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Original Research

Interleukin-22 derived from cervical cancer-associated fibroblasts accelerates senescence of normal fibroblasts and promotes expression of tumorigenesis-related factors in HeLa cells

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Summary

The present study aimed to evaluate the effect of interleukin-22 (IL-22), secreted by cervical cancer-associated fibroblasts (CAFs), on the senescence of normal fibroblasts (NFs) and the malignant characteristics of cancer cells. CAFs and NFs were isolated from clinical tissue samples and the degrees of senescence were compared. NFs were cultured with a CAF-conditioned medium and analyzed for the expression of senescence markers. HeLa cells were cultured with an exogenous IL-22 supplement and analyzed for the expression of tumor markers. Compared to NFs, CAFs showed a delayed growth and an elevated activity of senescence-associated β-galactosidase (SA β-gal). Expression of α-SMA, p16, and IL-22 was upregulated in the CAFs. Further, the CAF-conditioned medium promoted the senescence of NFs through the suppression of cell proliferation and the elevated expression of α-SMA and p16; whereas the addition of an IL-22 antibody reversed the senescence process. Exogenous IL-22 upregulated N-cadherin and downregulated E-cadherin in HeLa cells. The IL-22 supplement also increased the expression of VEGF, MMP2, and MMP9 in HeLa cells. In conclusion, IL-22 produced by CAFs accelerated the senescence of NFs and promoted the expression of tumorigenesis-related factors in HeLa cells.

Key words: Cervical cancer; Interleukin-22; Senescence; Cancer-associated fibroblasts; Oncogenesis.

Introduction

Cervical cancer is one of the leading gynecological cancers worldwide [1]. The introduction and widespread use of the Papanicolaou (Pap) test has decreased the incidence and mortality rate of cervical cancer dramatically [2]. However, due to limited access to health care or other nonfinancial barriers to screening, the incidence and mortality rate of cervical cancer remains substantial [3, 4]. Invasive cervical cancer is the fourth leading cause of cancer-related deaths in women worldwide and ranks even higher in the developing countries [5]. The etiology of cervical cancer is attributed to several risk factors, including persistent infection with high-risk genital human papillomavirus (HPV) strain, history of sexual activity, reduced inflammatory response, and weakened immune activity [6]. In addition to early screening and vaccination, investigation of its molecular mechanisms is important for identifying novel target drugs and improving the prognosis of cervical cancer.

HPV infection triggers cellular immune response, regulatory T cell infiltration, and T-helper (Th) cell differentiation in the host. Increasing evidence indicates that Th cells, a subgroup of lymphocytes, govern the progression of autoimmune dysfunction, inflammatory disorders, and malignant tumors [7, 8]. Among the subsets of Th cells, Th22 is acknowledged as a distinct subset compared to the other Th cells [9]. Th22 cells are characterized by an abundant production of interleukin-22 (IL-22) and tumor necrosis factor-α (TNF-α), and a limited secretion of IL-4, IL-17A, and interferon-γ (IFN-γ) [10, 11]. IL-22, the main cytokine released by Th22 cells, belongs to the IL-10 cytokine family [12]. Th22 cell population and IL-22 concentration were significantly elevated in the peripheral blood of cervical cancer patients compared to that of healthy controls [13]. These findings suggested that IL-22 may be involved in the pathogenesis of cervical cancer.

Cancer-associated fibroblasts (CAFs) are a special type of fibroblast found around tumor tissues. Alterations in the stromal microenvironment attributable to heterogeneous normal fibroblasts (NFs) and CAFs exert distinct functional effects on carcinogenesis [14, 15]. CAFs crosstalk with cancer cells through secretion of various cytokines and affect tumor proliferation, invasion, and metastasis. In mouse models, the pro-inflammatory signature generated by CAFs correlated with the pathogenesis of human breast, uterine, and ovarian cancers [10, 16]. Additionally, secretion of

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collagen by CAFs was also enhanced compared to NFs [17]. Senescence is a complex biological process which is influenced by genetic and external factors, including environmental factors, and results in dysfunction of tissues and organs [18]. The hallmarks of senescence include decreased cell proliferation, alterations in cellular morphology, increased senescence-associated β-galactosidase (SA-β-gal) activity, and overproduction of inflammatory cytokines [19]. CAFs show a higher proportion of senescent cells compared to NFs and thereby create a stromal microenvironment that is conducive to the growth and malignant transformation of cancer cells [20, 21].

