

# Expression of immune regulatory factors, chemokines and growth factors in differentiated gastric cancer cells treated with an anticancer bioactive peptide combined with oxaliplatin

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**Abstract.** Gastric cancer is one of the most common malignant tumors of the digestive system. An anticancer bioactive peptide (ACBP) was previously shown to have an important role in inhibiting the differentiation of the MKN-45, N87 and GES-1 cell lines. However, to date, research on the effects of inflammatory factors in MKN-45, N87 and GES-1 cell lines after treatment with ACBP combined with oxaliplatin (OXA) has not been performed. To investigate the expression of immune regulatory factors, tumor growth factors and chemotactic factors in differentiated gastric cancer cells treated with ACBP combined with OXA, the expression of cytokines, including interleukin (IL)-1 $\beta$ , IL-1 receptor antagonist, IL-2, IL-4, IL-6-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, basic fibroblast growth factor (bFGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)- $\gamma$ , monocyte chemoattractant protein (MCP)-1, IFN- $\gamma$ -induced protein-10, macrophage inflammatory protein (MIP)-1 $\alpha$ , platelet-derived growth factor (PDGF)-BB, MIP-1 $\beta$ , regulated upon activation, normal T cell expressed and presumably secreted, TNF- $\alpha$  and VEGF, was assessed with cell experiments using the Bio-Plex ProT Human Cytokine 27-plex Assay. The results indicated that immune regulatory factor, tumor growth factor and chemotactic factor expression levels were different after treatment with ACBP alone or ACBP combined with OXA. IFN- $\gamma$ , IL-1 $\beta$ , IL-17, IL-9, IL-10, IL-15, bFGF, GM-CSF and PDGF-BB expression was

decreased in MKN-45 and N87 cells after ACBP treatment ( $P<0.01$ ) and ACBP+OXA treatment ( $P<0.01$ ) compared with the control cells, which indicated that ACBP inhibited tumor growth by regulating these cytokines, and the combination treatment inhibited tumor growth by regulating these cytokines. MIP-1 $\beta$ , MCP-1 and IL-13 expression was decreased in MKN-45 and N87 cells after the combination treatment compared with ACBP treatment alone, which indicated that ACBP combined with OXA was able to inhibit tumor growth by regulating these cytokines, while the mechanism of action of the ACBP and OXA is actually different, e.g. for OXA, this would be to cause DNA damage response. Therefore, the ACBP and OXA combination treatment may be closely associated with tumor progression and metastasis with immunological competence by MCP-1, MIP-1 $\beta$  and IL-13 expression.

## Introduction

Gastric cancer is one of the most common malignancies of the gastrointestinal system. Every year, 7 million patients die from cancer, and 1 in 10 patients die of gastric cancer. Gastric malignancies have the fourth highest incidence rates and the second highest mortality rates among all cancers. The incidence rate of gastric cancer is second only to lung cancer in China and the mortality rate ranks first among various cancers (1,2). At present, the primary conventional treatment for gastric cancer remains surgical resection supplemented by radiotherapy and chemotherapy, and chemotherapy has gradually become an important method for patients who miss the opportunity to undergo surgery and comprehensive treatment for gastric cancer (3). However, chemotherapy drugs that target and suppress cancer also exert substantial effects on normal cells, exhibit poor targeting and killing of tumor cells, and cause different degrees of toxicity; during the process of treatment, certain patients develop multiple drug resistance to different chemotherapy drugs and the dose of chemotherapy drugs must be increased, making the quality of life of patients decrease and causing patients to refuse chemotherapy or end treatment due to pain (4,5). Therefore, finding strategies to avoid or even solve these problems, reduce the pain experienced by patients

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and improve patient quality of life has become an urgent need in cancer treatment.

The anticancer active peptide is a novel biological agent with anticancer activities and numerous years of experiments have confirmed that it has obvious antitumor and tumor suppressor effects without toxic side effects (6). ACBP exerts a certain inhibitory effect on the growth of various tumor cells, such as human leukemia, colorectal cancer and mouse sarcoma cells, and it may enhance the antitumor effect of lymphocytes and sensitivity to chemotherapeutic drugs (7,8). It was previously found that ACBP exhibits antineoplastic activity and inhibits tumor growth in nude mice with dutch gallbladder carcinoma. It also increased the chemotherapeutic sensitizing effect and decreased side effects associated with chemotherapy (9). Oxaliplatin (OXA) is a commonly used chemotherapeutic drug in the treatment of gastric cancer, with obvious efficacy compared to other chemotherapeutic drugs, such as cisplatin and carboplatin, but its toxic effects are unavoidable, which limits its application in the treatment of gastric cancer (9).

