Effect of adriamycin combined with metformin on biological function of human tongue cancer SSC-15 cells

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Abstract. The effect of adriamycin (ADM) combined with metformin (MET) on the biological function of human tongue cancer SSC-15 cells was investigated. SCC-15 cells (ATCC® CRL-1623) were cultured *in vitro*. The close concentration of the median lethal dose (LD50) of ADM was 0.05 mg/l and the LD50 of MET was 10 mmol/l after 48 h of intervention. They were used for drug combination experiments. Cells without drug treatment were used as the control group, cells treated with ADM alone, MET alone and their drug combination (ADM+MET) as the experimental groups. CCK-8 was used to detect the cell survival rate, and flow cytometry to detect the apoptosis rate in each group, Transwell chamber to detect the invasion ability *in vitro* of cells and scratch-healing experiment to observe the migration ability of the cells. The survival rate of tongue cancer SCC-15 cells gradually decreased with the increase in ADM and MET concentrations and in intervention time (P<0.05). The apoptosis rate in the ADM, MET and ADM+MET groups was significantly higher than that in the control group (P<0.05). The apoptosis rate in the ADM+MET group was higher than that in the ADM and MET groups (P<0.05). The invasion and migration ability of cells in the ADM and MET groups were higher than those in the ADM+MET group (P<0.05). The cell membrane number and the migration rate of cells in the ADM+MET group were significantly lower than those in the ADM and MET groups (P<0.05). Both MET and ADM inhibit the growth, invasion and migration of tongue cancer SSC-15 cells, and induce their apoptosis. Thus, ADM and MET in combination is more effective than ADM alone and MET alone in inhibiting the growth, invasion and migration of tongue cancer cells as well as in inducing their apoptosis.

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Key words: adriamycin, metformin, human tongue cancer, SSC-15, cell survival, apoptosis, cell invasion and migration

Introduction

Tongue cancer is one of the most common oral malignant tumors, and its pathological type is mainly squamous epithelial cells. It has high malignancy and develops rapidly (1). At present, tongue cancer is still treated by surgical resection combined with radiotherapy and chemotherapy. However, there are rich blood and lymph node tissues in the tongue, so tongue cancer has a higher recurrence and metastasis rate, resulting in a poor prognosis and with less than 50% 5-year survival rate in patients (2). The surgical resection rate of tongue cancer has been significantly increased in recent years, but high recurrence rate and metastasis remain. There is related literature (3) reporting that the highest lymph node metastasis rate of tongue cancer is 80%. Therefore, it is of great clinical significance to improve the survival rate of patients to effectively improve the recurrence and metastasis rate of tongue cancer by radiotherapy and chemotherapy.

Adriamycin (ADM), a broad-spectrum aminoglycoside antitumor drug, is widely used in clinical chemotherapy due to its high anticancer activity (4). As a drug with non-specific cell cycle, ADM has a unique role in inhibiting the growth of tumor cells and promoting their apoptosis. It also increases the sensitivity of cancer cells to radiotherapy, so as to better exert its anticancer effect (5). ADM is the first choice drug for tongue cancer chemotherapy, but it also has myelosuppression, cardiotoxicity and other severe adverse reactions. This causes dose reduction and even discontinuation in many patients due to the fact that they are intolerant to those adverse reactions caused by ADM. As a result, the clinical efficacy is reduced (6). Metformin (MET) is a clinically common oral hypoglycemic drug for the treatment of type 2 diabetes. It is widely used in clinical practice because of its low price, good safety and less adverse reactions (7). There is a previous study (8) reporting that MET has an antitumor effect. In the investigation of epidemiology (9), it is also found that diabetic patients taking MET for a long time have a lower risk of cancer and tumor-related mortality than patients taking other hypoglycemic drugs. In the study by Wang *et al* (10), MET was found to inhibit the proliferation *in vitro* and *in vivo* of tongue cancer HSC-3 and HSC-4 cells. There is a study showing that chemotherapeutic drugs combined with MET enhance the efficacy in liver (11), ovarian (12) and breast cancer (13).