Multiple studies have reported novel mechanisms of CAFs in cervical cancer. Mixed culture of CAF and HeLa cells enhanced the proliferation and survival of irradiated HeLa cells [22]. Interactions between CAFs and cervical cancer cell line, CSCC7, promoted secretion of matrix metalloproteases (MMPs), and contributed to the remodeling of interstitial stroma [23]. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) produced by CAFs promoted proliferation of ME180 cervical cancer cells [24]. However, the potential effect of IL-22 during the development of cervical cancer remains unclear. A previous study demonstrated that IL-22 produced by CAFs enhanced the invasive ability of gastric cancer cells [25]. Therefore, the aim of present study was to evaluate the effect of IL-22 on senescence of NFs and malignant characteristics of cervical cancer cells.

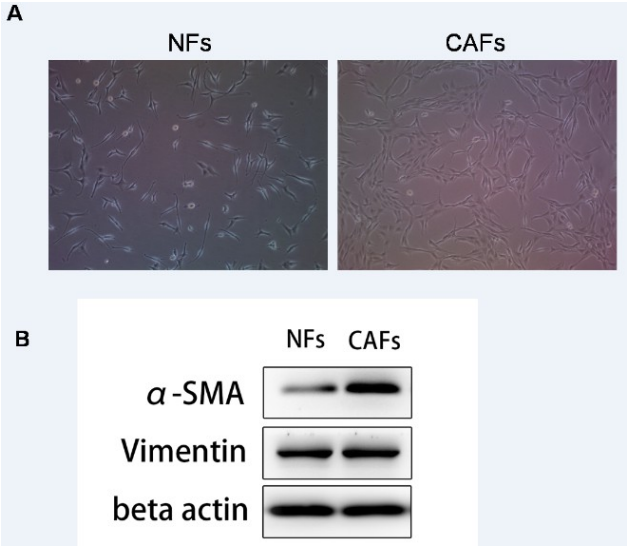

Figure 1. — Identification of isolated normal fibroblasts (NFs) and cervical cancer associated-fibroblasts (CAFs). (A) Cell morphology of NFs and CAFs observed under light scope (magnification $\times 100$). (B) Identification of fibroblast marker, Vimentin, and CAF marker, α smooth muscle actin (α-SMA), in NF and CAF cells by Western blotting.

Method

Cell Counting Kit-8 (CCK-8) assay kit, BCA protein detection kit, and enhanced chemiluminescence kit were supplied by Beyotime Institute of Biotechnology (Shanghai, China). IL-22 enzyme-linked immunosorbent assay (ELISA) kit was produced by Elabscience Biotech. Co. Ltd. (Wuhan, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Life Technologies (Carlsbad, CA, USA). Rabbit anti-p16 antibody was purchased from Abcam (Cambridge, UK); the other primary antibodies and secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

CAFs were isolated from fresh uterine cervical cancer tissue, which were surgically resected from diagnosed cervical cancer patients. NFs were isolated from uterine cervix tissues, which were resected from patients with benign gynecologic diseases. All the patients signed the informed content. The isolation was done by collagenase digestion according to the procedures as previously described [26]. Briefly, the tissues were washed twice with sterile PBS, chopped into small pieces, and digested with collagenase at 37°C for 2 hours. Then the samples were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2/95% air atmosphere at 37°C. Afterwards, the tissue blocks were maintained until the cells growing into confluent monolayer. Thirty minutes later, the non-adherent cells were removed. The attaching treatment was conducted to purify primary fibroblast cells. All CAFs and NFs less than 10 passages were applied to following experiments. The Western blotting analysis for vimentin and α-SMA were performed to examine the cellular identification of CAFs and NFs after 2-3 passages. The cell morphology was observed under a microscopy in a blinded manner.

