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Original Article

Esophageal cancer-associated fibroblasts promote proliferation, invasion, migration and resistance to chemoradiotherapy in esophageal squamous carcinoma cells

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Abstract: Correlation between tumor microenvironment and chemoradiotherapy resistance is unclear in esophageal squamous cell carcinoma (ESCC). We therefore sought to investigate the influence of esophageal cancer-associated fibroblasts (CAFs) on growth, invasion, migration, and chemoradiotherapy resistance in esophageal squamous cancer cells. For primary culture, CAFs were isolated from a patient who was pathologically confirmed esophageal squamous cancer after surgery. After 48 h culture of CAFs (which identified by immunohistochemical method), we collected the purified medium which we called CAFs medium. The CAFs medium was used to culture esophageal squamous cancer cells (KYSE-150) and the morphologic changes of cells in this medium were observed and compared with KYSE-150 cells cultured in normal medium (10% FBS RPMI-1640). The growth rate, migration ability and invasion ability of KYSE-150 cells were detected by MTS assay, wound-healing assay and transwell assay. The effect of CAFs on the sensitivity of radiotherapy and chemotherapy in KYSE-150 cells was determined by MTS assay and colony forming assay. Results showed that a dramatic morphological change was observed in KYSE-150 cells that were cultured in the CAFs medium. Compared with normally cultured KYSE-150 cells, proliferation activity, migration ability and invasion ability of KYSE-150 cells cultured in CAFs medium were significantly enhanced. MTS assay and colony forming assay also showed that CAFs medium efficiently decreases the chemosensitivity and enhance the radiotherapy resistance in KYSE-150 cells ($P < 0.05$). Altogether, our study suggested that primary cultured esophageal CAFs can promote proliferation, migration, invasion and enhance resistance to chemoradiotherapy in esophageal squamous cancer.

Keywords: Esophageal squamous carcinoma, cancer-associated fibroblasts, chemoradiotherapy resistance

Introduction

A stable micro environment is extremely important to maintain the normal physiological function of the body. When the micro environment is broken, normal cells may change into cancer cells. The microenvironment of the tumor has a profound impact on the survival, growth, invasion and metastasis in the process of tumor formation [1]. The main interstitial component of tumor microenvironment are fibroblasts.

The fibroblast is one of the core components of extracellular matrix cells. It is one of the most basic cellular tissue matrix, which participates in the synthesis and secretion of extracellular matrix (ECM) and extracellular matrix enzymes. It plays a regulatory role in the differentiation of epithelial cells through cell adhesion and release of growth factors [2]. After tumorigenesis, normal fibroblasts change their own characteristics in the course of tumor development and become cancer-associated fibroblasts (CAFs) that can secrete a variety of growth factors for tumors [3, 4].

Esophageal cancer is one of the most common malignant tumor in China. Esophageal squa-

mous cell carcinoma has been the principal pathological type of esophageal cancer in Asian countries, including China [5]. Although the overall survival rate of patients with esophageal cancer has been improved, the overall prognosis is not a good, especially in the patients with late-stage disease [6]. Inoperable patients or patients who are temporarily inoperable may only be treated with radiotherapy or chemotherapy. But the sensitivity of esophageal squamous cell carcinoma to radiotherapy and chemotherapy is not particularly good. Therefore, there is an urgent need to examine the mechanism of resistance to chemotherapy and chemotherapy in esophageal squamous cell carcinoma.

This study aims to establish a model for primary cultured esophageal squamous cell carcinoma and interstitial fibroblasts, then investigate the effect of esophageal cancer associated fibroblasts on the proliferation, migration, invasion and resistance to radiotherapy and chemotherapy in esophageal squamous cancer cells.

Materials and methods

Primary culture of esophageal cancer associated fibroblasts

To isolate stromal fibroblasts, tumor tissues were obtained from a pathologically diagnosed ESCC patient who had not been treated with any preoperative chemo-radiotherapy before esophagectomy. Clinical specimens were obtained with the permission of patients and used in accordance with the guidelines of Committees for Ethical Review of Research at the First Affiliated Hospital of Guangxi Medical University. Upon resection, tissues specimens were cut into the smallest pieces as possible, rinsed with PBS solution and then digested with 1 mg/ml collagenase type II for 2 h at 37°C in 5% CO_2/95% air. The suspension was filtered through a 200-mesh sterile copper net. The filtrate was centrifuged at 1000 rpm for 5 min and cell precipitation was collected. Then it was seeded into 25 cm^2 culture flask in 5 ml RPMI-1640 medium supplemented with 10% fetal bovine serum. After 30 min incubation, the medium was replaced with fresh medium to remove un-adherent cells (mainly tumor cells) and then pure fibroblasts were obtained. After 2-3 generations, a unique colony of stromal fibroblasts were obtained and cultured for further study. The fibroblasts isolated from tumor tissues in our study were identified as cancer-associated fibroblasts (CAFs).