Currently, there are few reports on the effect of MET combined with chemotherapeutic drugs on the proliferation, apoptosis, invasion and migration of human tongue cancer cells.

Therefore, in this study, the effects of ADM alone, MET alone and ADM+MET groups (their drug combination) on the proliferation, apoptosis, invasion and migration ability of human tongue cancer SCC-15 cells were respectively investigated, to explore the effect of traditional chemotherapeutic drug ADM combined with new antitumor drug MET on the biological function of human tongue cancer cells, so as to provide a more optimized solution for reducing the postoperative metastasis and recurrence rate of tongue cancer in clinical practice.

Materials and methods

Experimental instruments and reagents. The DMIL LED inverted microscope was purchased from Leica, Microsystems GmbH (Wechsler, Germany), microplate reader SpectraMax M5 from Meigu (Shanghai, China), flow cytometer CytoFLEX LX from Beckman Coulter, Inc. (Brea, CA, USA), sterile plus sample gun head from Axygen Scientific, Inc. (Union City, CA, USA), human tongue squamous cancer SCC-15 cell line from ATCC (cat. no. ATCC® CRL-1623; Manassas, VA, USA) and cryopreserved in this laboratory, DMEM-F12 medium from Gibco; Thermo Fisher Scientific Inc. (Waltham, MA, USA), fetal bovine serum (FBS), trypsin and phosphate buffer powder from HyClone; GE Healthcare (Chicago, IL, USA), sterile gun head from Axygen Scientific, Inc., CCK-8 reagent from Tongren Company (Tokyo, Japan), Transwell chamber from Corning Incorporated (Corning, NY, USA), Annexin V-FITC/PI apoptosis kit from Kaiji Biological Co., Ltd. (Jiangsu, China), MET powder and ADM powder from Baole Pharmaceutical Co., Ltd. (Shenzhen, China).

The study was approved by the Ethics Committee of Qianfoshan Hospital of Shandong Province (Jinan, China).

Cell recovery, culture, passage and cryopreservation. Relevant literature was consulted and the cryopreserved SCC-15 cell line was taken out from the liquid nitrogen container, and quickly placed in an incubator at 37˚C to melt the cryoprotectant. The melted cell sap was then transferred to a centrifuge tube under aseptic conditions, and the DMEM-F12 medium containing 10% FBS was added and cultured in the incubator at 5% CO_2 and 37˚C. The inverted microscope was used to observe the cell growth, and the solution was changed at an appropriate time. When cells were adherently grown in a culture flask to more than 80%, the old medium was first aspirated and discarded, then washed with 3 ml of PBS to remove residual serum and suspended dead cells. After that, trypsin was added for digestion. When cells became translucent and their morphology was round, they were observed under a microscope, then added to the DMEM complete medium containing FBS in order to terminate the digestion. The bottom of the flask was strongly tapped until cells were detached from the bottle wall, and the suspension was then transferred to the centrifuge tube, centrifuged at 800 x g for 5 min at 20˚C. Next, the complete medium was added to resuspend cells that were distributed evenly into 3 new culture flasks to complete the passage. Cells continued to be cultured in the incubator at 37˚C. The SCC-15 cell line in log phase was used to repeat the above process of cell digestion, washing and passage. After that, it was transferred to a 1.5 ml cryogenic vial and stored overnight in a -80˚C refrigerator. The cryogenic vial was transferred to the liquid nitrogen container the next day.

