Qilan Preparation Inhibits Proliferation and Induces Apoptosis by down-regulating microRNA-21 in Human Tca8113 Tongue Squamous Cell Carcinoma Cells

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Research

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Abstract

**Background:** Qilan preparation, a complex Chinese herbal medicine consisting of ingredients extracted from *Radix Astragali*, *Gynostemma Pentaphyllum*, *Rhizoma Chuanxiong* and selenium-rich green tea and known for ‘fortifying the spleen and boosting *qi*, quickening the blood and transforming stasis, and resolving toxins and relieving pain, is used for the prevention and management of oral diseases. The aim of this study was to examine the antitumor effects of Qilan preparation on oral squamous cell carcinoma (OSCC) *in vitro* and to explore its underlying mechanisms of action.

**Methods:** Human Tca8113 tongue squamous cell carcinoma (TSCC) cells were tested. Cell proliferation, cell cycle distribution and apoptosis were examined using cell counting kit-8 (CCK8) and flow cytometry (FCM). The expression of PTEN and PDCD4 were determined by western blot. Changes in miR-21 levels were quantified using TaqMan stem-loop real-time PCR. After miR-21 was transiently transfected into Tca8113 cells using Lipofectamine® 3000, cell proliferation, apoptosis and miR-21 and PDCD4 expression levels were measured.

**Results:** Qilan preparation inhibited Tca8113 cell growth in a dose- and time-dependent manner by inducing apoptosis and cell cycle arrest in S-phase, decreasing miR-21 levels and increasing PTEN and PDCD4 expression. MiR-21 overexpression reversed the Qilan preparation-induced suppression of cell proliferation and induction of apoptosis while also blocking the increase in PDCD4.

**Conclusions:** Our study revealed, for the first time, the ability of Qilan preparation to suppress TSCC cell growth and elucidated that Qilan preparation elicits its anti-cancer actions via either the miR-21/PDCD4 or PTEN pathway.