According to their functions, cytokines may be divided into interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs), chemokines and growth factors. Cytokines have various biological functions, including regulating innate immunity, adaptive immunity, hemopoiesis, cell growth and differentiation, as well as tissue damage repair. Numerous cytokines promote or restrict each other in the body, forming a complex cytokine immune regulation network. IL is a family of cytokines capable of bidirectional regulating factors of the immune system, mainly involved in the differentiation and activation of immune cells. Cytokines, such as IL-6, TNF- $\alpha$ , IL-10 and IFN- $\gamma$ -induced protein (IP)10, are key transcription factors involved in controlling the expression of proinflammatory cytokines and responses to infection (10). They have important roles in immune regulation, cell differentiation, cell apoptosis and cell cycle regulation. IL-1, IL-10, IL-12, family members have a key role in the differentiation and function of polarized innate and adaptive lymphoid cells (11). IL-2, an important immune regulator that is synthesized and released by lymphocytes by specific antigen stimulation, enhances the T-cell population by promoting T cells to transition from the G1 phase to the S phase of the cell cycle. It is currently suggested that IL-2 has anticancer effects. IL-4 is a cytokine with various biological functions, including stimulating B cells, promoting B-cell transformation into plasma cells and inducing B-cell IgE production, promoting T helper (Th) cells to transform into Th2 cells and inhibiting the production of Th1 cells.

Growing evidence suggests that IL-8 has pivotal roles in the pathogenesis of cancer through the modulation of the tumor immune response or the promotion of angiogenesis (12). IL-15 serves multiple functions, including dictating the T-cell response, regulating tissue repair and B-cell homing, modulating inflammation and activating NK cells (13). IL-17 has a well-recognized role in immune surveillance at mucosal and barrier surfaces but has also been increasingly implicated as a driver of immunopathology in settings of autoimmunity and chronic inflammation (14). IL-1 receptor antagonist (IL-1RA) can suppress tumors by promoting tumor angiogenesis through vascular endothelial growth factor (VEGF) production, and a study indicated that downregulation of IL-1RA is closely related to TNM staging and survival prognosis (15).

IL-9 exerts unprecedented antitumor immune effects not only by inducing innate and adaptive immune responses but also by directly promoting the apoptosis of tumor cells (16). Eotaxin-1/eosinophils appear to have a role in coronary artery disease independent of known risk factors (17).

IFN is a special kind of protein or glycoprotein produced by the body in response to various stimuli (including viruses). It can regulate innate immunity, activated antiviral properties and antiproliferative function. Furthermore, IFN- $\gamma$  is probably one of the most relevant cytokines for orchestrating the immune response in vertebrates (18).

Growth factors are a class of peptides that can affect the growth, differentiation and apoptosis process and regulate immunity of a variety of cells. They include a wide variety of substances, including insulin-like growth factor, epidermal growth factor and transforming growth factor- $\beta$ . VEGF is a growth factor with important pro-angiogenic activity, and it exerts mitogenic and anti-apoptotic effects on endothelial cells, increases vascular permeability and promotes cell migration (19). Furthermore, basic fibroblast growth factor (bFGF) lacks somnogenic activity (14), but it stimulates proliferation and hyaluronan production. Platelet-derived growth factor-BB (PDGF-BB) has a role in suppressing proliferation, angiogenesis and osteogenesis (20).