CCK-8 detection of inhibition in cell proliferation. The inhibition of ADM alone and MET alone in cell proliferation was compared. The cryopreserved SCC-15 cells in log phase were resuscitated according to the above steps, and incubated in a 96-well plate (four plates in total) for culture. The drug intervention group, the control group and the blank group were set up in each plate. The blank group was added only with medium, the control group and the drug intervention group with $3x10^5$ SCC-15 cells per well. After cells were adherently grown, the drug was added to the drug intervention group. First, each of 2 ml of ADM with concentrations of 0.01, 0.05, 0.1/l, 0.5 and 1 mg/l were added to two plates for ADM, 5 groups of MET with concentrations of 1, 5, 10, 20 and 40 mmol/l to two plates for MET, respectively. CCK-8 solution (10 μl/well) was respectively added at 24, 48 and 72 h after dosing. Then, cells continued to be cultured for 2 h in the incubator. The SpectraMax M5 microplate reader was used to measure the optical density (OD) value of each well at a wavelength of 450 nm in order to detect cell proliferation. The experiment was repeated 3 times. Next, the inhibition rate of cell growth was calculated according to the formula: cell survival rate (%) = (OD value in the experimental group - OD value in the control group)/(OD value in the control group - OD value in the blank group) x 100%. It was concluded that the 50% inhibiting concentration (IC_{50}) of ADM was close to 0.05 mg/l and the IC_{50} of MET was close to 10 mmol/l after 48 h of intervention. Then, the above-mentioned incubation plate method was repeated, and the cell proliferation was compared with drug combination. The 96-well plate was grouped into the blank group, the control group, the ADM group, the MET group and the ADM+MET group. The blank group was added only with 100 μl of medium, other groups with $3x10^5$ SCC-15 cells per well, respectively. After cells were adherently grown, 2 ml of ADM 0.05 mg/l, MET 10 mmol/l and ADM 0.05 mg/l plus MET 10 mmol/l were respectively added to the ADM group, the MET group and the ADM+MET group. After cultured for 48 h, CCK-8 solution (10 μl/well) was added. After cultured for 2 h, the OD value of each group was measured. The experiment was repeated 3 times. The cell survival rate *in vitro* was calculated according to the above given formula.

Flow cytometry detection of apoptosis. The cryopreserved SCC-15 cells in log phase were taken out, and 4 flasks of them were cultured in the incubator. Cells ($3x10^5$) were collected from each flask. Flask 1 was used as the control group with only medium added, flask 2 as the ADM group with 4 ml of ADM 0.05 mg/l added, flask 3 as the MET group with 4 ml of MET 10 mmol/l, and flask 4 as the ADM+MET group with 2 ml of ADM 0.05 mg/l plus 2 ml of MET 10 mmol/l. After 48 h of dosing, a single cell suspension was prepared, washed with PBS and centrifuged twice at 1,580 x g for 5 min at 4˚C. The supernatant was removed, and cells were resuspended. Annexin V-FITC (5 μl) and 5 ml of PI were added, and incubated for 20 min at room temperature in the dark. The CytoFLEX LX flow cytometer was used to detect apoptosis. The experiment was repeated 3 times.

Transwell chamber detection of cell migration ability in vitro. Cells in log phase were incubated at 37˚C in a 24-well plate based on the above method, with approximately 3,000 cells

Table I. CCK-8 detection of the effect of ADM with different concentrations on survival rate of SCC-15 cells at different time-points (%).

Concentration mg/l	24 h	48 h	72 h	F value	P-value
0	100±0	100±0	100±0	-	-
0.01	93.1±2.3[c]	77.3±2.7[a,c]	61.2±3.1[a-c]	103.2	<0.001
0.05	85.6±2.9[c]	60.8±3.3[a,c]	53.9±3.7[a-c]	75.82	<0.001
0.1	78.7±3.4[c]	49.6±2.1[a,c]	40.2±3.2[a-c]	138.3	<0.001
0.5	63.5±3.1[c]	37.3±3.8[a,c]	24.1±3.8[a-c]	94.04	<0.001
1	54.5±5.8[c]	21.2±5.3[a,c]	13.7±3.5[a-c]	57.38	<0.001
F value	80.06	221.0	282.0	-	-
P-value	<0.001	<0.001	<0.001	-	-

Single factor analysis of variance, repeated measurement analysis of variance and pairwise comparison after treatment were used. [a]P<0.001, compared with the 24 h group at the same concentration; [b]P<0.001 compared with the 48 h group at the same concentration; [c]P<0.05 compared with 0 mg/l at the same time.