**Background**

Accumulating evidence suggests that traditional Chinese medicine (TCM) is a novel avenue for the successful treatment of cancers via the ‘Fuzheng Quxie (support right and dispel evil)’ therapeutic rule. A major therapeutic approach of TCM is using a mixture of herbs containing different types of plants and/or minerals that can engage multiple targets and exert synergistic therapeutic effects. Qilan preparation, also known as Chinese medicinal 1023 co., has been independently researched and developed by our team according to the basic theories of TCM and following the prescription principles of Monarch, Minister, Assistant and Guide, which consist of ingredients extracted from Astragali Radix (AR; *Huang Qi* in Chinese; roots of *Astragalus membranaceus* (Fisch.) Bunge), *Gynostemma Pentaphyllum* (Thunb.) Makino (G. *pentaphyllum*; *Jiao Gu Lan* in Chinese; a perennial liana herb belonging to the *Cucurbitaceae*), Chuanxiong Rhizoma (CR; *Chuan Xiong* in Chinese; rhizomes of *Ligusticum chuanxiong* Hort) and selenium-rich green tea (leaves of *Camellia sinensis* (L.) Kuntze) and are used for the prevention and management of oral diseases, especially oral epithelial dysplasia [1]. These active compounds have been used as traditional popular folk medicine by the Chinese population for centuries and are widely used in Chinese clinics as cancer adjuvants. AR is one of the best-known tonifying
medicinal herbs in China, Japan, Korea and other Asian countries. Traditionally, *Fuzheng Guben* (support right and secure the root) prescriptions are widely used to treat cancers for thousand years and AR is always a main compound of the prescriptions and exhibits good anti-tumor synergy effects [2, 3]. *G. pentaphyllum* is a well-known edible and medicinal plant in southern China, Japan, India and Korea, which is firstly recorded in Materia Medica for Famines (*Jiuhuang Bencao* in Chinese) written in the Ming Dynasty and used to exhibit anti-cancer actions [4] including for tongue squamous cell carcinoma (TSCC) [5]. CR is a traditional plant herb that has the efficacy of *Huoxue Xingqi* (quicken the blood and move qi) and *Qufeng Zhitong* (expel wind and relieve pain) in TCM (National Pharmacopoeia Committee, 2005). Recently, CR has been identified as an anti-tumor agent with multiple effects and low toxicity that is closely related to the traditional efficacy [6, 7]. The ingestion of herbal teas is common in many countries with ancient medicinal cultures and the consumption of green tea may help prevent cancers in humans due to the presence of (−) epigallocatechin-3-gallate (EGCG) [8], the most abundant catechin in green tea. Based on the above findings, it is plausible that these properties could contribute to the anti-tumor effects of the Qilan preparation herbs mixture. Our previous study demonstrated that Qilan preparation has tumor-inhibiting and cancer-blocking effects in 4-nitro-quinoline-1-oxide(4NQO)-induced rat tongue carcinogenesis models [9]. As a Chinese herbal medicine preparation with complex effects and composition, it is necessary to completely understand the mechanisms of its anticancer effects.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNA molecules that mainly regulate gene expression in a post-transcriptional manner and are believed to play important roles in the tumorigenesis microenvironment. MicroRNA-21 (miR-21), a genuine oncogene [11], is overexpressed in a number of cancers including solid tumor like oral squamous cell carcinoma (OSCC). Recent studies have revealed that miR-21 is up-regulated in OSCC and considered to act as an apoptosis inhibitor by partly silencing tropomyosin 1 (TPM1) [12]. MiR-21 also modulates cisplatin chemosensitivity and increases tumor cell invasion in OSCC by targeting programmed cell death 4 (PDCD4)[10]. Additionally, miR-21 levels are inversely correlated with phosphatase and tensin homologue (PTEN), an miR-21 target gene, in OSCC [12, 13]. PDCD4, a suppressor gene, can inhibit cancer cell growth via cell cycle arrest and the induction of apoptosis, which is under-expressed in OSCC tissues compared with normal oral tissues. PTEN functions as a protein phosphatase and has been implicated in the inhibition of cell cycle progression and the induction of cell apoptosis, which are also down-regulated in OSCC [14]. More interestingly, recent findings indicate that miRNAs can substantially modulate sensitivity and resistance to anticancer drugs including TCM[15], suggesting that miRNAs may be effective targets of anticancer agents.

Based on these observations, we hypothesize that Qilan preparation might exert cytotoxic effects on OSCC cells via either the miR-21/PTEN or miR-21/PDCD4 pathways. To test this hypothesis, we examined the association between miR-21/PTEN/PDCD4 expression and the effects of Qilan preparation on human Tca8113 TSCC cell proliferation, cell cycle and apoptosis *in vitro*.

**Methods**
1. **Plant materials**

The four herbal medicines were screened individually. AR (Batch NO. 140304), *G. Pentaphyllum* (Batch NO. 140305) and CR (Batch NO. 140211) were purchased from the Xiamen YanLaiFu Pharmaceutical Co., LTD., Fujian, China. Selenium-rich green tea (Batch NO. 140316) was purchased from Enshi ChunGui tea industry Co., LTD, Hubei, China. They were authenticated carefully by one of the authors (Professor Yiqi Huang). The voucher specimens (AR NO. Q1-20140526, *G. Pentaphyllum* NO. Q2-20140526, CR NO. Q3-20140526, selenium-rich green tea NO. Q4-20140526) were deposited at the herbarium of the Xiamen Medicine Research Institute, Fujian, China.

2. **Extraction and identification of Qilan preparation**

In brief, the four herbs (740 g) were chopped into pieces and decocted with 8-fold volume of distilled water three times for 1 h each. After combination, the filtered and mixed suspensions from three decoctions were concentrated at 740 mg/mL under reduced pressure. Finally, the concentrated solution was diluted to a 100 mg/mL stock solution in basic medium and stored at 4°C after filtered through 0.45 μm and 0.22 μm filters. Fresh working drugs were prepared at the following concentrations in basic medium: (1) 1.56 mg/mL, (2) 3.12 mg/mL, (3) 6.25 mg/mL, (4) 12.5 mg/mL and (5) 25 mg/mL. The same volume of basic medium was used as control or for the 0 mg/mL group.