Identification of CAFs (immunohistochemical method)

The cultured CAF cells were crawled and then fixed by carbinol. Immunohistochemical Kit (Santa, USA) was used to complete the immunohistochemistry according to the instructions. Primary antibodies (CK, vimentin, and alphasma) were purchased from Mai Biotechnology Company.

CAFs medium preparation and KYSE-150 cells cultured

CAFs were seeded into 75 cm^2 culture flask in 15 ml RPMI-1640 medium complemented with 10% fetal bovine serum. After 48 h culture, when cells were grown at approximately 80% confluences, the culture medium was collected and centrifuged at 3000 rpm at 4°C for 30 min. The supernatant was harvested as CAFs medium and kept at -80°C until use. KYSE-150 cells (the human esophageal squamous cell carcinoma, purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were cultured in normal medium (RPMI-1640 medium (American Corning company) supplemented with 10% fetal bovine serum) and CAFs medium at 37°C in 5% CO_2/95% air respectively. After a total of 14 days of incubation (medium was changed once every 2-3 days), the observation of morphological changes of KYSE-150 cells was conducted.

MTS assay

Cell proliferation ability was determined using MTS assay. KYSE-150 cells cultured by normal medium and KYSE-150 cells cultured by CAFs medium (2×10^4/ml cells) were plated into 96-well plate and incubated overnight. Then cells were exposed to different treatments. After incubation for indicated time intervals, the medium was replaced with fresh culture medium containing MTS mixture (Shanghai Gene Chemical Technology Co., Ltd). The plates were incubated for additional 1 h. Then the OD value of each hole at 492 nm

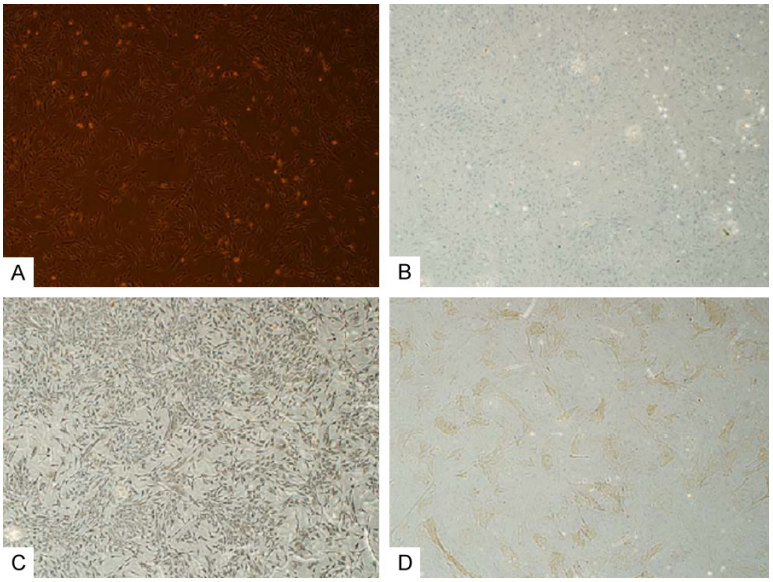

Figure 1. Esophageal CAFs primary culture and identification. A. Primary cultured CAFs (×10). B. The expression of CK in esophageal cancer associated fibroblasts (×10). C. The expression of Vimentin in esophageal cancer associated fibroblasts (×10). D. The expression of α-SMA in esophageal cancer associated fibroblasts (×10).

wavelength was measured in the microplate reader.

Wound-healing assay

Migration ability was determined using a wound-healing assay. Cells were plated into the 6-wells plates. After the cells reached sub-confluence, the cells were wounded by scraping the monolayer and photographs were taken immediately (time zero) and 6, 12, 24 h after wounding respectively. The distance migrated by the monolayer to close the wounded area during each time period was measured. Results were expressed as a migration index. All assays were conducted in triplicate.

Transwell invasion assay

Cell invasion assays were performed using transwell filters (Costar, USA) coated with matrigel (3.9 μg/μl, 60-80 μl) on the upper surface of the polycarbonic membrane (6.5 mm in diameter, 8 μm pore size). Cells (10^4/well) were seeded in the upper compartment of the chamber. Following incubation for 24 h, the cells that had migrated from the matrigel into the pores of the inserted filter were fixed with 4% formaldehy, stained with 0.01% crystal violet for 30 min, and photographed under light microscope.