To prepare CAF-conditioned media, CAFs were inoculated in a 100 ml flask and grown to 80% confluent. The medium was switched to serum-free DMEM and the cells were cultured for another 48 hours. The culture supernatant was collected, centrifuged to remove dead cells, filtered for sterilization, and stored at -20ºC for further use.

The cell proliferation rate was determined using CCK-8 kit. 1×10^4 CAF and NF cells were seeded onto 96-well plates, and cell proliferation was measured every day for continuous 20 days. The cells were incubated with CCK-8 solution at room temperature for 1 hour. After that the optical density (OD) was measured at 450 nm using by a spectrophotometer (Model 680, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction.

The SA-β-gal staining expression was determined by cellular senescence assay kit (OZ Bioscience, San Diego,

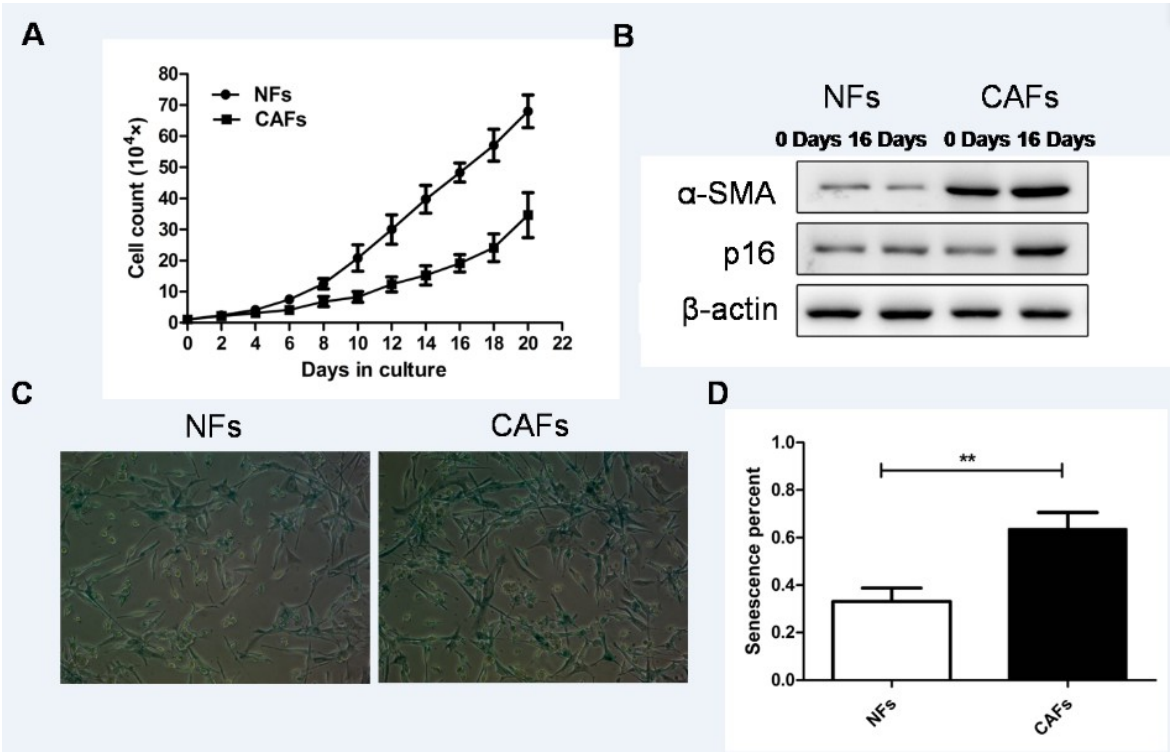

Figure 2. — Comparison of senescence-related characteristics in normal fibroblasts (NFs) and cancer associated-fibroblasts (CAFs). (A) Cell proliferation curve of NF and CAF cells. (B) Expression of CAF marker, α smooth muscle actin (α-SMA), and senescence marker, p16, in NFs and CAFs by Western blotting. (C-D) Senescence-associated β-galactosidase (SA β-gal) activity at pH 6.0 in NFs and CAFs (magnification $\times$100). $^{**}P < 0.01$.