The quality of Qilan preparation was measured by thin layer chromatography (TLC). Briefly, solution of the extracts was spotted on TLC plate using appropriate spray reagents and UV absorbances according to procedures described by the Chinese Pharmacopeia 2005 (National Pharmacopoeia Committee, 2005). The standards of astragaloside A, ginsenoside Rb1 and CR contrast material were used as positive references.

3. **Cell culture and transfection**

Human TSCC cell line Tca8113 was purchased from the ShangHai SBO Medical Biotechnology Co., LTD. (Shanghai, China). Cells were cultured in RPMI1640 (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. For functional analyses, Tca8113 cells were transfected with miR-21 mimics or the negative control RNA (NC RNA, which had the same number of basic groups but a different sequence compared with the miR-21 mimic) (GenePharma, Shanghai, China) using the Lipofectamine®3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, after incubating the cells overnight at 70% confluency, the cells were washed twice with PBS and resuspended in antibiotic-free medium. For each well in the 6-well plate, miR-21 mimic (60 nmol/L), NC (60 nmol/L) and 7.5 μL of the Lipofectamine®3000 transfection reagent was separately added to 125 μL of Opti-MEM®I medium (Gibco, Grand Island, NY, USA) and then mixed together to form the transfection complexes. The transfection complexes were added to the cells and incubated for 6 h before adding fresh medium. According to the optimum reagent amount, the multiplication factor (determined by the area ratio) was
used to determine the amount of reagent needed for new plate formats. Twenty-four hours after transfection, the cells were exposed to Qilan preparation at a concentration of 6.25mg/mL or 0mg/mL for an additional 24h and then harvested for the following experiments. All of the experiments were repeated independently at least three times.

4. **Cell viability assay**

Cells were seeded onto 96-well plates (Nunc, Denmark) at a density of $1 \times 10^4$ cells per well, incubated overnight, and then exposed to different concentrations of Qilan preparation in triplicate for 12, 24 or 48 h, respectively. Cell viability was quantified using CCK-8 (Dojindo, Kumamoto, Japan). The results represent the average of three independent experiments. Cell viability was calculated using the following formula: cell viability = \[ \frac{(OD_{treatment}-OD_{blank})}{(OD_{control}-OD_{blank})} \] \times 100%.

5. **Cell cycle analysis**

Cells were seeded onto 6-well plates (Nunc, Denmark) with $1 \times 10^6$ cells per well and treated with various concentrations of Qilan preparation (1.56, 3.125, 6.25, 12.5, or 25mg/mL) for 12h. The cells were then collected and washed twice with cold PBS. After fixing the cell in cold 70% ethanol at 4°C overnight, the cells were washed twice with cold PBS and then resuspended gently in propidium iodide (PI) staining solution (Beyotime, Shanghai, China), which consisted of 10µL RNase A, 25µL PI staining solution and 0.5mL buffer, and then incubated at 37°C for 30 min in the dark. The samples were analyzed by flow cytometry (BD Accuri C6, San Diego, CA, USA) within 1h of staining, and the percentage of cells in the G0/G1, S and G2/M phases were determined using FCS Express 4 plus.

6. **Cell apoptosis assay**

Apoptosis analysis was performed according to the manufacturer's instructions for the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA). Transfected or non-transfected Tca8113 cells were cultured in 6-well plates and were exposed to different concentrations of Qilan preparation for 24 h. Then, the cells were harvested with 0.25% Trypsin without EDTA (Gibco, Grand Island, NY, USA), washed twice with cold PBS and resuspended in 100 µL of binding buffer containing 5 µL of FITC Annexin V and 5 µL of PI working solution at a concentration of $1 \times 10^6$ cells/mL. After incubation at room temperature for 15 min in the dark, 400 µL of the binding buffer was added to each sample. Apoptosis was analyzed by flow cytometry (BD Accuri C6, San Diego, CA, USA) for at least 10,000 events within 1h of staining.

