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miRNA-155 as a Novel Target for Isoliquiritigenin to Induce Autophagy in Oral Squamous Cell Carcinoma

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Abstract

BACKGROUND: The most common obstacle facing chemotherapeutic agents is the development of drug resistance to cancer cells by dysregulation of autophagy and apoptosis. Targeting miRNAs by a natural flavonoid such as Isoliquiritigenin (ISL) is a novel strategy to reverse drug resistance.

AIM: The aim of the present study was to evaluate ISL impacts on apoptosis and autophagy in oral squamous cell carcinoma (OSCC) through the expression levels of related two microRNAs: miRNA-21 and miRNA-155.

MATERIALS AND METHODS: The expression levels of both miRNAs were analyzed using quantitative real-time PCR and the effect of ISL on apoptosis was evaluated using annexin assay. In addition, the expression of the autophagy marker (ATG7) was measured using immunofluorescence.

RESULTS: Our results showed that ISL significantly downregulated both miRNA-21 and miRNA-155 with a fold change of 22.01 and 52.35, respectively. It also induced apoptosis in the cancer cells with high percentage (51.3%). Moreover, ATG7 was highly expressed after ISL treatment.

CONCLUSION: From this study, we can conclude that ISL has an apoptotic and autophagic effect on OSCC through the downregulation of miRNA-21 and miRNA-155, major regulators of PI3K/Akt pathway which can provide novel targets for OSCC therapy.

Introduction

Oral squamous cell carcinoma (OSCC) accounts for about 95% of all head and neck cancer cases which considered to be one of the most common types of cancers worldwide [1]. According to the Global Cancer Statistics, 2020 more than 350.000 new cases and more than 150.000 deaths were associated with lip and oral cavity sites cancer [2]. Moreover, 25–40% of the intraoral malignancies of the oral cavity consist of tongue squamous cell carcinoma [3]. Tobacco and heavy alcohol consumption are the principal etiologic factors for the development of OSCC [4], [5].

All the three main treatment modalities, surgery, radiation, and chemotherapy are used either alone or in combination to treat OSCC regarding some aspects. Mostly, tumor characteristics include site, proximity to bone, and the depth of invasion. Other factors such as the tumor size, lymph node involvement, and rise of metastasis, the age of the patient, co-morbidities, compliance to treatment, and the desire to make lifestyle changes, should be also taken into consideration. Despite advanced existing diagnostic and treatment modalities, the overall survival after being diagnosed with OSCC remains very low, around 50%, regardless of the stage at which it was determined [6]. However, promising findings of microRNAs (miRNAs) functions and their role in the occurrence and development of OSCC by silencing targeted mRNA, in the past few years highlighted their importance and pointing novel anti-cancer strategies [7], [8]. The Phosphoinositide 3-kinase (PI3K)/AKT signaling pathway regulates, the transcriptional activity of FOXOs (PI3K/AKT/FOXO pathway), which control the transcription of genes involved in cell proliferation, growth, survival, cell cycle, and apoptosis [9]. Moreover, the same pathway (PI3K/AKT) activates the rapamycin (TOR) kinase-containing protein complex, the autophagy induction regulator [10]. It was found that the downregulation of miRNA-21 and miRNA-155 had suppressed the PI3K/AKT/FOXO1 signaling pathway, and had a crucial role in autophagy through inhibition of the PI3K-AKT-mTOR pathway in cancer cells [7], [11], [12].

Besides, a possible inhibitor for these miRNAs to suppress the multistage carcinogenesis processes could be flavonoid Isoliquiritigenin (ISL), which has been isolated from the root of the liquorice plant (Glycyrrhiza uralensis). Both natural and synthetic ISL antioxidant was distinguished by their

ability to inhibit viability, proliferation, and migration of cancer cells, and their functions in apoptosis and autophagy [13], [14]. Recent studies showed that downregulation of miRNA-25 by ISL effectively inhibits the proliferation and invasion of oral cancer cells and also promotes autophagic cell death [10]. Inhibition of miRNA-155 expression reduced tumor volume, triggered apoptosis and showed promising results for therapeutic application [15]. However, the inhibition capacity of synthetic ISL in OSCCs on miRNA-155 has not been investigated yet.