CA, USA) according to the manufacturer's protocol. Briefly, NF cells (2500 cells/well) were seeded in six-well plates. The cells were divided into 4 groups and cultured with the following media: CAF-conditioned medium group, CAF-conditioned medium + IL-22 antibody (50 μg/ml) group, IL-22 (100 ng/ml) group as positive control, and serum-free DMEM group as negative control. The cells were cultured for continuous 5 days. After washing with PBS twice, the cells were incubated with 1 ml staining mixture solution overnight at 37°C in the dark for 15 minutes. After staining, the blue-stained cells were considered as senescent cells under light microscopy and the proportion of SA-β-gal-positive cells was calculated. For each evaluation, at least 200 cells per field of vision were counted in more than 3 fields.

CAF and NF cells were seeded on 96-well plates at a density of 1×10^4 cells/well. 24 hours later, the culture medium was changed to the normal DMEM medium without FBS. After another 48 hours of incubation, the IL-22 content in the supernatant of CAFs and NFs was measured using the commercial available ELISA kits according to the manufacturer's instruction. The absorbance values were read at 490 nm with a microplate spectrophotometer, and the corresponding concentration was calculated by the standard curve.

The cells were cultured as described in the SA-β-gal staining assay. The cells were harvested and lysed in modified RIPA buffer (Beyotime). The protein concentrations were determined by BCA assay kit. The individual cell lysates were separated by 10% SDS polyacrylamide gel (SDS-PAGE) and electro-blotted to polyvinylidene fluoride (PVDF) filter membranes. The membrane was blocked with 5% nonfat milk for 2 hours and washed with TBST for three times. PVDF membranes were incubated overnight at 4°C with the primary antibodies against α smooth muscle actin (α-SMA, 1:1000), vimentin (1:1000), p16 (1:1000), and β-actin (1:1000). β-actin was loaded as internal control. After washing, the blots were further treated with an HRP-conjugated mouse anti-rabbit IgG (1:2000). Finally, the reactive bands were detected by enhanced chemiluminescence method using a gel imaging system (Bio-Rad, Richmond, CA, USA).

Cervical cancer HeLa cells (2×10^4 cells/well) were seeded in 12-well plates and culture with conventional medium supplemented with IL-22 (100 ng/ml). After incubation for 4 days, the expression of E-cadherin, N-cadherin, vascular endothelial growth factor (VEGF), MMP2, and MMP9 in HeLa cells was detected by immunofluorescence assay. The HeLa cells were washed with PBS and fixed with paraformaldehyde at room temperature for 30 minutes.

After rehydrating, the plates were permeabilized for 5 minutes with 0.2% Triton X-100 in PBS, and further blocked with 2% BSA. Thereafter, the cells were incubated with corresponding primary antibodies against E-cadherin (1:100), N-cadherin (1:200), VEGF (1:100), MMP2 (1:100), and MMP9 (1:200) at 4°C in the dark overnight. After exposure to fluorescent secondary antibody (1:100) at 37°C for 1.5 h, the images were visualized with a fluorescent microscopy (LSM 780, Carl Zeiss, Germany).

The results of the current study are expressed as mean value ± SD. Differences between two groups were analyzed by Student *t*-test. Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA) with Tukey multiple comparison test. $P < 0.05$ was considered as significant.