7. **RNA isolation, reverse transcription and TaqMan real-time PCR.**

Transfected or non-transfected Tca8113 cells were cultured in 6-well plates and exposed to different concentrations of Qilan preparation for 24 h. Total RNA was isolated using the TRIzol Reagent
(Invitrogen, Carlsbad, CA, USA). A TaqMan stem-loop real-time PCR method was used to assess miR-21 expression using kits from Applied Biosystems (Foster City, CA, USA). For each sample, we calculated the ΔC_T value (target-reference). The fold change between the treated sample and the normal miR-21 control was calculated with the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = \Delta C_T$ (target-reference, in treated samples) - $\Delta C_T$ (target-reference, in control samples). Real-time PCR was repeated in triplicate for each sample in the independent experiments. The mature miR-21 expression levels were quantified using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with RNU44 as the normalization control [16, 17].

8. Western blot

Transfected or non-transfected Tca8113 cells were cultured in 60-mm dishes and were exposed to different concentrations of Qilan preparation for 24 h. The cells were lysed in cell lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO4, 1 μg/mL leupeptin, and 1 mM PMSF) for 5 min at 4°C and centrifuged at 12,000 g (Sigma 1-15 PK, German) for 30 min at 4°C. Protein concentration was determined with a BCA kit (Beyotime, Shanghai, China). Equal amounts of each sample protein were then subjected to a 12% SDS-PAGE gel for electrophoresis and then transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking for 1 h at room temperature with a 5% skim milk solution, the membranes were prepared with Tris-buffered saline with 0.1% Tween-20 (TBST) and exposed to primary antibodies against PDCD4 and PTEN (all diluted 1:1000, Proteintech, Wuhan, China) at 4°C overnight. After washing three times with TBST for 5 min each, the membranes were incubated with the secondary antibody conjugated to horseradish peroxidase (all diluted 1:5000, Proteintech, Wuhan, China). After the membranes were washed three more times with TBST for 5 min each and then the proteins were detected using the enhanced chemiluminescence (ECL) Western Blot Detection Kit. Band intensities were measured with the AlphaView SA software (Alpha Innotech, San Leandro, CA, USA). Relative PDCD4 and PTEN protein expression was calculated relative to GAPDH.

9. Statistical analysis

Data are presented as the mean ± SD of three independent experiments. Statistical significance was determined by Student’s test or one-way ANOVA followed by the LSD or Dunnett’s T3 multiple comparison test assuming homogeneity of variances or non-assumed homogeneity of variances using the SPSS 19.0 software. Descriptive and variance homogeneity tests were also performed. *, #, and ^ indicate statistical significance with P< 0.05.

Results

The qualitative results of Qilan preparation by TLC revealed the presence of astragaloside A and ginsenoside Rb1 which are rich in AR and G. Pentaphyllum (Fig 1 A). Compared with CR contrast material, the Qilan preparation had the same color spots on the same position of TLC plate (Fig 1 B).
1. **Effects of Qilan preparation on Tca8113 cell viability**

Cell viability, determined by the CCK-8 assay, was significantly reduced at 1.56, 3.12, 6.25, 12.5 and 25 mg/mL Qilan preparation (P<0.05) after treatment for 12, 24 and 48 h, respectively (Fig 2). Qilan preparation exerted an inhibitory effect on Tca8113 cells in a dose- and time-dependent manner.

2. **Qilan preparation induced apoptosis and S-phase cell cycle arrest in Tca8113 cells**

Apoptosis is characterized by DNA fragmentation, cell shrinkage and nuclear condensation, and phosphatidylserine flipping from the inner to the outer leaflet of the plasma membrane. The decrease in cell viability due to the Qilan preparation could be explained, in part, by the increased apoptosis determined by Annexin V-FITC and PI staining using flow cytometry. In the scatter plot from the double variable flow cytometry, the Q3 quadrant (FITC - / PI -) shows living cells, the Q2 quadrant (FITC + / PI +) shows late apoptotic cells and the Q4 quadrant (FITC + / PI -) represents early apoptotic cells. In our study, the apoptosis rates include both the early and the late apoptotic cells. As assessed by flow cytometry and shown in Fig 3A and B, the amount of apoptotic cells was significantly increased from 15.04±1.67% to 69.74±4.63% when treated with concentrations of the Qilan preparation ranging from 3.12 to 25 mg/mL for 24 h compared with the control group (4.71±1.65%, P<0.05). In addition, the reduction in the cell viability was associated with the S-phase cell cycle arrest. As shown in Fig 3C and D, when cancer cells were treated with 3.12, 6.25, 12.5 or 25 mg/mL Qilan preparation for 12h, respectively, the number of cells in S-phase steadily increased from 38.64±1.17% to 65.40±1.96% compared with the untreated control cells (35.81±1.16%, P<0.05). These data indicate that the Qilan preparation was able to reduce cell viability by inducing apoptosis and blocking cell cycle progression.

