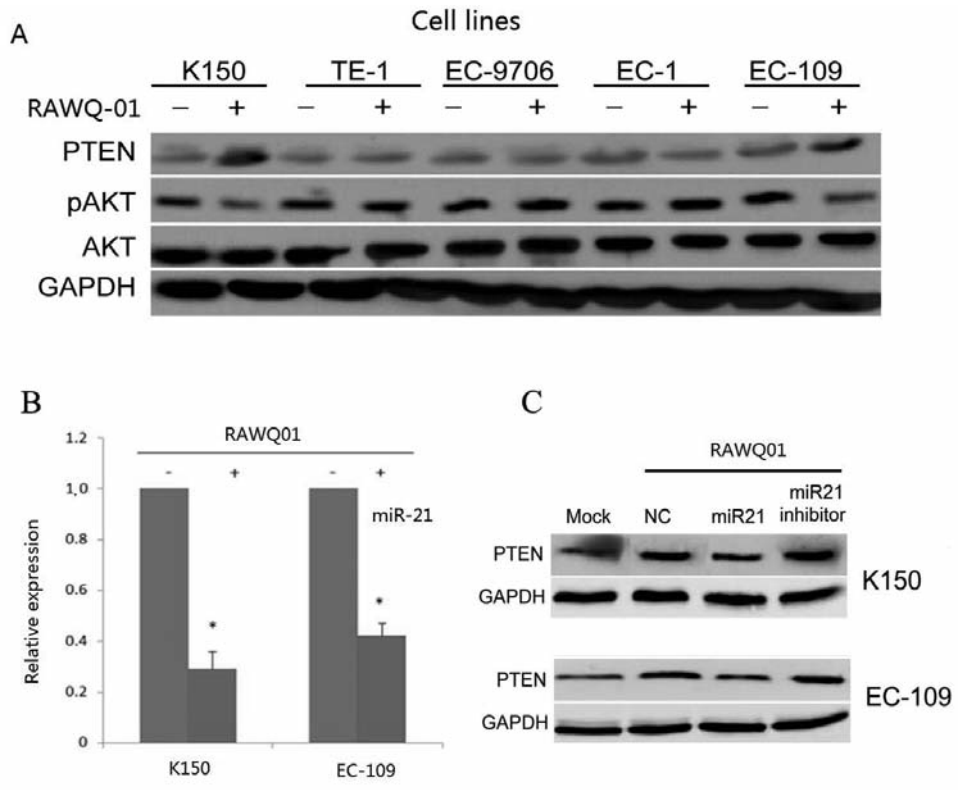

Figure 2. *Arene ruthenium compound RAWQ01 down-regulated mir-21 and triggered the activity of PTEN in K150 and EC-109 cells. Effect of RAWQ01 treatment on the A: protein expression of PTEN, pAKT and total AKT in esophageal carcinoma cells (K150, TE-1, EC-109, EC-9706 and EC-1) and B: Mir-21expression in K150 and EC-109 cells (B). The levels of PTEN protein detected by western blotting after transfection of mir-21 mimics, miR-21 inhibitor and scramble control (NC) in K150 and EC-109 cells (C).*

in carcinogenesis and the progression of ESCC and up-regulation of PTEN and inhibition of the PI3K/AKT pathway may be a promising approach for novel chemotherapeutic agents. In the present study, we found that RAWQ01 increased PTEN expression and reduced (pAKT) expression in K150 and EC-109 cell. Furthermore, RAWQ01 was found to induce ESCC cell growth inhibition and apoptosis induction. It seems that RAWQ01 may serve as a candidate for chemotherapeutic agents.

However, the mechanism by which Rawq01 up-regulate PTEN is unknown. Recently, Hu *et al*. examined a panel of 33 primary ESCC tumor samples and 20 corresponding morphologically-normal tissues for mutations in all nine exons of the *PTEN* gene by means of PCR-SSCP and direct DNA sequencing methods (47). Only one out of 33 esophageal squamous cell carcinomas showed an aberrant SSCP band, suggesting that PTEN mutations do not play a major role in the carcinogenesis of ESCC. Therefore, we hypothesized that *PTEN* function of inactivation in K150 and EC-109 cells might be associated with abnormal post-transcriptional action and PTEN function may be re-activated by Rawq01.

MicroRNAs have emerged as central regulators of cancer, and the aberrant expression of microRNAs may contribute to PTEN inactivation (45). Several studies have confirmed that *PTEN* is the target gene of miR-21 (30, 31, 48-50). Furthermore, the expression of mature miR-21 was found to be higher in ESCC than in normal epithelium. High levels of miR-21 were associated with lymph node positivity in patients with ESCC (51). Therefore, we hypothesize that Rawq01 may up-regulate PTEN expression through miR-21 inhibition. Our results shown that Rawq01 significantly down-regulated miR-21 expression in ESCC cells. In addition, Rawq01 combined with miR-21 inhibitor led to a significant increase of PTEN protein compared to Rawq01 treatment alone, while miR-21 mimics combined with Rawq01 did not lead to an significant change in PTEN protein expression, indicating that PTEN is a target of miR-21 in Rawq01-treated cells. Overexpression of PTEN significantly suppressed growth and induced apoptosis in esophageal cancer cell lines and inhibited the growth of subcutaneous tumor xenografts by significantly reducing tumor size *in vivo* (52).