The number of cells invading the matrigel was counted from three randomly selected visual fields, each from the central and peripheral portion of the filter. All assays were conducted in triplicate.

Drug sensitivity test

The concentration of cisplatin (DDP) was diluted to 100, 80, 50, 20, 10, 5 μg/ml. The concentration of paclitaxel was diluted to 5, 2.5, 1, 0.5 μg/ml. KYSE-150 cells cultured by normal medium and KYSE-150 cells cultured by CAFs medium (5000 cells per pore) were plated into 96-well plates. After 12 hours cultured, the original medium was discarded and each well was added with normal medium containing different concentrations of cisplatin or paclitaxel. After 48 hours cultured, MTS experiments were carried as mentioned above.

Radiation sensitivity determination and clone forming test

KYSE-150 cells cultured by normal medium and KYSE-150 cells cultured by CAF medium (1×10^6 cells) were plated into 6-well plate (400 cells were inoculated per hole). When the cells are fully adherent (usually 12-24 hours), they were treated with radiotherapy (6 MeV 4 Gy) and continuously cultured in an incubator. When cell clones visible by naked eye appeared in 6-well plate (usually for 14 days), clone forming test was performed. Cells were fixated with 4% PFA (paraformaldehyde; Sigma, USA) for 30 mins and stained with 0.01% crystal violet for 30 mins. The number of clones larger than 10 cells was counted under the microscope (low magnification). All assays were conducted in triplicate.

Statistical method

Data were presented as means ± SD. The difference among groups was determined by ANOVA analysis and comparison between two groups was analyzed by the Student's t-test. SPSS17.0 statistical software was used for sta-

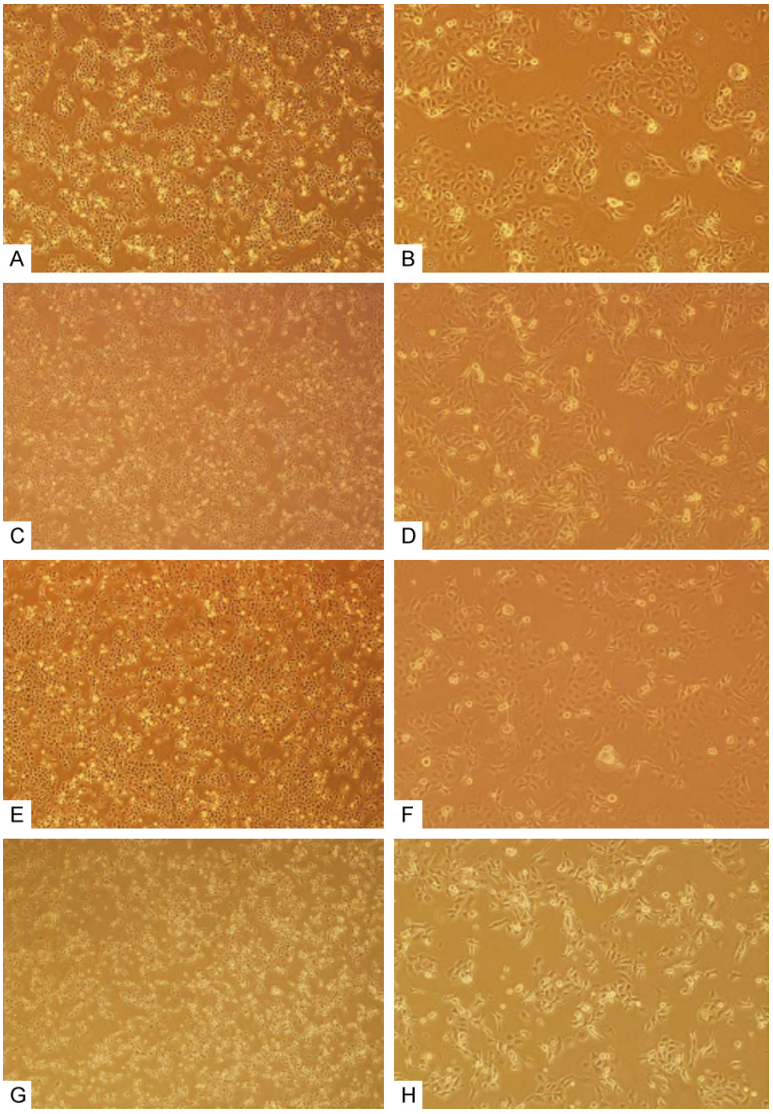

Figure 2. Effect of esophageal CAFs medium on the morphology of KYSE-150 cells. A. KYSE-150 in normal medium (×10). B. KYSE-150 in normal medium (×40). C. KYSE-150 in CAFs medium (×10), 2 days. D. KYSE-150 in CAFs medium (×40), 2 days. E. KYSE-150 in CAFs medium (×10), 7 days. F. KYSE-150 in CAFs medium (×40), 7 days. G. KYSE-150 in CAFs medium (×10), 14 days. H. KYSE-150 in CAFs medium (×40), 14 days.

tistical data processing. All *P*-values were two sided, and a *P*-value of < 0.05 was considered statistically significant.