3. **Effects of the Qilan preparation on the expression of miR-21 and its target proteins, PTEN and PDCD4, in Tca8113 cells**

Previous studies have shown that miR-21 is an anti-apoptosis factor in OSCC; thus, we examined the potential implication of miR-21 on the actions of the Qilan preparation. Using a TaqMan assay, we discovered a dose-dependent down-regulation of miR-21 after treatment with the Qilan preparation at concentrations ranging from 3.12 to 25 mg/mL compared with the control group (P<0.05, Fig 4A).

The inhibition of miR-21 expression was associated with an increase in PDCD4 and PTEN (Fig 4B and C), two miR-21 targets. Peak stimulation of PDCD4 (2.48±0.12-fold) and PTEN (1.51±0.06-fold) occurred at 6.25 mg/mL and 12.5 mg/mL, respectively, with no further increases observed at the higher concentrations. There were still significantly increasing PDCD4 and PTEN levels at the higher concentrations compared with the control group (P<0.05, Fig 4B and C). As tumor suppressor genes, PDCD4 and PTEN function as pro-apoptosis factors and are targeted by miR-21, at least partly explaining
the apoptosis-inducing effect of the Qilan preparation. All of these data suggest a functional role of the Qilan preparation mediated by miR-21.

4. **Downregulation of miR-21 may contribute to Qilan preparation-induced cell growth inhibition and apoptosis induction**

To investigate the involvement of miR-21 down-regulation in the effect of the Qilan preparation Tca8113 cells, functional analyses were performed to test the effects of miR-21 on cell viability and apoptosis. There were five groups including Control, NC RNA, Qilan preparation, NC RNA + Qilan preparation and miR-21 + Qilan preparation group. Basic medium was used as a blank control (Control group). NC RNA group was used as a negative control. Qilan preparation group was used as a positive control. As shown in Fig 5A, we over-expressed miR-21 by approximately 14-fold compared to the NC RNA group and 29-fold compared to the NC RNA + Qilan preparation group with a miR-21 transfection mimic for 24h before treatment with the Qilan preparation. Based on the results from the earlier experiments, we used 6.25mg/mL as the effective concentration for the reverse test. A CCK-8 and apoptosis assay and Western blot were performed to determine the response of Tca8113 cells to miR-21 and the Qilan preparation. As shown in Fig 5B, C and D, over-expression of miR-21 partially reversed the anti-proliferative and pro-apoptosis effects of the Qilan preparation. Compared with the NC RNA+ Qilan preparation group (55.75±3.63%), the cell viability of miR-21+Qilan preparation group was increased to 71.08±8.72%, but was still lower than the NC RNA group (97.83±5.63%, P<0.05). In contrast, the percentage of cells undergoing apoptosis in the miR-21 + Qilan preparation group decreased to 13.27±0.44% compared with the NC RNA+ Qilan preparation group (22.72±1.52%), but was still higher than the NC group (P<0.05). Similar to these results, the expression level of the PDCD4 protein in the miR-21 + Qilan preparation group was between that of the NC RNA group and the NC RNA + Qilan preparation group (P<0.05) (Fig 5E and F). These data confirm the finding that miR-21 is a potential target of the Qilan preparation and that over-expression of miR-21 partly antagonized the anti-tumor actions of the Qilan preparation in human Tca8113 cells *in vitro*.