The aim of the study was to (i) investigate the effects of ISL on the cytotoxicity, proliferation, and invasion, (ii) evaluate ISL impacts on apoptosis and autophagy in OSCC, through the expression levels of related micro RNAs, miRNA-21, and miRNA-155.

Materials and Methods

Reagents and cell line cultivation

ISL was obtained from Chemscene with molecular formula of $C_{15}H_{12}O_4$ and molecular weight 256.25. A stock solution (100 mM) was composed in DMSO and aliquoted. Cisplatin was used as positive control drug. HNO97 cells are tongue squamous cell carcinoma cells which were imported from the "American Type Culture Collection." The cells were seeded in 96-well culture plate. In 200 μL of Dulbecco's Modified Eagle Medium (DMEM), (Gibco, Thermo Scientific, Germany) consisting of 10% fatal bovine serum (Gibco, Thermo Scientific, Germany) and 1% of penicillin G sodium (10.000 UI), streptomycin (10 mg) and amphotericin B (25 μg) (PSA) (Gibco, Thermo Scientific, Germany), an average 1×10^4 HNO97 cells were seeded. Culture plates were incubated at 37°C in an atmosphere of 5% CO_2 for 24 h to reach the 70 % confluence.

Assessment of cell viability by cell proliferation assay (MTT)

The cell cytotoxicity assay was carried out using the Vybrant® MTT Cell Proliferation Assay Kit, cat no: M6494 (Thermo Fisher, Germany). Serial concentrations of ISL drug "100 μM, 10 μM, 1 μM, 0.1 μM, and 0.01 μM" were evaluated. In addition, the carrier solvent (0.1% DMSO) was used for control cells. The dose of Cisplatin was adjusted to 5 μM according to the calculated IC_{50} of Cisplatin in Genomics of Drug Sensitivity in Cancer (Drug: Cisplatin - Cancerrxgene - Genomics of Drug Sensitivity in Cancer). The HNO97 cells (8×10^3 cells per well) were seeded in 96-well culture plates in and incubated at 37°C with 5 % CO_2 for 48 h in DMEM media, then 100 μL of media was removed and replaced by new media. Twenty μL of 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/mL) (Invitrogen, Thermo Scientific, Germany) was added to each well and the plates were incubated at 37°C and 5 % CO_2 for 4 h. Cell viability was determined by measuring the optical density at 570 nm on a spectrophotometer (ELx 800; Bio-Tek Instruments Inc., Winooski, VT, USA). The IC_{50} was calculated using the GraphPad prism software 9.

Assessment of apoptosis using annexin/PT staining by flowcytometry

The HNO97 treated cells were harvested at 48 h post-transfection. Staining was performed following trypsinization, FITC-Annexin V and Propidium iodide (PI). To separate apoptotic cells (early), the Dead Cell Apoptosis Kit with Annexin V FITC and PI, for flow cytometry (Invitrogen, cat no: V13242) was used. PI has stained necrotic cells with red fluorescence. After the treatment with both probes, apoptotic cells showed green fluorescence, while dead cells showed red and green fluorescence together, and finally live cells showed a little or no fluorescence. The Navios software (Beckman Coulter) was used to analyze flow cytometry data.

Testing the HNO97 migration and invasion potential by Boyden chamber assay

To access the invasion and migration potential of treated HNO97 cells, an *in vitro* cell migration assay was conducted. The cells were suspended in 200 μL serum-free medium and inoculated into the upper cavity with a Boyden of 8 mm caliber (ThermoFisher Scientific, Germany) after the transfection for 24 h. At 37°C, they were incubated for 24 h. Then the upper lumen cells were taken out with cotton swabs, the lower surface cells were fixed with methanol, and images were captured by inverted microscope.