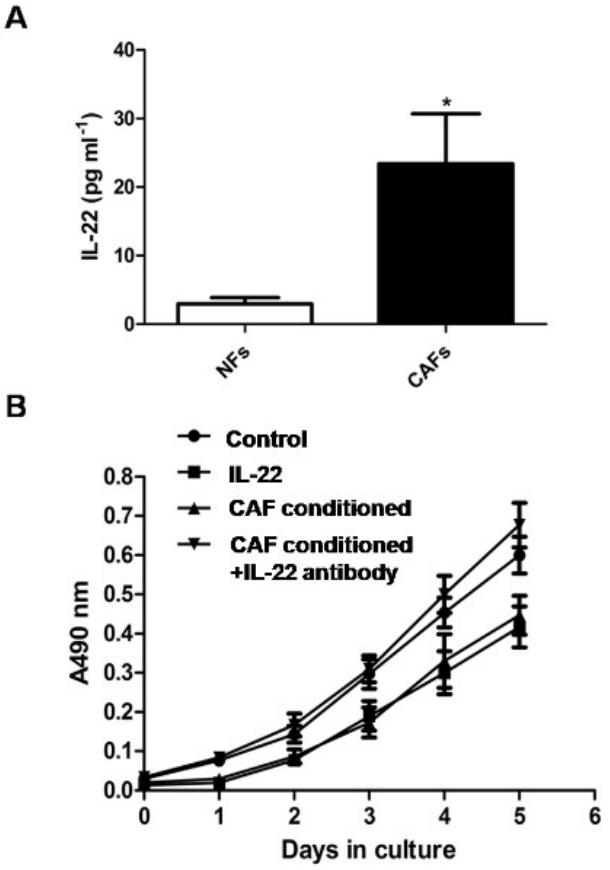

Figure 3. — Effect of interleukin-22 (IL-22) produced by cancer associated-fibroblasts (CAFs) on cell proliferation of normal fibroblasts (NFs). (A) IL-22 content in the culture supernatants of CAFs and NFs. (B) Cell growth curve of NFs treated with conventional medium (control), CAF-conditioned medium, CAF-conditioned medium + IL-22 antibody, and conventional medium + IL-22. *$P < 0.05$.

Results

Following the isolation of CAFs and NFs, their cell morphology was observed using a microscope. As shown in Figure 1A, both CAFs and NFs displayed an elongated spindle shape, while the CAFs had a more flattened morphology. To verify the identity of the CAFs and NFs, defining markers, including vimentin and α-SMA, were analyzed by Western blotting. As shown in Figure 1B, the fibroblast marker, vimentin, was expressed in both NFs and CAFs. However, a strong expression of α-SMA was observed only in the CAFs. The results suggested that CAFs and NFs were successfully isolated.

Next, we compared the proportion of senescent cells in the CAFs and NFs of the same passage number. First, we analyzed the growth curve of the cells. The cell count of NFs increased faster than those of CAFs during the 20-day culture period (Figure 2A). Cells were harvested on days 0 and 16 for western blot analysis of α-SMA and the senescence marker, p16. The results showed that the expression of α-SMA was markedly upregulated in the CAFs on days 0 and 16 as compared to the NFs during the same period. The level of p16 was notably elevated in CAFs on day 16 compared to on day 0, and in NFs on day 0 or 16(Figure 2B). SA-β-gal staining revealed that the CAFs appeared larger and more senescent than the NFs under light microscopy. The percentage of senescent cells in the CAFs was also significantly higher than that in the NFs (Figures 2C and 2D). These results suggested that CAFs exhibited a higher rate of cellular senescence and an enhanced senescent phenotype compared to the NFs.

The level of IL-22 in the culture supernatant of NFs and CAFs was determined using ELISA method. The secretion of IL-22 from the CAFs was higher than from the NFs (Figure 3A). The results suggested that IL-22 production may be positively associated with senescence of fibroblasts. To further examine the effect of IL-22 on the senescence of fibroblasts, NFs were cultured with CAF-conditioned medium and the growth curve was analyzed. As shown in Figure 3B, NFs cultured with CAF-conditioned medium showed slower cell proliferation rate compared to the control; whereas addition of IL-22 antibody to the culture media blocked the inhibitory effect and promoted growth of the NFs. Addition of IL-22 to conventional DMEM caused similar effect as CAF-conditioned media to inhibit cell proliferation. The results demonstrated that IL-22 secreted by CAFs could suppress the growth of NFs.