**Discussion**

In TCM theories, an imbalance between *Yin* and *Yang* is the essential reason for cancer and TCM could achieve successful treatment by restoring the dynamic balance of body's internal environment based on the *Fuzheng Quxie* rule. According to modern medicine, cancer is well known as a disease of apoptosis deregulation and cell cycle dysfunction. Thus, the molecular players in apoptotic pathways might be successful targets for anticancer drug design [18]. Several reports have documented that TCM inhibits the growth and proliferation of various cancer cells through inducing apoptosis and/or cell cycle arrest[19]. Our present data reinforce this point and confirm the cytotoxicity of the Qilan preparation in human Tca8113 TSCC cells. We found that Qilan preparation treatment significantly inhibited Tca8113 cell viability in a dose- and time-dependent manner as determined by the CCK-8 assay. Moreover, Qilan...
preparation caused a dose-dependent induction of apoptosis and an accumulation of S phase cells, suggesting that the induction of apoptosis and the blockage of cells in S-phase are underlying mechanisms for Qilan preparation-mediated growth inhibition. We hypothesize that Qilan preparation regulates growth arrest in S-phase by preventing proper DNA repair that causes the cell to undergo apoptosis. However, further study is necessary to determine the exact mechanism.

Medina PP et al. [11] demonstrated that miR-21 was a genuine oncomiR and that tumors could become addicted to oncomiRs, suggesting that the pharmacological inactivation of miRNAs such as miR-21 could be used to treat human cancers. This demonstration of the oncomiR addiction of miR-21 has far-reaching consequences for oncotherapy and suggests that miR-21 is an important therapeutic target for anti-tumor agents. Because over-expression of miR-21 affected tumor progression by down-regulating various target tumor suppressor genes in OSCC[20], it is reasonable to hypothesize that Qilan preparation could regulate miR-21 expression in OSCC. In this study, we found that Qilan preparation treatment significantly decreased the abundance of miR-21 in Tca8113 cells \textit{in vitro}; similar results were observed in the cell viability and apoptosis assays. This corroborates with previous data that showed that miR-21 up-regulation indicates poor prognosis and is an apoptosis inhibitor in TSCC [12]. To further confirm that miR-21 plays a role in the Qilan preparation mechanism in Tca8113 cells, we over-expressed miR-21 prior to Qilan preparation treatment and found that the cell viability and apoptosis were partially but significantly reversed. Because TCMs can have multiple targets and multifaceted functions, it is possible that there are other currently unknown target genes of the Qilan preparation responsible for the anticancer effects aside from just miR-21. However, our data suggest an anticancer function of the Qilan preparation mediated by miR-21.

It has been suggested that a single miRNA can simultaneously regulate a great number of target genes [21]. MiR-21 regulated genes include PDCD4, PTEN, TPM1 and CDK2AP1 in OSCC. PDCD4, a novel tumor suppressor gene, was originally identified to be up-regulated in apoptotic cells [22] and has also been reported to be involved in apoptosis in cancer cell lines when those cells are exposed to anticancer agents[23]. PDCD4, which is under-expressed in OSCC, also appears to have an important role in the inhibition of cell cycle progression at S-phase [24]. PTEN functions as a protein phosphatase and mediates cell growth and apoptosis via the phosphatidylinositol-3,4,5-trisphos-phate (PIP$_3$) pathway [25, 26]. Thus, we examined the contributions of the PDCD4 and PTEN proteins, and found that Qilan preparation treatment increased the expression of these two proteins in Tca8113 cells. Restoration of miR-21 levels significantly but incompletely blocked the increase in PDCD4 levels. One explanation for this observation is that one gene may be regulated by many miRs and that miR-21 may only be one of many factors that the Qilan preparation affects. Wang et al. [27] demonstrated that ursolic acid (UA), a naturally occurring pentacyclic triterpene, exhibited potent proliferation inhibition and apoptosis induction in the human glioblastoma cell line U251 by suppressing the TGF-\(\beta\)/miR-21/PDCD4 pathway. Berberine, a naturally occurring isoquinoline alkaloid that can be extracted from many medicinal herbs, has been reported to induce apoptosis in human multiple myeloma cells by decreasing miR-21 levels [28]. Another report revealed that Matrine, one of the major alkaloids extracted from \textit{Sophora flavesens}, could inhibit
breast cancer growth via the miR-21/PTEN/Akt pathway in MCF-7 cells [29]. In accordance with previous studies, our data show that miR-21 is a molecular target involved in the regulation of apoptosis in cancer cell lines and reinforces the notion that miRNAs, such as miR-21, can act as mediators of herbal medicines, by regulating target genes.