RNA preparation and genes expression in HNO97 cells after treatment

Total mRNA was extracted using miReasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription reaction using mi Script RT Kit (Qiagen, Hilden, Germany). The quantification of miRNA-155 and miRNA-21 genes expression levels was amplified from miRNA extract using a miScript (Hs_miR-21 and miR-155a) Primer Assays. The miRNAs genes were amplified by miScript Syber green Master mix (Qiagen, Hilden, Germany). The RUN6 Primer Assay was used as a housekeeper gene. All samples were analyzed using the 5 plex Rotor-Gene PCR Analyzer (Qiagen, Germany).

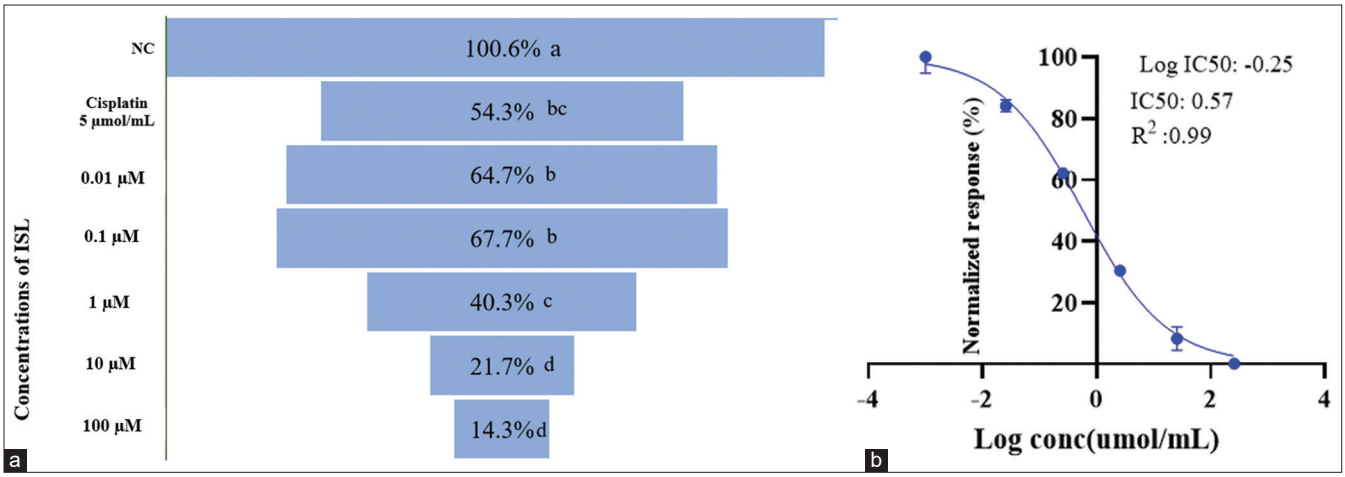

Figure 1: (a) - MTT assay results for HNO97 cells treated with different concentration of ISL, (b) The illustration of linear regression curve for the log dose of ISL versus the normalized response in HNO97 cells. IC_{50}: Half maximum cytotoxic effect. Statistical differences are indicated with different letters for each bar at level of $p < 0.05$

Assessment of ATG7 protein expression on HNO97 cells using immunofluorescence staining

The HNO97 cells were treated with ISL and examined for the expression of ATG7 using specific polyclonal antibody. Cells were fixed with warm 4% formaldehyde. Then, they were immune-stained with Rabbit anti-ATG7 Antibody, cat no: (PA5-35203) primary antibody (Invitrogen; ThermoFisher Scientific, Hilden; Germany), incubated overnight at 4°C. The cells were washed with PBS and incubated with Goat Anti-Rabbit IgG H&L secondary antibody-Alexa Flour 488 (Invitrogen; ThermoFisher Scientific, Hilden; Germany). The slide was covered with Prolong Gold Antifade Reagent (Abcam, Cambridge, UK) and mounted overnight at room temperature. LABOMED Fluorescence microscope LX400, cat no: 9126000; USA