TNF is a pro-inflammatory cytokine. It has a pro-inflammatory effect and can induce necrosis of tumor cells. In addition, it is also involved in the occurrence of fever and inflammation. Macrophage inflammatory protein (MIP)-1 $\beta$  and TNF- $\alpha$  are produced by individual unstimulated and lipopolysaccharide-stimulated human macrophages (21), and elevated circulating levels of MIP-1 $\beta$  may be associated with a lower risk of rheumatoid arthritis (RA) (22). Chemokines are cytokines with chemotactic properties for inflammatory cells and other cell types. Regulated upon activation, normal T cell expressed and presumably secreted (RANTES), MCP-1 (also known as MCAF), IL-8 and IL-13 have fundamental roles in histamine and serotonin generation and cell function in mast cells (23). MIP-1 $\alpha$  is an important chemokine and a prognostic biomarker in both solid and hematological malignancies (24). IFN- $\gamma$ -induced protein 10 (IP-10) participates in the formation of the proinflammatory immune microenvironment during early pregnancy by regulating the distribution of immune cells and promoting the production of proinflammatory cytokines (25). Specifically targeting the crosstalk between T cells and myeloid cells through granulocyte-macrophage colony-stimulating factor (GM-CSF) holds promise for the development of therapeutics to combat chronic tissue inflammation (26). Therefore, in the present study, the levels of 25 cytokines in four differentiated gastric cancer cell lines were measured after treatment with ACBP combined with OXA. Gaining better insight into the host immune response and inhibition of gastric cancer cell proliferation may be the basis for the identification of immunotherapeutic targets, particularly for severe cases in which ACBP combined with OXA treatment is not sufficient.

## Materials and methods

**Cells and materials.** The anticancer active peptide used in the present study, anticancer bioactive peptide (ACBP) is a

low-molecular-weight active substance that was obtained from the Clinical Medical Research Center, Affiliated Hospital of Inner Mongolia Medical University (Hohhot, China). The poorly differentiated human gastric adenocarcinoma cell line of MKN-45, the highly differentiated human gastric adenocarcinoma cell line NCI-N87 and the immortalized and non-tumorigenic human gastric mucosal epithelial cell line GES-1 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 medium and high-glucose DMEM were purchased from Hyclone (Cytiva). Penicillin, streptomycin, fetal bovine serum and 0.25% trypsin were from Gibco (Thermo Fisher Scientific, Inc.).

The Bio-Plex ProT Human Cytokine 27-plex Assay (cat. no. M500KCAFOY; Bio-Rad Laboratories, Inc.) is a multiplex assay that detects cytokines including IL-1 $\beta$ , IL-1RA, IL-2, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, bFGF, GM-CSF, IFN- $\gamma$ , MCP-1 (MCAF), IP-10, MIP-1 $\alpha$ , PDGF-BB, MIP-1 $\beta$ , RANTES, TNF- $\alpha$  and VEGF. OXA was obtained from Jiangsu Hengrui Pharmaceutical Co., Ltd.

**Cell recovery.** MKN-45 cells were cultured in high-glucose DMEM supplemented with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 10% fetal bovine serum. N87 cells and GES-1 cells were cultured in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 10% fetal bovine serum. The MKN-45, N87 and GES-1 cells were mixed well and centrifuged at 85 x g at 25°C for 5 min. The supernatants were discarded and the cells were resuspended in 1 ml complete medium. Then, the cells were plated in 10-cm dishes, complete medium was added to a final volume of 10 ml; the cells were then mixed and placed in a 37°C incubator with saturated humidity and 5% CO<sub>2</sub>.

**Cell treatment and cytokine assay.** MKN-45, N87 and GES-1 cells were digested, suspended, counted and seeded in 96-well plates at 5,000 cells per well. After 24 h, the cells were divided into four groups: OXA group (11.7  $\mu$ g/ml), ACBP group (18.8  $\mu$ g/ml), combined drug group (5.85  $\mu$ g/ml OXA + 9.4  $\mu$ g/ml ACBP) and control group. Cell supernatants were collected 48 h after treatment and the protein concentrations were determined. In the present study, the Bio-Plex ProT Human Cytokine 27-plex Assay was used for determining the levels of the 25 cytokines, and the expression levels of multiple cytokines were detected. Cytokines, including IL-1 $\beta$ , IL-1RA, IL-2, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, bFGF, GM-CSF, IFN- $\gamma$ , MCP-1 (MCAF), IP-10, MIP-1 $\alpha$ , PDGF-BB, MIP-1 $\beta$ , RANTES, TNF- $\alpha$  and VEGF, were diluted to 1X and added to plates at volumes of 50  $\mu$ l per well at room temperature for 30 min. They were washed twice with 100  $\mu$ l wash buffer and 50  $\mu$ l of the collected cell supernatant was added to each well. The plates were sealed and incubated in the dark at room temperature with shaking for 30 min. The cells were washed 3 times with 100  $\mu$ l wash buffer. Subsequently, 25  $\mu$ l of 1X antibodies were added per well. The plates were sealed and incubated in the dark at room temperature with shaking at room temperature for 30 min. The plates were washed 3 times with 100  $\mu$ l wash buffer and 50  $\mu$ l 1X streptavidin-phycoerythrin was then added to each