Numerous studies have suggested that some traditional medicine extracts can induce tumor regression by arresting cell cycle at S phase[30]. Consistent with these previous papers, our cell cycle analysis data showed that the Qilan preparation caused a strong S-phase arrest in Tca8113 cells after 12 h treatment; however, the exact molecular mechanisms remain to be clarified. Previous results showed that reintroduction of PDCD4 could induce S-phase arrest of cell cycle in cancer cell lines [24, 31]. In our study, Qilan preparation induced S-phase arrest and increased the PDCD4 levels. However, overexpression of miR-21 partially reversed the up-regulation of PDCD4, but the portion of S-phase arrested cells induced by treatment with the Qilan preparation did not change (data not shown). The reason may be due to cell-type specificity of the PDCD4 actions [32]. For example, Jansen et al. [33] reported that PDCD4 may enhance geldanamycin-induced G2-M arrest in UO-31 renal cancer cells. In a different ovarian cancer cell line, overexpression of PDCD4 induced cell cycle arrest as exemplified by an increased number of cells in the G2 or S phases [31]. However, in the colon carcinoma cell line RKO, PDCD4 did not alter cell cycle progression [34]. Taken together, it is possible that PDCD4 is not the direct molecular target of S-phase cell cycle arrest due to Qilan preparation treatment and further studies are needed to determine the exact mechanism of action.

**Conclusions**

We demonstrated in this study that Qilan preparation exhibits anti-proliferative and pro-apoptotic activities, as well as inducing S-phase cell cycle arrest in human Tca8113 TSCC cells. These results are produced, at least in part, by the preparation's actions on the miR-21/PDCD4 or PTEN pathway, as it shown in figure 6. These findings help us to understand the effects and molecular mechanisms of the anti-cancer activity of Qilan preparation, which will aid in its application in the clinic.

**Abbreviations**

OSCC, oral squamous cell carcinoma; TSCC, tongue squamous cell carcinoma; CCK8, Cell Counting Kit-8; FCM, flow cytometry; TCM, traditional Chinese medicine; AR, Astragali Radix; G. pentaphyllum, *Gynostemma Pentaphyllum* Makino; CR, Chuanxiong Rhizoma; EGCG, (−) epigallocatechin-3-gallate; 4NQO, 4-nitro-quinoline 1-oxide; miRNAs, MicroRNAs; miR-21, microRNA-21; TPM1, tropomyosin 1; PDCD4, Programmed cell death 4; PTEN, phosphatase and tensin homologue; TLC, thin layer chromatography; NC, negative control; PI, propidium iodide; PVDF, polyvinylidene fluoride; PIP3, phosphatidylinositol-3, 4, 5- trisphos-phate; UA, ursolic acid.

**Declarations**
Ethics approval and consent to participate:
Not applicable.

Consent for publication:
Not applicable.

Availability of data and materials:
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:
The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Authors' contributions:
JMD contributed to operation of the whole experiment and approval of the article. WLC mainly assisted in the operation of the experiment. ZXM and YDX wrote and revised the manuscript. ZLC and LZ provided the overall idea for the experiment and revised the paper in the later stage. All authors have read and approved the manuscript before submission.