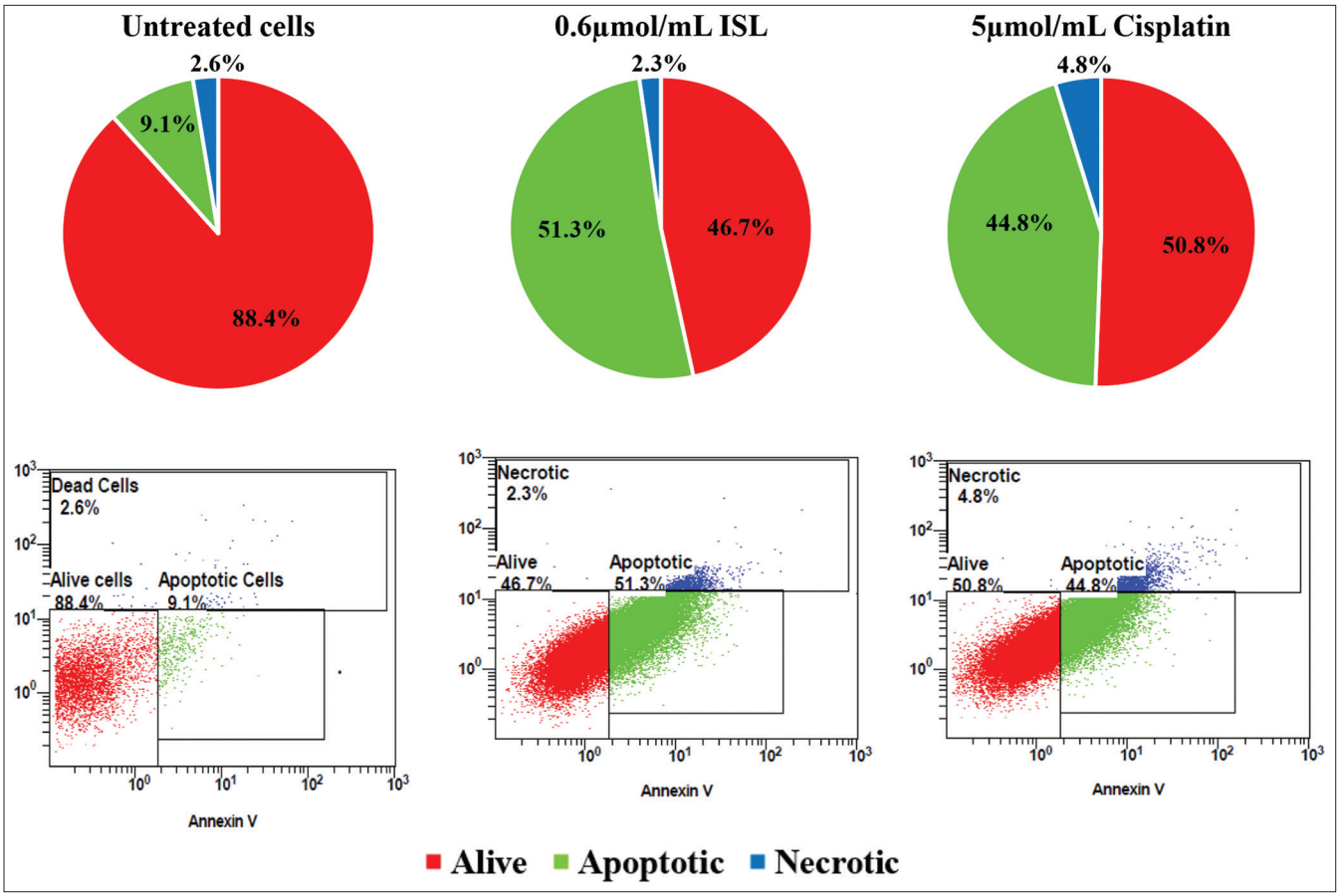

Figure 2: Apoptotic effect of ISL on OSCC cells using annexin v assay. The figure showing percentages of different types of cells in the three groups (negative control cells at the left, ISL treated cells in the center, and positive control cells in the right)

was used for the microscopic examination. In brief, the florescence score of each sample was calculated as the sum of each intensity (0–3) multiplied by the percentage of positive cells (0–100%). The score ranged from 0 to 300. The median value of florescence score was calculated.