Table II. CCK-8 detection of the effect of MET with different concentrations on survival rate of SCC-15 cells at different time-points (%).

Concentration mg/l	24 h	48 h	72 h	F value	P-value
0	100±0	100±0	100±0	-	-
1	91.1±1.4[c]	72.3±12.7[a,c]	62.2±2.4[a-c]	11.45	<0.050
5	85.3±2.9[c]	52.8±2.3[a,c]	42.9±3.1[a-c]	190.0	<0.001
10	74.7±2.4[c]	49.6±2.4[a,c]	31.2±3.0[a-c]	209.1	<0.001
20	61.7±4.3[c]	31.2±2.2[a,c]	23.1±3.9[a-c]	96.75	<0.001
40	42.5±2.5[c]	25.2±4.3[a,c]	11.7±2.5[a-c]	69.22	<0.001
F value	195.4	70.14	400.9	-	-
P-value	<0.001	<0.001	<0.001	-	-

Single factor analysis of variance, repeated measurement analysis of variance and pairwise comparison after treatment were used. [a]P<0.001, compared with the 24 h group at the same concentration; [b]P<0.001 compared with the 48 h group at the same concentration; [c]P<0.05 compared with 0 mg/l at the same time.

per well. They were divided into the control group (only medium added), the ADM group (2 ml of ADM 0.05 mg/l added), the MET group (2 ml of MET 10 mmol/l added) and the ADM+MET group (2 ml of ADM 0.05 mg/l plus MET 10 mmol/l added). After 48 h of drug intervention, cells were diluted to a density of $3x10^4$ cells/ml with a serum-free DMEM-F12 medium. The diluted cells (200 μl) were added to the upper chamber, 600 μl of DMEM-F12 medium containing 20% FBS to the lower chamber. After 24 h, the chamber was rinsed with PBS and fixed with 4% paraformaldehyde solution for 10 min. It was rinsed again with PBS after taken out and stained with 0.5% crystal violet for 10 min at 20˚C. Then, it was taken out and rinsed with PBS again until clarification. Finally, the cell invasion of 5 visual fields was randomly calculated with a microscope, and the average value was calculated. The experiment was repeated 3 times.

Scratch-healing experiment for observation of cell migration ability in vitro. Cells in log phase were prepared into a single cell suspension. Diluted to $3x10^5$ cells/ml based on the above method, they were added to a 6-well plate, grouped based on the above method and cultured by adding the medium and the drug. After 48 h, 200 μl of sterile plus sample gun head was used to scratch the cells in the middle of the plate in order to form a cell-free area. The cells were rinsed with PBS and a new medium was added for culture. At 0 h (W_0) and 24 h (W_{24}) after the cells had been scratched, the width of the cell-free area of the scratches at three different positions was calculated under the microscope. Cell migration index = $(W_0 - W_{24})/W_0$ x 100%.

Statistical analysis. In this study, SPSS19.0 software [Boyizhixun (Beijing) Information Technology Co., Ltd., (Beijing, China)] was used to statistically analyze the data, and GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) to plot all the illustrations in this experiment. Measurement data were expressed as mean ± standard deviation. Single factor analysis of variance was used for

Table III. Apoptosis rates of SSC-15 cells after 48 h of ADM alone, MET alone and their drug combination (%).

Groups	Control group	ADM group	MET group	ADM+MET group
Apoptosis rate	4.12±1.02	13.47±2.57[a]	13.56±2.13[a]	25.17±2.33[a,b]
Cell membrane number	65.7±5.6	31.5±6.7[a]	29.2±7.1[a]	8.2±1.3[a,b]
Cell migration	78.6±1.24	59.5±1.78[a]	58.7±3.22[a]	39.5±2.31[a,b]

[a]P<0.001, compared to the control group; [b]P<0.001, compared to ADM and MET groups.