well. The wells were sealed, incubated in the dark at room temperature with shaking at 600 mm/sec for 10 min and washed 3 times with 100  $\mu$ l wash buffer. The pellet was resuspended in 125  $\mu$ l assay buffer, sealed and incubated in the dark at room temperature with shaking at 600 mm/sec for 30 sec. The biotin-labeled antibody and streptavidin-phycoerythrin conjugates were part of the assay kit. After a further wash, the assay buffer was added to wells to re-suspend the beads, and the fluorescence was measured using an automatic immunoassay analyzer (Bio-Plex® 200 System; Bio-Rad Laboratories, Inc.). Finally, the cytokine concentration was calculated from the standard curve (27).

**Statistical analysis.** Statistical analyses were performed using SPSS version 15.0 (SPSS, Inc.). Values are expressed as the mean  $\pm$  standard deviation. One-way analysis of variance was used for multiple-group comparisons and Bonferroni's post-hoc test was used for subsequent pairwise comparisons. P<0.05 was considered statistically significant.

## Results

**Expression of immune regulatory factors.** As indicated in Fig. 1, the levels of IL-2, IL-4, IL-7, IL-10, IFN- $\gamma$  and GM-CSF in the supernatants of MKN-45 gastric cancer cells were decreased after treatment with ACBP compared with the control group (P<0.01). The IL-12, IFN- $\gamma$ , GM-CSF, VEGF, IL-10 and IL-13 levels were decreased in the supernatants of the N87 gastric cancer cell line after treatment with ACBP compared with the control (P<0.01). The IL-2, GM-CSF and IFN- $\gamma$  levels in the supernatants of GES-1 cells were increased by ACBP treatment compared with the control (P<0.01). In addition, the levels of IL-2, IL-12, IL-7, IL-10, IL-13, GM-CSF and IFN- $\gamma$  and VEGF in the supernatants of MKN-45 and N87 cells were decreased by ACBP and combination treatment compared with the control (P<0.01). However, the IL-4, IL-7, IFN- $\gamma$  and GM-CSF levels were increased in the supernatants of GES-1 cells after combination treatment compared with the control (P<0.01). Furthermore, the IL-2 and IL-13 expression levels were not significantly different from those in the control group in GES-1 cells.

Compared with ACBP, IL-2 and GM-CSF levels were significantly increased in MKN-45 cells after treatment with ACBP combined with OXA, while the IL-4, IL-7, IL-10, IL-12, IL-13, IFN- $\gamma$  and VEGF levels were significantly decreased (P<0.01). After N87 cells were treated with the combination of ACBP and OXA, IL-4 and IL-7 levels were increased (P<0.01), IL-2, IL-13 and GM-CSF levels were decreased (P<0.01, P<0.05) and IL-10, IL-12, IFN- $\gamma$  and VEGF levels were not significantly different compared with the ACBP group. Furthermore, after combination treatment, the IL-7, IL-10, IL-12, IL-13, IFN- $\gamma$ , GM-CSF and VEGF levels were significantly increased (P<0.01 or P<0.05) in the supernatants of the GES-1 cells compared with the ACBP group.

Compared with OXA, the levels of IL-4, IL-7, IL-10, IL-12, IL-13, IFN- $\gamma$ , and VEGF in the supernatants of MKN-45 were significantly increased (P<0.01) after treatment with ACBP. In addition, the levels of IL-7 in the supernatants of MKN-45 were significantly increased, while the levels of IL-2, IL-10, IL-13, IFN- $\gamma$ , and GM-CSF and VEGF expression were