Next, we performed SA-β-gal staining and western blotting to further analyze the senescence markers in the NFs cultured in the CAF-conditioned medium. As shown in Figures 4A and 4B, the ratio of SA-β-gal-positive cells was markedly higher in the CAF-conditioned medium and the

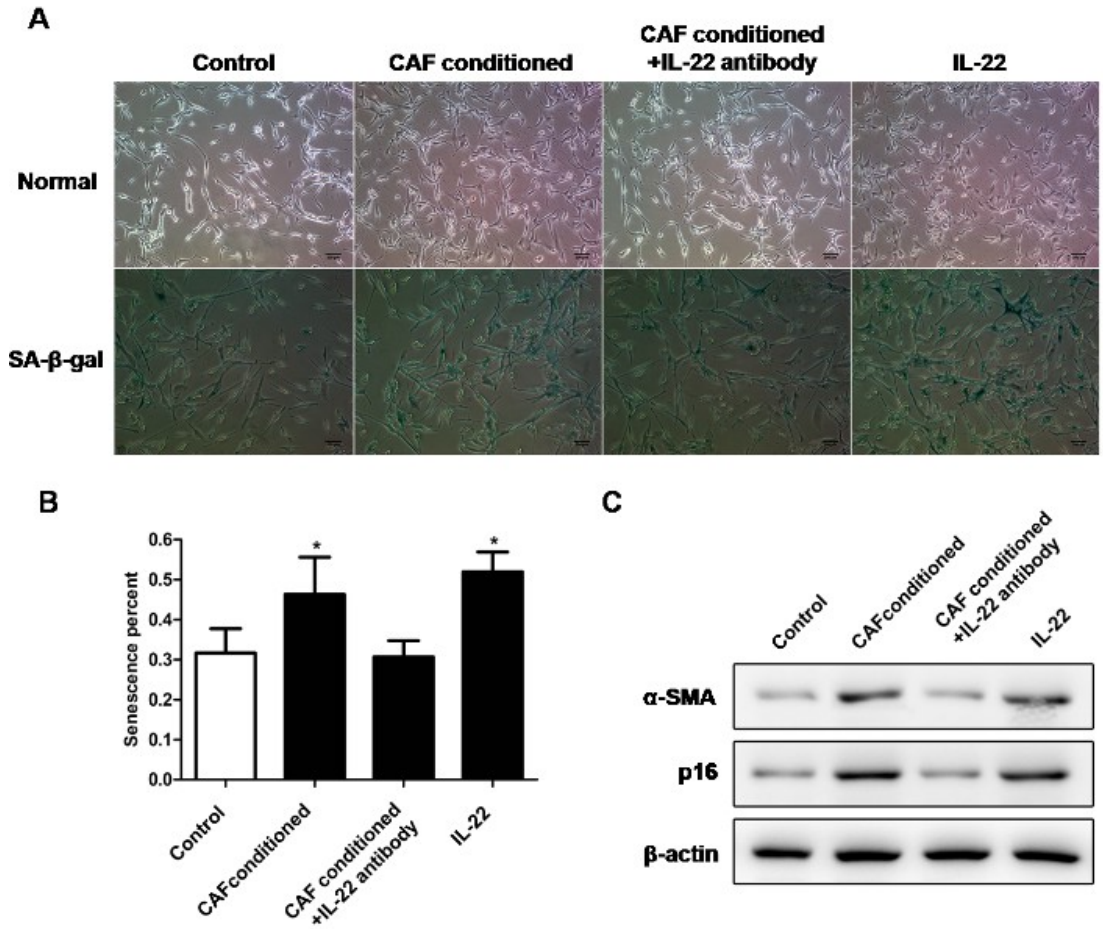

Figure 4. — Effect of interleukin-22 (IL-22) produced by cancer associated-fibroblasts (CAFs) on cell senescence of normal fibroblasts (NFs). NFs were treated with conventional medium (control), CAF-conditioned medium, CAF-conditioned medium + IL-22 antibody, and conventional medium + IL-22. (A-B) Senescence-associated β-galactosidase (SA β-gal) activity at pH 6.0 (magnification $\times$100). *$P < 0.05$. (C) Expression of CAF marker, α smooth muscle actin (α-SMA), and senescence marker, p16.