Results

Esophageal CAFs primary culture and identification

After primary culture, CAFs could be seen to adhere to the wall for the second day. After 1-2 weeks, they were found to cover the bottom of the culture bottle (**Figure 1A**). The growth of CAFs after subculture was faster than that of primary cells. Morphology of primary cultured CAFs was spindle shaped, and some star shaped, arranged in disorder. After isolation and purification of esophageal CAFs, immunohistochemical staining was conducted on cells. The expression of a broad spectrum cytokeratin was negative in esophageal CAFs (**Figure 1B**). It showed that they were non epithelial derived cells. The expression of vimentin was positive in esophageal CAFs (**Figure 1C**). It showed that they were in accordance with the characteristics of the fibroblasts. The expression of alpha smooth muscle actin in esophageal CAFs was positive, and the positive rate was above 30%-40% (**Figure 1D**). It suggested that the fibroblasts in CAFs were activated and possessed some characteristics of myofibroblasts.

Effect of esophageal CAFs medium on the morphology of KYSE-150 cells

Compared with normal cultured KYSE-150 cells, the morphologic changes of KYSE-150 cells cultured in CAFs medium were observed. KYSE-150 cells are typical cancer cells with distinct atypia and nest like growth. They are class circle, polygon and have large nuclear and less cytoplasm. With the extension of incubation time, more and more KYSE-150 cells cultured in CAFs medium showed obvious protrusions on the cell surface, and change to spindle shape and even star shape. Cell growth can be superimposed and they grow more vigorously. It suggested that substance secreted by CAFs can significantly change the morphology of KYSE-150 cells (**Figure 2**).

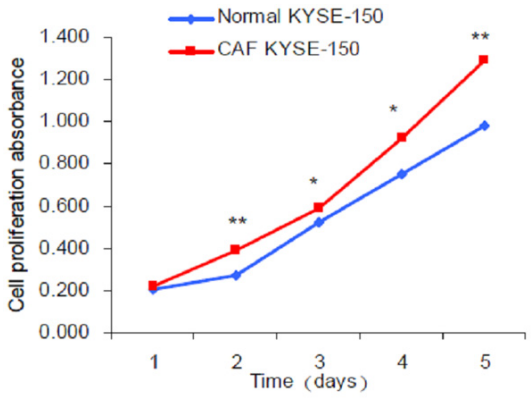

Figure 3. Growth and proliferation of CAF KYSE-150 compared with normal KYSE-150 (MTS test). *P < 0.05, **P < 0.01 using Student's t-test.

Effect of esophageal CAFs on proliferation in KYSE-150 cells

The growth curve of KYSE-150 cells cultured in CAFs medium (CAF KYSE-150) and KYSE-150 cells cultured in a common medium (Normal KYSE-150) was measured and plotted. The growth and proliferation activity of CAF KYSE-150 was significantly higher than that of normal KYSE-150. The differences of growth were statistically significant (**Figure 3**). This indicates that CAFs secretion can dramatically accelerate the growth of KYSE-150 and enhance its proliferative activity.

Effect of CAFs on migration in KYSE-150 cells

Wound-healing assay was carried out on KYSE-150 cells cultured in CAFs medium and KYSE-150 cells cultured in normal medium. After 6 h, 12 h and 24 h, the results were observed. Healing speed of CAF KYSE-150 was faster than that of normal KYSE-150 (P < 0.05) (**Figure 4**). It suggested that migration ability of CAF KYSE-150 was stronger than that of normal KYSE-150. These results indicate that CAFs secretion can dramatically enhance the migration of KYSE-150 cells.

Effect of CAFs on invasion in KYSE-150 cells

In order to detect the CAFs on invasion of esophageal carcinoma, we used transwell invasion assay and found that KYSE-150 indubated with CAFs medium significantly enhanced esophageal squamous cell carcinoma KYSE-150 invasion ability in vitro. (P < 0.05) (**Figure 5**). It suggested that CAFs secretion also can dramatically enhance the invasion of KYSE-150 cells.