Statistical analysis

Data collected are expressed as the mean ± standard deviations. All data are the result of three replicates. Means were compared using Fisher's Least Significant Difference test. Statistically significant difference was considered at level of $p < 0.05$.

Results

The cytotoxic effect of ISL on HNO97 cells using MTT assay

To determine the half maximal cytotoxic effect (IC_{50}) of ISL on HNO97 cells, first cytotoxic effect of different ISL concentrations on cancer cells viability using MTT assay was performed (Figure 1). A concentration dependent decrease pattern in the viability of cancer cells using different doses of ISL was happened (Figure 1a). The largest drop in cell viability was found between 0.1 μM and 1 μM concentrations which was the range of IC_{50} concentration. Furthermore, a significant decrease in the viability of cells was found between 1 μM and 10 μM concentrations. However, no significant change was detected between 10 and 100 μM concentrations. The IC_{50} was calculated (equal to 0.57) using the linear regression curve (Figure 1b).

Determination of apoptotic effect of ISL on OSCC cells using annexin v assay

The results showed that ISL had the best apoptotic effect on OSCC cells over control cells and positive control (cisplatin treated cells) with almost more than half the percentage of cells (51.3%) showed apoptosis. There was a significant change in the percentage of apoptotic cells between control, cisplatin and ISL treated groups. In addition, a significant difference in the percentage of apoptotic cells was detected between ISL and cisplatin treated cell. However, no significant difference in the percentage of necrotic cells was found between all treatments (Figure 2).