IL-22 group; while the CAF-conditioned medium + IL-22 antibody group showed a lower ratio of SA-β-gal positive cells. Additionally, the expression level of α-SMA and p16 was clearly upregulated in the CAF-conditioned medium group compared to the control group (Figure 4C). Nevertheless, the levels of these proteins were markedly reduced in the CAF-conditioned medium + IL-22 antibody group compared to the CAF group. These results further confirmed the role of IL-22 in inducing senescence in NFs.

Previous studies reported that co-culture with CAFs enhanced the proliferation and invasion of cervical cancer cells [22, 23]. Therefore, we investigated the effect of exogenous IL-22 on the expression of tumorigenesis-related proteins in cervical cancer HeLa cells (Figure 5). When cultured in IL-22 supplemented medium, HeLa cells expressed higher levels of the epithelial marker, E-cadherin, and lower levels of the mesenchymal indicator, N-cadherin. These changes in the protein expression patterns indicated that exogenous IL-22 promoted epithelial mesenchymal transition (EMT). Additionally, the expression of the angiogenesis marker, VEGF, was reduced compared to that observed in the control group. The collagenases, MMP2 and MMP9, were also downregulated in the IL-22 group compared to the control group.

Discussion

In the current study, we examined the effect of cervical CAFs on the senescence of NFs and showed that IL-22 secreted by the CAFs was increased and promoted senescence of NFs. In addition, exogenous IL-22 unregulated the expression of proteins involved in EMT, angiogenesis, and extracellular matrix remodeling. NFs play an important role in maintaining epithelial homeostasis, and inhibit the formation of epithelial tumor. Our study suggests that the tumor suppressing effect was reduced in senescent CAFs and that this may be mediated by secretion of IL-22.

Senescence is a critical and irreversible cellular program characterized by changes in the behavior, function,

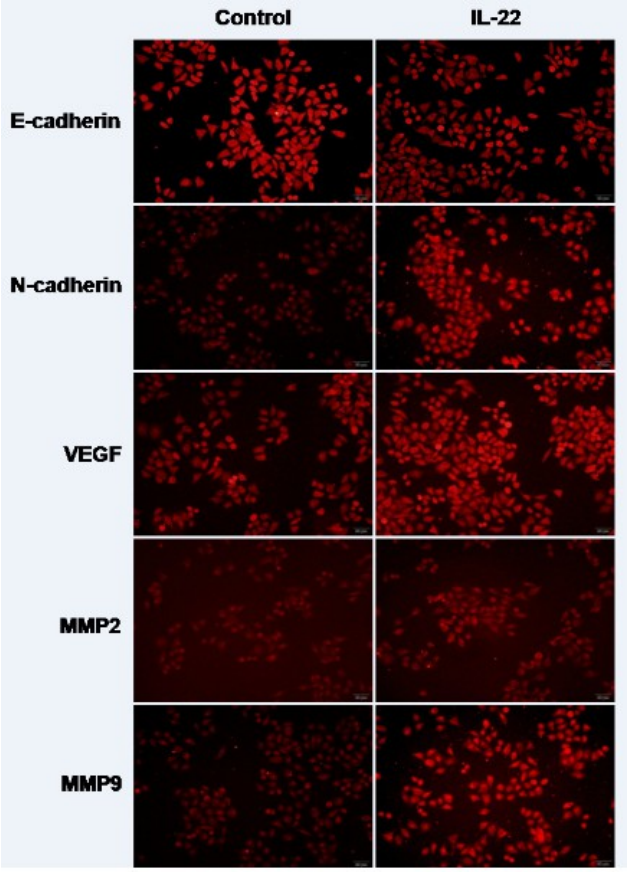

Figure 5. — Effect of interleukin-22 (IL-22) on expression of tumorigenesis-related factors in cervical cancer HeLa cells. HeLa cells were cultured with conventional medium or conventional medium supplemented with IL-22. Expression of E-cadherin, N-cadherin, vascular endothelial growth factor (VEGF), matrix metalloprotease 2 (MMP2), and MMP9 were detected by immunofluorescence analysis.