Effect of CAFs on resistance to cisplatin and paclitaxel in KYSE-150 cells

The survival rate of cells was calculated with the number of cells growing at 0 ug/ml concentration as the baseline. As shown in **Figure 6A** and **6B**, the cell viability of CAF KYSE-150 was higher than that of normal KYSE-150 after the cisplatin or paclitaxel was administered to these two cell lines with different concentrations at 48 h, and the difference was statistically significant (P < 0.05).

Effect of CAFs on radiotherapy resistance in KYSE-150 cells

CAF KYSE-150 and normal KYSE-150 were respectively subjected to plate colony forming assay and cell radiotherapy sensitivity test under 4 Gy radiation dose (**Figure 7A**). Clone formation test showed that the cloned formation rate of CAF KYSE-150 was about 51%, while that of the normal KYSE-150 was about 50%. The two are similar (non radiotherapy cell colony formation rate = the number of colonies formed/total number of inoculated cells).

For radiotherapy sensitivity test, the cell survival rate after radiotherapy = clone formation number under 4 gy radiation/(total number of inoculated cells under 4 gy X non radiotherapy cell colony formation rate). The cell survival rate after radiotherapy in CAF KYSE-150 was 9.48%, while that in normal KYSE-150 was only 2.67%. The difference was statistically significant (P = 0.036) (**Figure 7B**).

Discussion

In this study, we successfully isolated and cultured primary CAFs from esophageal carcinoma. The primary culture techniques and methods of esophageal cancer cells, esophageal CAFs, and breast CAFs were reviewed [7-9]. It's not clear how many cell types can be converted into CAFs. Most CAFs originate from fibroblasts in normal parts of the body, which are stimulated and transformed by some cytokines [10, 11]. CAFs specific molecular markers have not yet been detected [2]. Activated myofibroblast with expression of alpha-sma and non