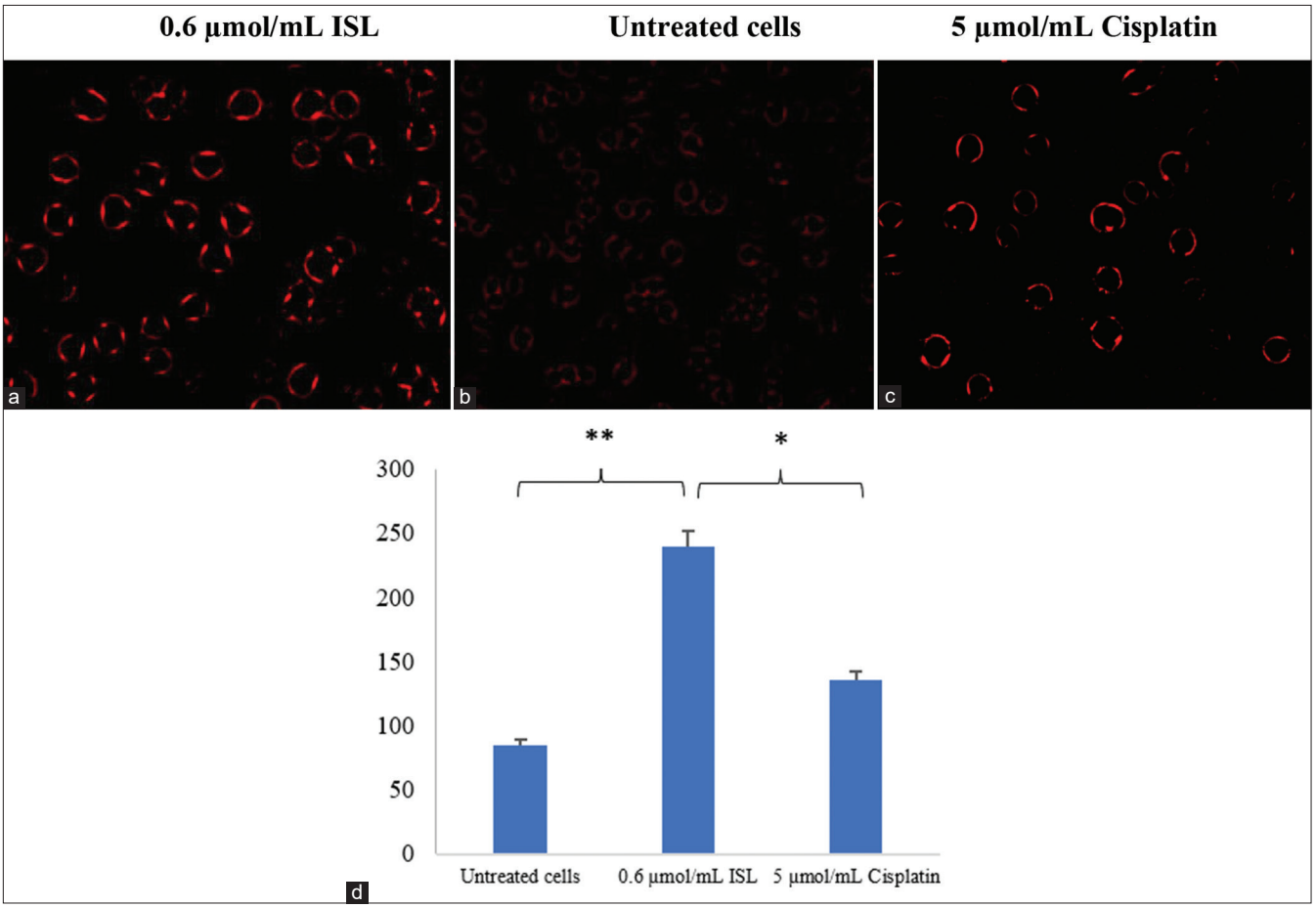

*Figure 3: (a-c) The three images showing the difference in the fluorescence intensity between the ISL treated group and the control groups. (d) The graph showing a significant difference in the fluorescence score between ISL treated group and control groups. *Indicates a significant statistical differences at, while **Indicates a highly significant differences*

The expression of ATG7 autophagy marker using immunofluorescence staining

ISL caused the highest expression of ATG7 (autophagy protein marker) with the highest fluorescent intensity with a value of 3 and a fluorescence score of 240. The most fluorescent intensity was found in the cells treated with IC_{50} of ISL (Figure 3a), which was more than negative (Figure 3b), and positive (Figure 3c) control treated cells. A significant difference was found in the fluorescence score between ISL treated cells and cisplatin treated cells, while a highly significant difference between ISL group and negative control group. However, no significant difference in the fluorescent intensity was found between negative control and cisplatin treated cells (Figure 3d).

ISL significantly decreased the expression of miRNA 21 and miRNA 155, the major regulatory miRNAs of apoptosis and autophagy respectively

IC_{50} concentration of the ISL caused downward regulation and suppressed the expression of miRNA 21 gene which is one of the most important regulatory miRNAs of apoptosis with a cycle threshold 30.07 and fold change 22.01 compared to the RUN housekeeping gene. The expression of miRNA-21 in ISL treated cells was also less than cisplatin treated cells. miRNA-155, one of the key role genes in autophagy, was also significantly down regulated by treating HNO97 cells with ISL with a cycle threshold 30.82 and fold change 52.35 (Figure 4a and b).

The effect ISL on invasion and migration potential of HNO97 cells

The percentage of the migrated cells was significantly decreased in ISL treated cells (45 %) in comparison with the negative control which had a percentage of 85% migrated cells, and cisplatin treated cells which had percentage of 65% migrated cells as shown in Figure 5. The images showed the differences in migration potential between groups.

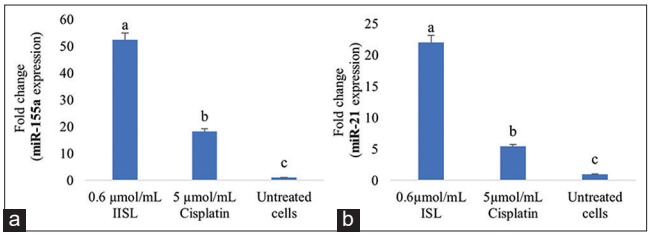

Figure 4: (a) The graph showing a significant change in the fold change difference in expression of miRNA-155 between ISO treated and control cells (positive and negative). (b) The second graph showing the differential fold change in expression profile of miRNA -21 between different groups. Statistical differences are indicated with different letters for each bar at level of $p < 0.05$