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**Figures**

![Figure 1](image)
The qualitative results of Qilan preparation by TLC. Solution of the Qilan preparation was spotted on TLC plate using appropriate spray reagents and UV absorbances according to procedures described by the Chinese Pharmacopeia 2005. The standards of astragaloside A, ginsenoside Rb1 and CR contrast material were used as positive references. Qilan preparation without AR, G. Pentaphyllum, or CR were used as negative references. (A) The qualitative results of chemical markers by TLC in (1) astragaloside A, (2) ginsenoside Rb1, (3) Qilan preparation, (4) Qilan preparation without AR and (5) Qilan preparation without G. Pentaphyllum. (B) The qualitative results of chemical markers by TLC in (1) CR contrast material, (2), (3), (4) Qilan preparation and (5) Qilan preparation without CR.
Figure 2

Effects of Qilan preparation on Tca8113 cell viability in vitro. Tca8113 cells were treated with various concentrations of the Qilan preparation (0, 1.56, 3.12, 6.25, 12.5, and 25 mg/mL), and cultured for 12, 24, or 48 h. Cell number was determined by the CCK-8 assay. Data are represented as the mean ± SD for three experiments per group. #, *, and ^ P<0.05 vs the 0 mg/mL group for 12, 24, or 48 h.

Figure 3

The effects of Qilan preparation on Tca8113 cell apoptosis and cell cycle. Tca8113 cells were treated without (control) and with Qilan preparation (1.56, 3.12, 6.25, 12.5, and 25 mg/mL) for 24 h. The cells were stained with FITC-conjugated Annexin V and PI for flow cytometry. (A) The cell populations shown in the upper (Annexin V+/PI+) and lower (Annexin V+/PI-) right represent the apoptotic cells. (B) The percentage of apoptotic cells, *P<0.05 vs Control. Tca8113 cells were treated without (Control) and with Qilan preparation (1.56, 3.12, 6.25, 12.5, and 25 mg/mL) for 12 h. Next, the cells were stained with PI for flow cytometry. (C) and (D) The cell cycle distribution (G0/G1, S, and G2/M) was based on 2N and 4N DNA content for the DNA content analysis using the FCS Express 4 plus software. The data are represented as the mean ± SD for three experiments per group. *P<0.05 vs Control for S-phase percentage. #P<0.05 vs Control for G1/G0-phase percentage.
Figure 4

Effects of Qilan preparation on miR-21 expression and PDCD4 and PTEN in Tca8113 cells. Expression of miR-21, PDCD4 and PTEN were separately detected by stem-loop a Taqman assay and Western blot following treatment without (Control) and with Qilan preparation (1.56, 3.12, 6.25, 12.5, and 25 mg/mL) for 24 h. (A) The fold change in relative expression of miR-21 to RUN44. The fold change in relative expression of (B) PDCD4 or (C) PTEN to GAPDH. The data are represented as the mean ± SD for three experiments per group. *P<0.05 vs Control.
Figure 5

Downregulation of miR-21 partially contributes to Qilan preparation-induced cell growth inhibition and apoptosis induction. The miR-21 mimic and negative control RNA (NC RNA, which had the same number of basic groups but a different sequence compared with the miR-21 mimic) were introduced into the Tca8113 cells for 24 h. Transfected or non-transfected cells were treated with 6.25mg/mL of the Qilan preparation for 24 h. Thus, there were five groups including Control, NC RNA, Qilan preparation, NC RNA +
Qilan preparation and miR-21+Qilan preparation group. Basic medium was used as a blank control (Control group). NC RNA group was used as a negative control. Qilan preparation group was used as a positive control. Significant differences were observed in the miR-21 expression levels between the miR-21+Qilan preparation, the NC RNA and the NC RNA+Qilan preparation groups. After the level of miR-21 was increased, (B) the cell viability increased, but the (C and D) apoptosis ratio and (E) PDCD4 expression levels decreased. The data are represented as the mean ± SD for three or four experiments. There were no significant differences between Control and NC RNA groups. * P<0.05 vs the NC RNA group and # P<0.05 vs the NC RNA + Qilan preparation group.

Figure 6

Qilan preparation inhibits proliferation and induces apoptosis by down-regulating microRNA-21 in human Tca8113 tongue squamous cell carcinoma cells Qilan preparation inhibited Tca8113 cell growth by inducing apoptosis and cell cycle arrest in S-phase, decreasing miR-21 levels and increasing PTEN and PDCD4 expression. MiR-21 overexpression reversed the Qilan preparation-induced suppression of cell proliferation and induction of apoptosis while also blocking the increase in PDCD4.

Supplementary Files

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