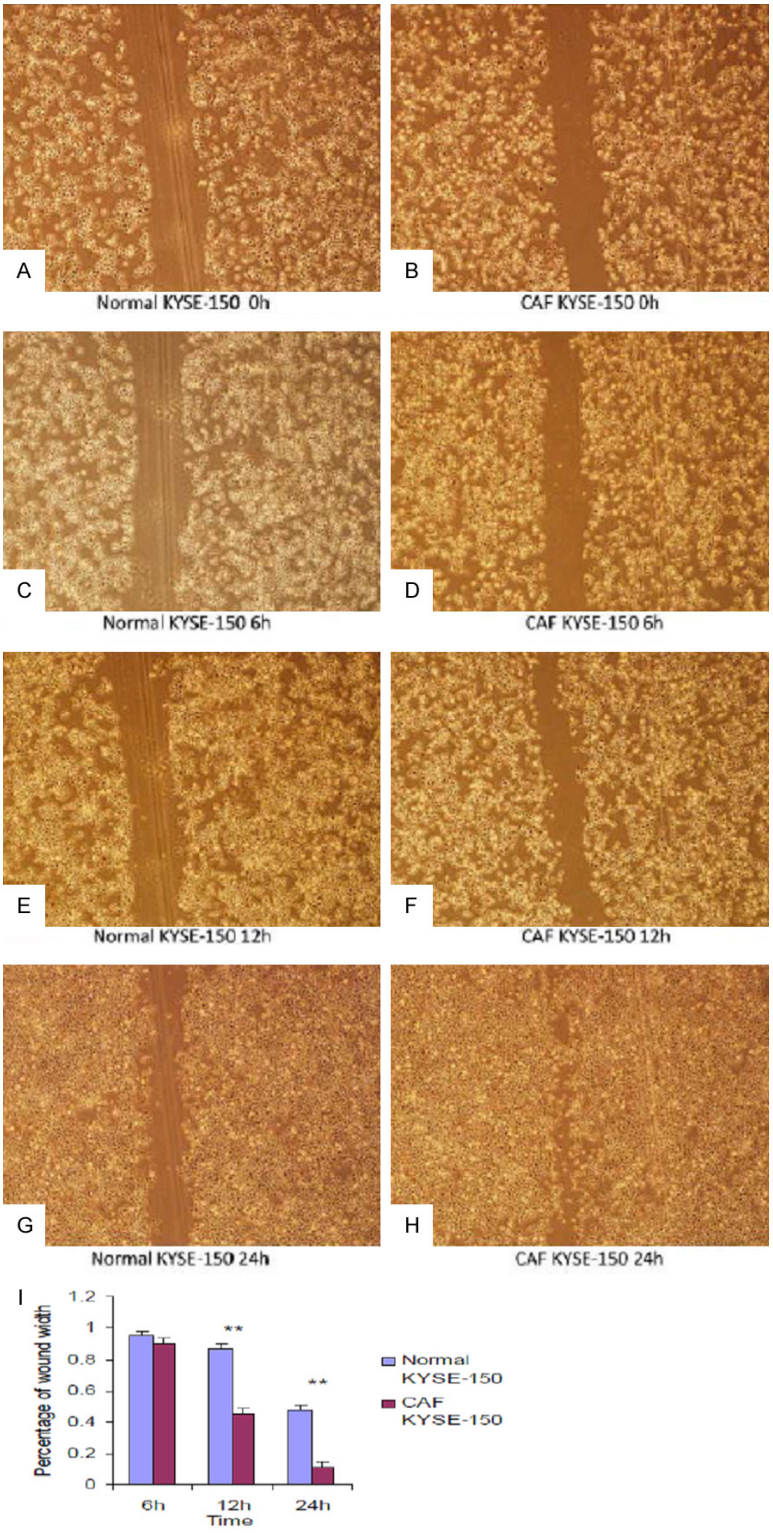

Figure 4. Migration ability of CAF KYSE-150 compared with normal KYSE-150. Wound-healing assay was carried out on KYSE-150 cultured in CAF medium and KYSE-150 cultured in common medium. The healing rate of CAF KYSE-150 was significantly faster than that of normal KYSE-150 at 6 h, 12 h and 24 h ($P < 0.05$). A. Normal KYSE-150 0 h. B. CAF KYSE-150 0 h. C. Normal KYSE-150 6 h. D. CAF KYSE-150 6 h. E. Normal KYSE-150 12 h. F. CAF KYSE-150 12 h. G. Normal KYSE-150 24 h. H. CAF KYSE-150 24 h. I. Measurement of migration distance.

muscle fibroblasts without the expression of alpha-sma form CAFs together [1]. At the same time, it has been found that CAFs also expresses proteins such as FAP, fsp-1 and other related molecules [1]. In our study, immunohistochemical staining showed that the expression of vimentin was positive in esophageal CAFs, and the expression of cytokeratin was negative. This suggests that primary cultured esophageal CAFs retains the characteristic molecular markers of some fibroblasts. At the same time, the positive expression rate of alpha smooth muscle actin in esophageal CAFs is about 30-40%. It suggested that CAFs have the characteristics of differentiation into smooth muscle cells. This is similar to the expression of molecular markers in muscle fibroblasts (MFS), and similar studies have been made abroad [12]. This proved that the primary cultured CAFs was consistent with the characteristics of CAFs in the previous study [12].

Role of CAFs in carcinogenesis and progression of cancer has been studied. The most remarkable research results still come from cell co-culture experiments. In one study, the prostate cancer cells were injected into immuno-deficienct mice with CAFs from a prostate cancer patient, and metastatic tumors were eventually developed. At the same time, the prostate cancer cells and normal prostate tissue fibroblasts were injected into mice, but no metastasis tumor was found [13]. In the study of pancreatic cancer and breast cancer, when tumor cells and cancer-associ-

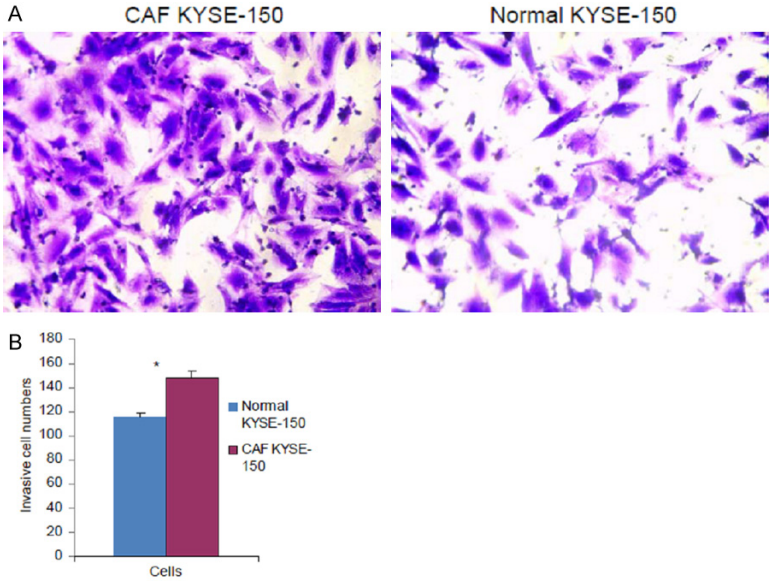

Figure 5. CAFs enhance esophageal carcinoma cells invasion in vitro. Cells were incubated with CAFs medium or Normal medium as described in the materials and methods section, and cells invasion ability was detected by transwell invasion assay. A. The filters were stained with crystal violet and inspected under a microscope. B. Quantitative measurement of invaded cells. Scale bars in microscope is 100 um. Data are representative of each group or expressed as mean ± SEM from three separate experiments and *P < 0.05 using Student's t-test.