Discussion

Cancer represents one of the most death causes worldwide. The emergence of drug resistance is the main reason for the failure of cancer treatment using chemotherapy. Using natural compounds targeting several cancer types with fewer side effects has gained much attention recently to overcome that issue [16]. Different studies have reported that natural compounds and their semi-synthetic analogs showed promising results against various malignancies [17], [18]. Extracts from liquorice have been widely used in the traditional Chinese medicines for centuries. ISL (20, 40, 4-trihydroxychalcone, ISL) is the most essential

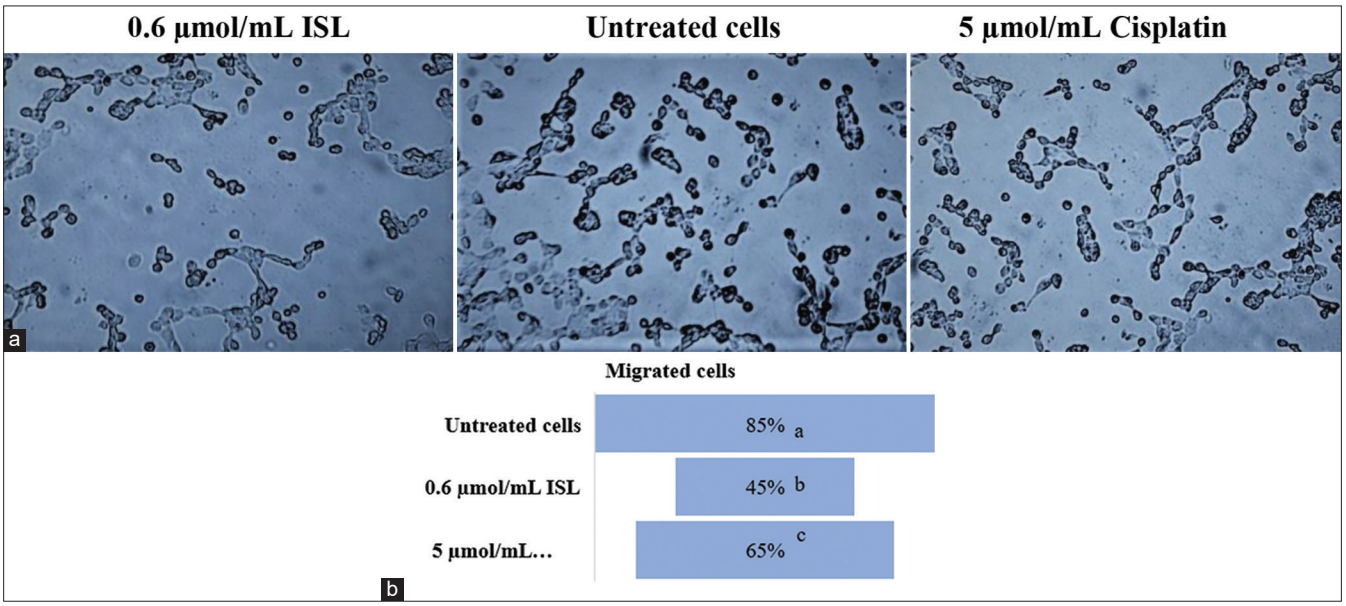

Figure 5: (a) Microscopic images illustrating the HNO97 migrated cells after treatment with 0.6 µmol/mL of ISL compared to HNO97 cells treated with Cisplatin (positive control) and untreated cells (negative control). (b) The graph showing the percentages of migrated cells in different groups. Statistical differences are indicated with different letters for each bar at level of $p < 0.05$

bioactive compound in liquorice extracts. Potential results have been obtained when using ISL even alone or in combination with other drugs [19]. ISL significantly inhibited cancer cells viability but has little side effects on normal cells [19]. In our study, we used cisplatin which was a traditional chemotherapeutic agent as a positive control with a well-known cytotoxic as well as side effects.