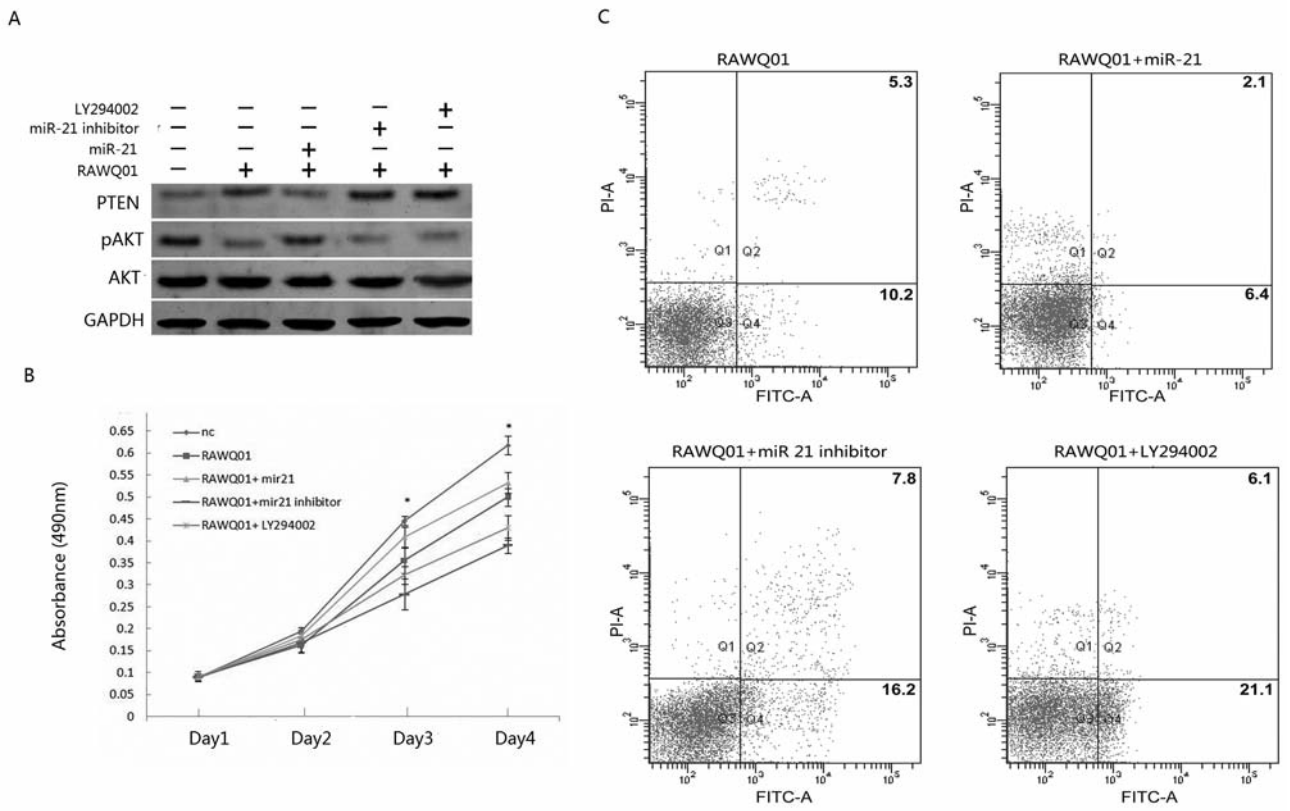

Figure 3. *Inhibiton of mir-21 expression, or the AKT pathway enhances the antitumor effect of RAWQ01 on EC-109 cells. Cells were treated with RAWQ01 alone or combined with mir-21 mimics, miR-21 inhibitor or PI3K inhibitor LY294002. A: The levels of PTEN, pAKT and total AKT protein were detected by western blotting. Cell viability (B) and apoptosis (C) were assessed by MTT and flow cytometry assays, respectively.*

Given that Rawq01 down-regulates miR-21 and inhibits the AKT pathway, we investigated whether inhibition of miR-21 expression or the AKT pathway could enhance the antitumour capability of Rawq01 in ESCC cells. Several studies have showen that miR-21 inhibition is a potential chemotherapy adjunct in the treatment of TMZ-resistant glioblastoma multiforme (51). Our results showed that both the miR-21 inhibitor and the AKT pathway inhibitor LY294002 enhance the inhibitory effect of Rawq01 on ESCC cells.

In summary, we have demonstrated that the effect of miR-21 on PTEN/AKT signaling pathway is abrogated by the novel compound Rawq01. We have also provided evidence that the miR-21inhibitor and the AKT pathway inhibitor LY294002 enhance the antitumour capability of Rawq01. Future *in vivo* studies will further clarify the role of miR-21 on PTEN/AKT signaling pathway by Rawq01 treatment. Our data may be useful in future development of chemosensitizing strategies through manipulation of miRNA expression.

Competing interests

The Authors declare that they have no competing interests.

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