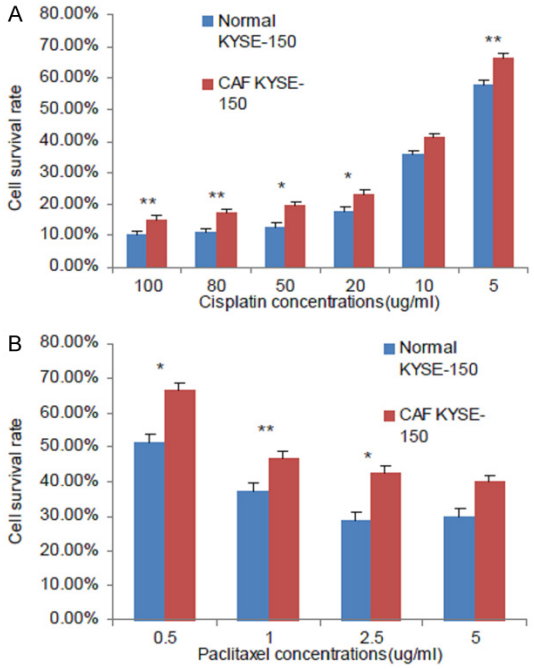

Figure 6. The impacts of chemotherapeutic drug with different concentrations on two kinds of KYSE-150. A. The impacts of cisplatin with different concentrations on two kinds of KYSE-150 (ordinate is cell survival rate). B. The impacts of paclitaxel with different concentrations on two kinds of KYSE-150 (ordinate is cell survival rate). *P < 0.05, **P < 0.01 using Student's t-test.

ated fibroblasts were co-cultured, the growth rate and invasive ability of tumor cells have been improved [14, 15]. In addition, it has been found that in esophageal squamous cell carcinoma, CAFs can promote tumor angiogenesis and secrete cytokines which play a key role in the aggregation of vascular endothelial cells [16]. CAFs also provides biomechanical support for tumor growth. In some tumors, such as squamous cell carcinoma (SCC), fibroblasts should be continuously supplemented in order to maintain their invasiveness. 3D co-culture experiments showed that CAFs are always like trail-breakers, which encroach on collagen layer together with SCC and promote the invasion and metastasis in squamous cell carcinoma [17]. Results of our study showed that proliferation rate and cell migration and invasion ability of CAF KYSE-150 cells were significantly higher than those cultured in normal medium. This is similar to the current research results [13-15]. These results suggest that esophageal CAFs can significantly increase the growth rate, migration and invasion ability of esophageal cancer cells by secreting related cytokines or related proteins. This was confirmed by more and more studies [18]. In addition, we observed that after 2 days of culture in CAFs medium, the cellular morphology of KYSE-150 changed markedly (from irregular deformation to long narrow spindle shape, even to star shape). The phenotypic changes are similar to epithelial mesenchyme transition (EMT). Whether phenotype changes are EMT remains to be confirmed by further studies, and the EMT may lead to resistance to chemoradiotherapy [19-21].

CAFs as the main component of the tumor microenvironment may lead resistance to biochemical and radiation therapy in tumor tissues, by activating autocrine or paracrine signaling pathways, regulating the DNA damage repair response of tumor cells, and promoting the proliferation of connective tissue [22, 23]. It has been reported that the host fibroblast can release growth factors that cause tumor resis-