Various studies have reported the remarkable anti-tumor impacts of ISL including proliferation suppression, apoptosis induction, cell cycle arrest, angiogenesis, and migration inhibition in various types of cancer [20], [21], [22]. ISL is used either alone or in combination to target various types of cancers, such as breast cancer [23], colorectal cancer [24], lung cancer [25], prostate cancer [26], gastric cancer [19], bladder cancer [27], melanoma [28], and hepatocellular carcinoma [29]. In addition, further investigation is required as the specific molecular mechanisms of ISL in the treatment of cancer are not well understood.

Our results showed that ISL had a cytotoxic effect on tongue squamous cell carcinoma cells with IC_{50} 0.57 μM which was lower than the predetermined used IC_{50} of cisplatin 5 μM which means that ISL is a more potent cytotoxic than cisplatin. The viability values of MTT assay also declared a significant difference between the second and third concentration which was the range of the presumed IC_{50}. Moreover, the low value of IC_{50} of ISL shows that it has no effect on normal cells, as it was reported by Wu *et al.* where the human endometrial stromal cells (T-HESCs; as a control) and human endometrial cancer cell lines (Ishikawa, HEC-1A, and RL95-2 cells) were compared. Their results indicated that ISL inhibits the growth of cancer cells at concentrations below 27 μM, but has little effect on normal cells [30].

Although the anticancer activity of ISL has been reported to be associated with some molecular mechanisms, the definitive target proteins and/or genes bound by ISL remain unknown, still is different from traditional chemotherapy drugs with only one or few targets in most circumstances. The PI3Ks in mammalian cells form a family which share the primary biochemical function to phosphorylate the 3-hydroxyl group of phosphoinositides [31]. PI3K is activated by diverse growth factor receptors and oncogenes, and the rise of PI3K signaling is considered a characteristic of cancer [32], [33]. The growth factor regulated serine/threonine kinase (Akt/PKB) is a key downstream target of PI3K, and a central medium for the PI3K pathway, which has multiple downstream effectors. Considering the role of PI3K/Akt/mTOR pathway in ISL-related apoptosis and autophagy induction and migration inhibition of cancer cells, it might be an important target for ISL. As Wan *et al.* said that hypoxia induced of miRNA 155 causing autophagy through targeting multiple proteins in the mTOR pathway so targeting miRNA155 may be beneficial for inducing autophagy in cancer cells [12]. Results demonstrated significant downregulation of miRNA 155 compared to negative and positive control group. A confirmatory result for the induction of the autophagy in ISL treated tongue squamous cell carcinoma cells was the high expression of autophagy marker ATG7 in ISL treated in comparison to non-treated and cisplatin treated cells. To the best of our knowledge, this was the first study to detect the effect of ISO on miRNA 155 and autophagy in tongue squamous cell carcinoma cell line.

Liu *et al.* revealed that miRNA 21 had a critical role in regulating PI3K/Akt/FOXO1 through targeting PDCD4 suppressing apoptosis in tongue squamous cell carcinoma and miRNA 21 inhibitors highly up regulating apoptosis which means that miRNA21 may be a novel agent in treatment of TSCC [9]. The real-time PCR analysis showed that miRNA21 was downregulated by ISO treatment in comparison to cisplatin treated and control cells which suggesting the role of isoliquiritigenin in apoptosis induction. The annexin assay confirmed the apoptotic effect of ISO with high percentage of apoptotic cell in ISL treated on expense of necrotic and alive cells in comparison to cisplatin treated and control cells which mean that ISL is a major regulator of apoptosis in TSCCs. To evaluate the effect of ISL on one of PI3K/Akt/FOXO1 pathway effects, we measured the invasion and migration potential of TSCC cells by Boyden chamber invasion assay. ISL significantly suppress the power of invasion in TSCC compared to cisplatin treated cells.

Conclusion

From our study, we can conclude that ISL has an apoptotic and autophagic effect on tongue squamous cell carcinoma cells through downregulation of miRNA 21 and miRNA 155 the major regulators of PI3K/Akt signaling pathway which provide novel targets for OSCC therapy.

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