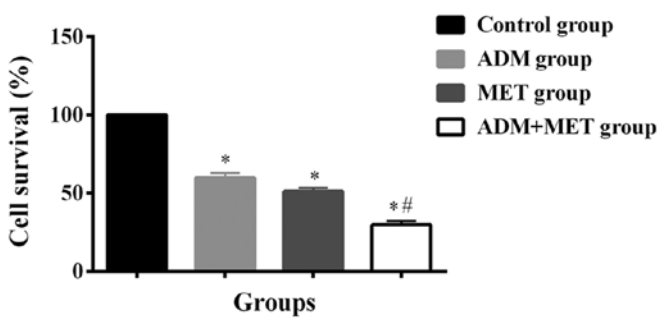

Figure 1. The effect of ADM and MET in combination on cell survival rate. CCK-8 assay showed that the cell survival rate of ADM, MET and ADM+MET groups was significantly lower than that of the control group. That of the drug of ADM+MET group was significantly lower than that of ADM and MET groups (P<0.05). There was no significant difference between ADM and MET groups (P>0.05). *P<0.05, compared to the control group; #P<0.05, compared to ADM and MET groups.

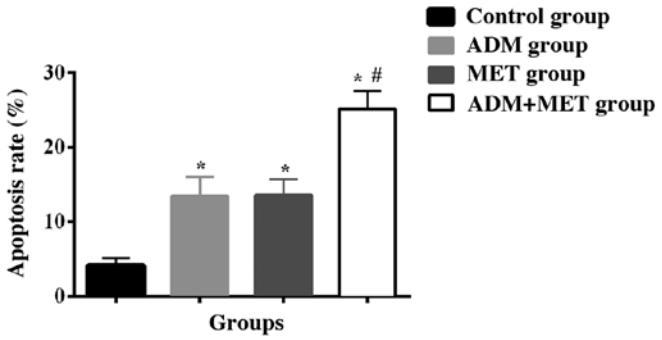

Figure 2. The apoptosis rates of tongue cancer SCC-15 cells after 48 h of treatment of each group of drugs. Flow cytometry detection showed that apoptosis rate of ADM, MET and ADM+MET groups was significantly higher than that of the control group (P<0.05). There was no significant difference between ADM and MET groups (P>0.05). The apoptosis of ADM+MET group was higher than that of ADM and MET groups (P<0.05). *P<0.05, compared to the control group; #P<0.05, compared to ADM and MET groups.

comparison among multiple groups, and repeated measurement analysis of variance was used for analysis at different time-points in the group. LSD test was used for pairwise comparison after the event and t-test for analysis between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of ADM and MET single drug intervention on proliferation of tongue cancer SCC-15 cells. CCK-8 assay was used to detect the cell proliferation in each group. It was found that the survival rate of tongue cancer SCC-15 cells gradually decreased with the increase in ADM and MET concentrations and in intervention time (P<0.05) (Tables I and II).

Effect of ADM and MET in combination on proliferation of tongue cancer SCC-15 cells. According to the above experimental results, it was calculated that the close concentration of the LD50 of ADM was 0.05 mg/l and the LD50 of MET was 10 mmol/l after 48 h of intervention. They were used for drug combination experiment. The cell survival rate at 48 h was 100% in the control group, 59.8±3.1% in the ADM group, 51.2±2.1% in the MET group, and 29.7±2.3% in the ADM+MET group. There was no significant difference in the cell survival rate between the ADM and MET groups (P>0.05). The cell survival rate in the ADM+MET group was significantly lower than that in the ADM and MET groups (P<0.05) (Fig. 1).