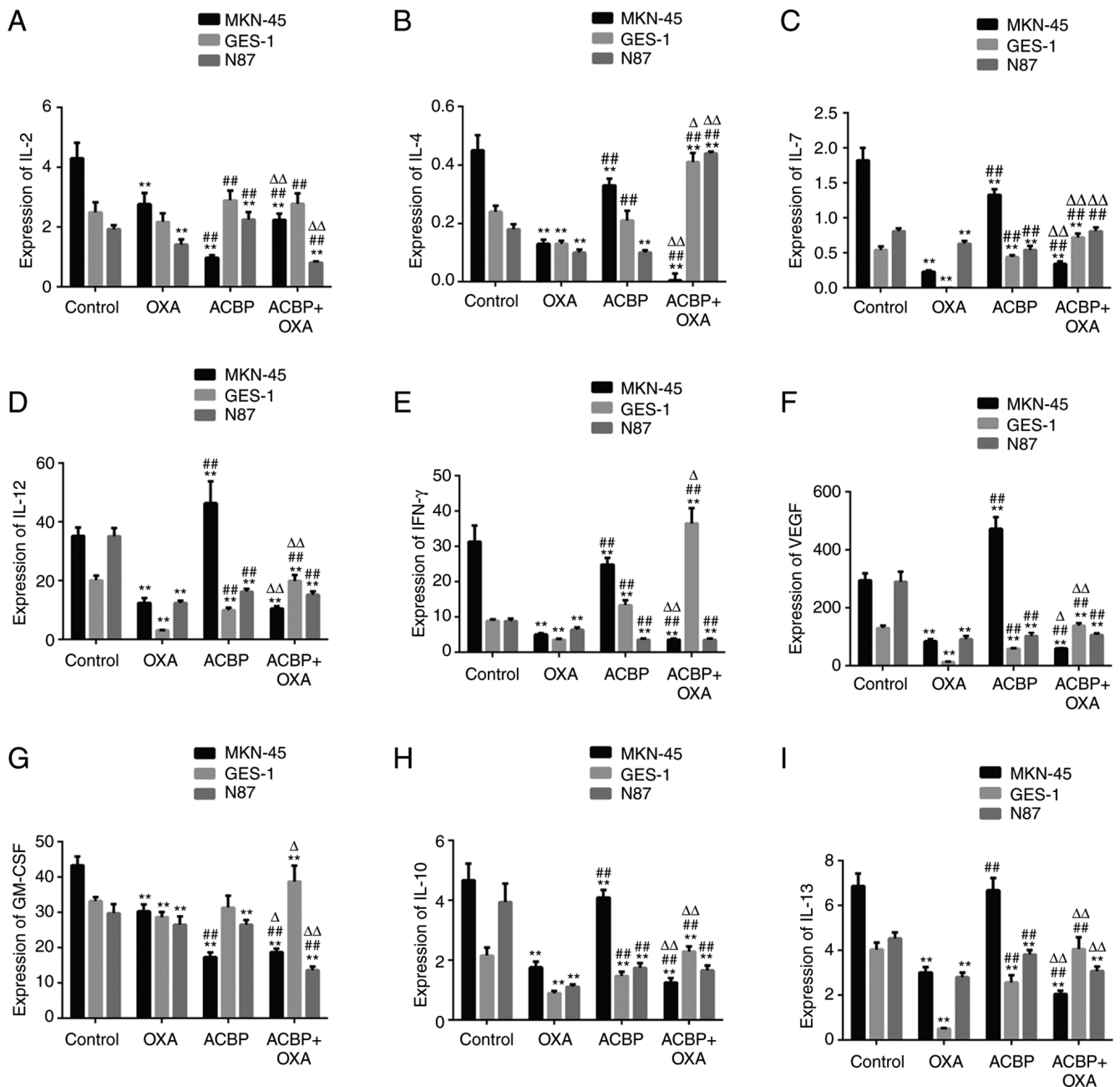


Figure 1. Expression of immune regulatory factors. The levels of (A) IL-2, (B) IL-4, (C) IL-7, (D) IL-12, (E) IFN- $\gamma$ , (F) VEGF, (G) GM-CSF, (H) IL-10, (I) IL-13 in the supernatants of GES-1, MKN-45 and N87 gastric cancer cells were decreased after treatment with ACBP or ACBP combined with OXA. \*\*P<0.01 vs. control group; ##P<0.01 vs. OXA group, ΔP<0.05, ΔΔP<0.01 vs. ACBP group. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; OXA, oxaliplatin; ACBP, anticancer bioactive peptide.