and morphology of cells and tissues [27]. Various stimuli, such as oncogene activation, DNA lesion, cytotoxic stimulation, and inflammatory stress, can lead to senescence. Senescent cells typically display an enlarged morphology and an enhanced SA-β-gal activity. The specific molecular characteristics of senescent cells include the upregulation of p16 and α-SMA [28, 29]. All these features were observed in the CAFs isolated in this study, indicating that the CAFs were more senescent than the NFs. The higher degree of senescence in the CAFs led to an increase in the secretion of the inflammatory and malignancy-related factors, and an acquisition of the senescence-associated secretory phenotype (SASP) [30, 31]. SASP is accompanied by the secretion of a large number of paracrine cytokines including inflammatory factors, growth factors, and collagenases, among others [32]. A previous study reported that CAF-derived IL-22 promoted gastric cancer cell invasion indicating that IL-22 was a novel SASP factor [25]. In the present investigation, the morphological features, SA-β-gal activity, and p16 expression confirmed that IL-22 from the cervical CAFs promoted senescence of the NFs. However, this induction of senescence could also be due to other SASP factors, in addition to IL-22. Although the addition of IL-22 antibody in the CAF-conditioned medium blocked the senescence-promotion effect, the effects of other paracrine cytokines cannot are ruled out completely.

IL-22, a member of the IL-10 cytokine family, represents the most important effector molecule of the Th22 cells [12]. IL-22 signaling is critical for a variety of malignancies including liver, lung, gastric, pancreatic, and colon cancers [33]. IL-22 induced hepatic stellate cell (HSC) senescence through the activation of STAT3 signaling [34]. IL-22 inhibited liver fibrosis by inducing HSC senescence through the TGF-β1/Notch pathway [35]. In breast cancer, IL-22 was shown to be a cancer promoting factor through regulation of the PI3K/Akt pathway [36]. IL-22 increased epithelial cell transformation and promoted mammary cancer through IL-22R [16]. It may be noted that the IL-22 receptor is a heterodimer composed of IL-22R1 and IL-10R2 subunits [37].

Elevated IL-22 level is associated with tumorigenesis and has been used as a diagnostic and prognostic marker in several studies. In non-small cell lung cancer patients, high level of IL-22 in the serum and bronchoalveolar lavage fluid was linked with shorter overall survival [38, 39]. In contrast, Souza *et al*. reported that serum IL-22 level was higher in cervical cancer patients with low-grade squamous intraepithelial lesion compared to patients with high-grade squamous intraepithelial lesion [40]. The conflicting data from various studies indicate that further studies are clearly needed to understand the significance of IL-22 as a diagnostic marker. In this study, we only performed in vitro experiments to investigate the role of exogenous IL-22 on HeLa cells. The importance of EMT, angiogenesis, and extracellular matrix remodeling in cervical cancer has been reported in several large studies previously [41-43]. Our results suggest that IL-22 regulates these pathophysiological processes. The effects of IL-22 in vivo will need to be further evaluated, in future studies.

Conclusion

The present study demonstrated that CAF-derived IL-22 induced senescence of NFs and exogenous IL-22 stimulated the expression of malignancy-related factors in cervical cancer cells. The results suggested that IL-22 mediates the crosstalk between cervical cancer cells and its surrounding stroma. Currently, our understanding of the mechanisms of IL-22 in cervical cancer is limited. Our research provides some preliminary data on the role of CAF-derived IL-22 in cervical cancer. Further investigations are war-

ranted to elucidate its underlying mechanism both in vitro and in vivo.

Acknowledgement

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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