Effects of ADM alone, MET alone and their drug combination on apoptosis, invasion and migration ability of tongue cancer SCC-15 cells. Apoptosis rate in the ADM, MET and ADM+MET group was significantly higher than that in the control group (P<0.05). There was no significant difference in the apoptosis rate between the ADM and MET groups (P>0.05). The apoptosis rate in the ADM+MET group was higher than that in the ADM and MET groups (P<0.05). Transwell chamber was used to detect the cell invasion ability in each group after 24 h of routine culture. It was found that the cell membrane number was (65.7±5.6) in the control group, (31.5±6.7) in the ADM group, (29.2±7.1) in the MET group and (8.2±1.3) in the ADM+MET group. There was a difference in the cell invasion ability between the ADM, MET, ADM+MET groups and the control group (P<0.05), but there was no significant difference in the cell membrane number between the ADM and MET groups (P>0.05). The cell membrane number in the ADM+MET group was significantly less than that in the ADM and MET groups (P<0.05). After 24 h of cell scratching, the migration of cells was 78.6±1.24% in the control group, 59.5±1.78% in the ADM group, 58.7±3.22% in the MET group and 39.5±2.31% in the ADM+MET group. In addition, there was a difference in the cell migration ability between the ADM, MET, ADM+MET groups and the control group (P<0.05), but there was no significant difference in the migration between the ADM and MET groups (P>0.05). The migration in the ADM+MET group was significantly lower than that in the ADM and MET groups (P<0.05) (Tables III-V and Figs. 2-4).

Table IV. Number of invasive membrane cells after 48 h of ADM and MET alone and in combination.

Groups	Control group	ADM group	MET group	ADM+MET group
Number of invasive membrane cells	65.7±5.6	31.5±6.7[a]	29.2±7.1[a]	8.2±1.3[a,b]

[a]P<0.05, compared to the control group; [b]P<0.05, compared to ADM and MET groups.

Table V. Effects of ADM and MET alone and in combination on migration ability of SCC-15 cells (%).

Groups	Control group	ADM group	MET group	ADM+MET group
Cell migration index	78.6±1.24	59.5±1.78[a]	58.7±3.22[a]	39.5±2.31[a,b]

[a]P<0.05, compared to the control group; [b] P<0.05, compared to ADM and MET groups

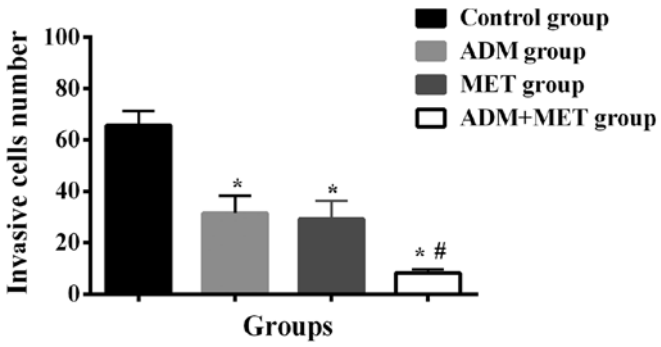

Figure 3. Number of invasive membrane cells after 48 h of ADM, MET and ADM+MET groups. Transwell chamber detection showed that there was a difference in the cell invasion ability between ADM, MET and ADM+MET groups and the control group (P<0.05), but there was no significant difference in the number of invasive membrane cells between ADM and MET groups (P>0.05). The number of invasive membrane cells in ADM+MET group was significantly less than that in ADM and MET groups (P<0.05). *P<0.05, compared to the control group; #P<0.05, compared to ADM and MET groups.

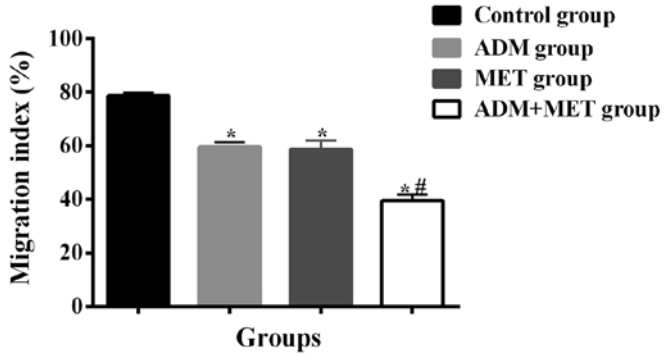

Figure 4. The effects of ADM, MET and ADM+MET groups on migration ability of cells. Scratch experiment showed that there was a difference in the cell migration ability between ADM, MET, ADM+MET and control groups (P<0.05), but there was no significant difference in the migration index between ADM and MET groups (P>0.05). The migration index of ADM+MET group was significantly lower than that of ADM and MET groups (P<0.05). *P<0.05, compared to the control group; #P<0.05, compared to ADM and MET groups.