decreased ( $P<0.01$ ) after treatment with ACBP combined with OXA. Furthermore, compared with OXA, the levels of IL-2, IL-7, IL-10, IL-12, IL-13, IFN- $\gamma$ , VEGF and GM-CSF in the supernatants of N87 were significantly increased or decreased ( $P<0.01$ ) after treatment with ACBP. The levels of IL-2, IL-4, IL-7, IL-10, IL-12, IFN- $\gamma$ , VEGF and GM-CSF in the supernatants of N87 were significantly increased or decreased ( $P<0.01$ ) after treatment with ACBP combined with OXA. Compared with OXA, the levels of IL-2, IL-4, IL-7, IL-10, IL-12, IL-13, IFN- $\gamma$  and VEGF in the supernatants of GES-1 were significantly increased ( $P<0.01$ ) after treatment with ACBP or ACBP combined with OXA, while the levels of GM-CSF showed no significant difference.

**Expression of tumor growth factors.** As presented in Fig. 2, the results showed that the levels of IL-9, IL-15, bFGF and PDGF-BB in the supernatants of the MKN-45 and N87 gastric cancer cell lines were significantly decreased after treatment with ACBP and combination of ACBP and OXA compared with the control group ( $P<0.01$ ). The IL-8 levels were significantly increased in the supernatants of the MKN-45 cells and GES-1 cells ( $P<0.01$ ) after ACBP treatment and combination treatment. The IL-1 $\beta$  and IL-15 levels were significantly decreased in N87 cells, and IL-1RA, IL-1 $\beta$ , IL-8, IL-9, IL-17, bFGF and PDGF-BB levels were significantly increased in the supernatants of GES-1 cells after treatment with ACBP and OXA compared with the control group ( $P<0.01$ ).