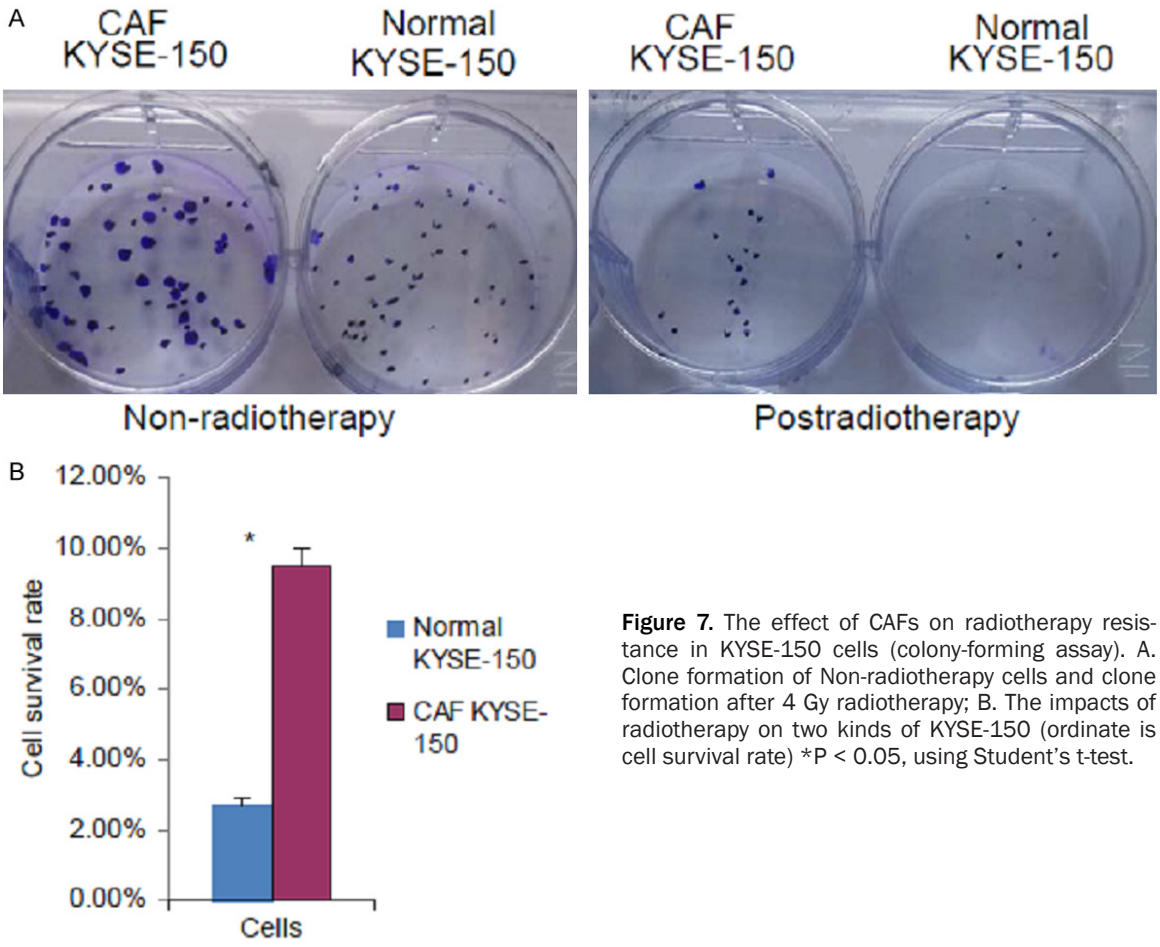

Figure 7. The effect of CAFs on radiotherapy resistance in KYSE-150 cells (colony-forming assay). A. Clone formation of Non-radiotherapy cells and clone formation after 4 Gy radiotherapy; B. The impacts of radiotherapy on two kinds of KYSE-150 (ordinate is cell survival rate) *P < 0.05, using Student's t-test.

tance to drugs [24]. A variety of cancer cell lines were cultured mixed with a variety of interstitial cells, and their drug resistance was improved to a certain extent, especially in combination with fibroblasts [24]. Interstitial cells can release more than 20 different growth factors to cause drug resistance, among which HGF is particularly effective [24]. Our experiments also showed that the sensitivity of CAF KYSE-150 to cisplatin, paclitaxel, and radiotherapy was greatly reduced compared with normal KYSE-150. In our study we did not co-culture esophageal cancer-associated fibroblasts with KYSE-150, but the culture medium from CAFs was used to culture esophageal cancer cell lines. We speculate that esophageal fibroblasts can secrete some substances (possibly the cytokines and extracellular matrix protein) that can reduce the chemotherapy sensitivity of esophageal cancer. Recent studies showed that IL6 and TGFβ1 derived from CAFs promote chemoresistance in esophagealsquamous cell carcinoma [25, 26]. In radiotherapy, CAF-secreted TGF-beta conferred radioresistance by regulating DNA damage response [27]. CXCL1 is another secretion from CAFs conferred radioresistance in esophageal squamous cell carcinoma [28]. The specific mechanism needs further study and discussion.

In conclusion, primary cultured esophageal CAFs can increase the proliferation activity, migration and invasion ability of esophageal squamous cells, and they also enhance resistance to chemoradiotherapy in esophageal squamous cancer.

Disclosure of conflict of interest

None.

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