Discussion

Tongue cancer, a mouth cancer that is one of the top ten common cancers in the world, has shown constantly rising incidence in recent years (14). The rich blood circulation and lymph nodes in the tongue are prone to cause tumor cell invasion in tongue cancer. The most common is the metastasis of tumor cells to lymph nodes (15). At present, low attention is paid by patients to tongue cancer that has high malignancy and rapid development. Therefore, many patients are in the advanced stage when diagnosed. Tongue cancer is mainly treated by operation combined with radiotherapy and chemotherapy (16). ADM is a widely used chemotherapeutic drug for the treatment of tongue cancer in clinical practice, its main function is to inhibit the spread and proliferation of tongue cancer cells (17). However, its long-term use causes severe toxic and side effects, such as kidney and liver damage and myocardial lesion. It also causes drug resistance in patients, thereby reducing the efficacy. Therefore, ADM has certain limitations in clinical application (18). MET is mainly used in the treatment of type 2 diabetes in clinical practice. Compared to traditional hypoglycemic drugs, it has the advantages of obvious efficacy, safety and low price (19). In recent years, an increasing number of studies have reported the antitumor effect of MET. Related experiments have also confirmed that it inhibits the proliferation of gastric cancer cells (20) and esophageal cancer cells (21). However, there are currently few studies on the effect of MET combined with chemotherapeutic drugs on human tongue cancer cells, especially on the effect of MET combined with chemotherapeutic drugs on the proliferation, apoptosis, invasion and migration ability of human tongue cancer cells. Therefore, in this study, the effect of ADM combined with MET on the biological function of human tongue cancer cells was investigated, in order to provide a more optimal treatment for tongue cancer patients.

First, the survival rates of tumor cells when ADM and MET were used alone and in combination were compared. It was found that the survival rate of tongue cancer SCC-15 cells gradually decreased with the increase in ADM and MET concentrations used alone and in intervention time (P<0.05). The cell survival rate in the ADM+MET group was significantly

lower than that in the ADM and MET groups (P<0.05). This indicates that ADM and MET in combination is more effective than ADM and MET alone in inhibiting the proliferation of tumor cells. It was also found that the combination of MET and ADM has a more significant effect on Ehrlich tumors in mice than ADM or MET alone (22). It was found that the apoptosis rate in the ADM, MET and ADM+MET groups was significantly higher than that in the control group (P<0.05). There was no significant difference in the apoptosis rate between the ADM and MET groups (P>0.05). The apoptosis rate in the ADM+MET group was higher than that in the ADM and MET groups (P<0.05). It shows that both ADM and MET induce apoptosis, and the combination of the two strengthens the apoptosis effect. The effects of ADM alone, MET alone and their drug combination on the invasion and migration ability of tongue cancer cells were investigated. It was found that both ADM alone and MET alone inhibited the invasion and migration ability of tumor cells, but the combination of the two was more effective in the inhibitory effect. In the study by Bao *et al* (23), it was confirmed that MET inhibits the invasion and migration of tumor cells. There is also a study (24) showing that conventional chemotherapeutic drugs assisted with MET are helpful to improve the efficacy, which is consistent with our findings.

In summary, both MET and ADM inhibit the growth, invasion and migration of tongue cancer SSC-15 cells, and induce their apoptosis. ADM and MET in combination is more effective than ADM alone and MET alone in inhibiting the growth, invasion and migration of tongue cancer cells, and in inducing their apoptosis. However, in this study, the effect of ADM combined with MET *in vivo* on the biological function of tongue cancer cells was not explored, and the effects of AMD and MET on multiple cell lines were not compared. These problems will be improved in future studies.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Author's contributions

JZ drafted this manuscript, collected and interpreted the data, revised the manuscript critically for important intellectual content and was responsible for the conception and design of the study. JZ read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Qianfoshan Hospital of Shandong Province (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The author declares no competing interests.

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