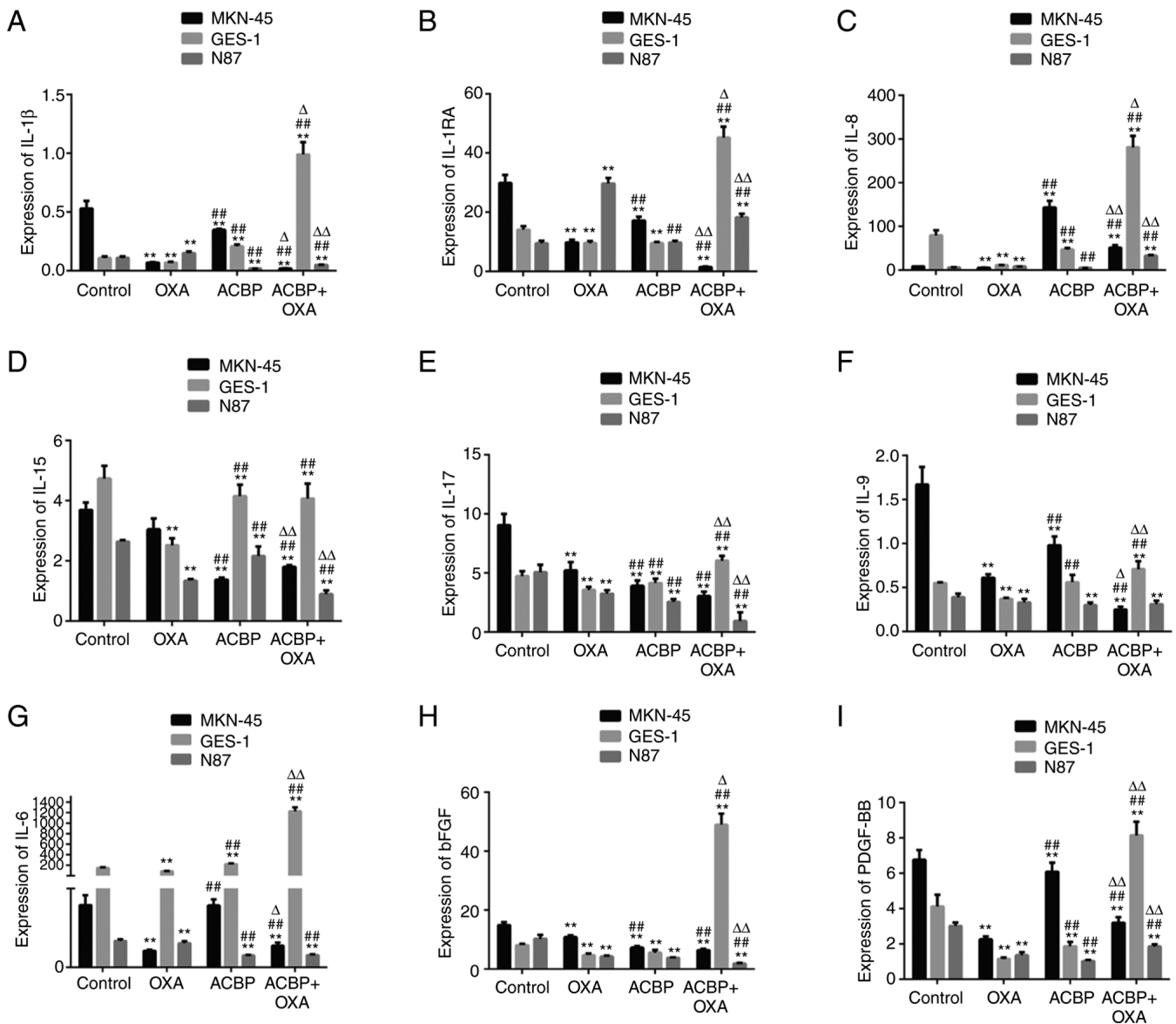


Figure 2. Expression of tumor growth factors. The levels of (A) IL-1 $\beta$ , (B) IL-1RA, (C) IL-8, (D) IL-15, (E) IL-17, (F) IL-9, (G) IL-6, (H) basic FGF and (I) PDGF-BB in the supernatants of GES-1, MKN-45 and N87 gastric cancer cells were decreased after treatment with ACBP or ACBP combined with OXA. \*\*P<0.01 vs. control group; ##P<0.01 vs. OXA group, ΔP<0.05, ΔΔP<0.01 vs. ACBP group. FGF, fibroblast growth factor; IL-1RA, interleukin 1 receptor antagonist; PDGF, platelet-derived growth factor; OXA, oxaliplatin; ACBP, anticancer bioactive peptide.

As shown in Fig. 2, the IL-1RA, IL-1 $\beta$ , IL-6, IL-9 and PDGF-BB levels were significantly decreased in the supernatants of MKN-45 cells after combination treatment compared with ACBP-treated cells (P<0.01), while the levels of IL-17 and bFGF showed no significant difference. After combination treatment, the IL-1RA, IL-1 $\beta$ , IL-6, IL-8, IL-9, IL-17 and PDGF-BB levels in the supernatants of the GES-1 cells were all significantly increased (P<0.01 or P<0.05) compared with the ACBP group. The results showed that the expression of IL-8 in the culture medium of the gastric cancer cell line MKN-45 was significantly increased (P<0.01). After treating MKN-45 cells with the combination of drug treatment, IL-8 was also elevated but not significantly increased when using the ACBP alone. The treatment of N87 cell with ACBP showed no significant change in IL-8 expression (P>0.01), while the expression level of IL-8 was significantly increased after combination of drug treatment (P<0.01). Compared with the OXA group, the IL-1 $\beta$ ,

IL-8, IL-6, IL-15, IL-17 and PDGF-BB levels in the supernatants of MKN-45, GES-1 and N87 cells were significantly different after ACBP treatment (P<0.01). The IL-1RA, IL-1 $\beta$ , IL-8, IL-6, IL-15, IL-17, bFGF and PDGF-BB levels in the supernatants of MKN-45 cells, GES-1 and N87 after ACBP combined with OXA treatment exhibited significant differences compared with OXA treatment (P<0.01).

**Expression of chemotactic factors.** As indicated in Fig. 3, the levels of MCP-1 (MCAF), Eotaxin, MIP-1 $\alpha$  and MIP-1 $\beta$  were significantly decreased in the supernatants of MKN-45 cells after treatment with ACBP compared with the control (P<0.01), while IP-10, TNF- $\alpha$  and RANTES showed no significant difference (P>0.05). The Eotaxin, IP-10 and RANTES levels were significantly increased in the supernatants of N87 cells after ACBP treatment (P<0.01). The MCP-1 (MCAF), MIP-1 $\alpha$  and MIP-1 $\beta$  levels were not significantly different in N87 cell

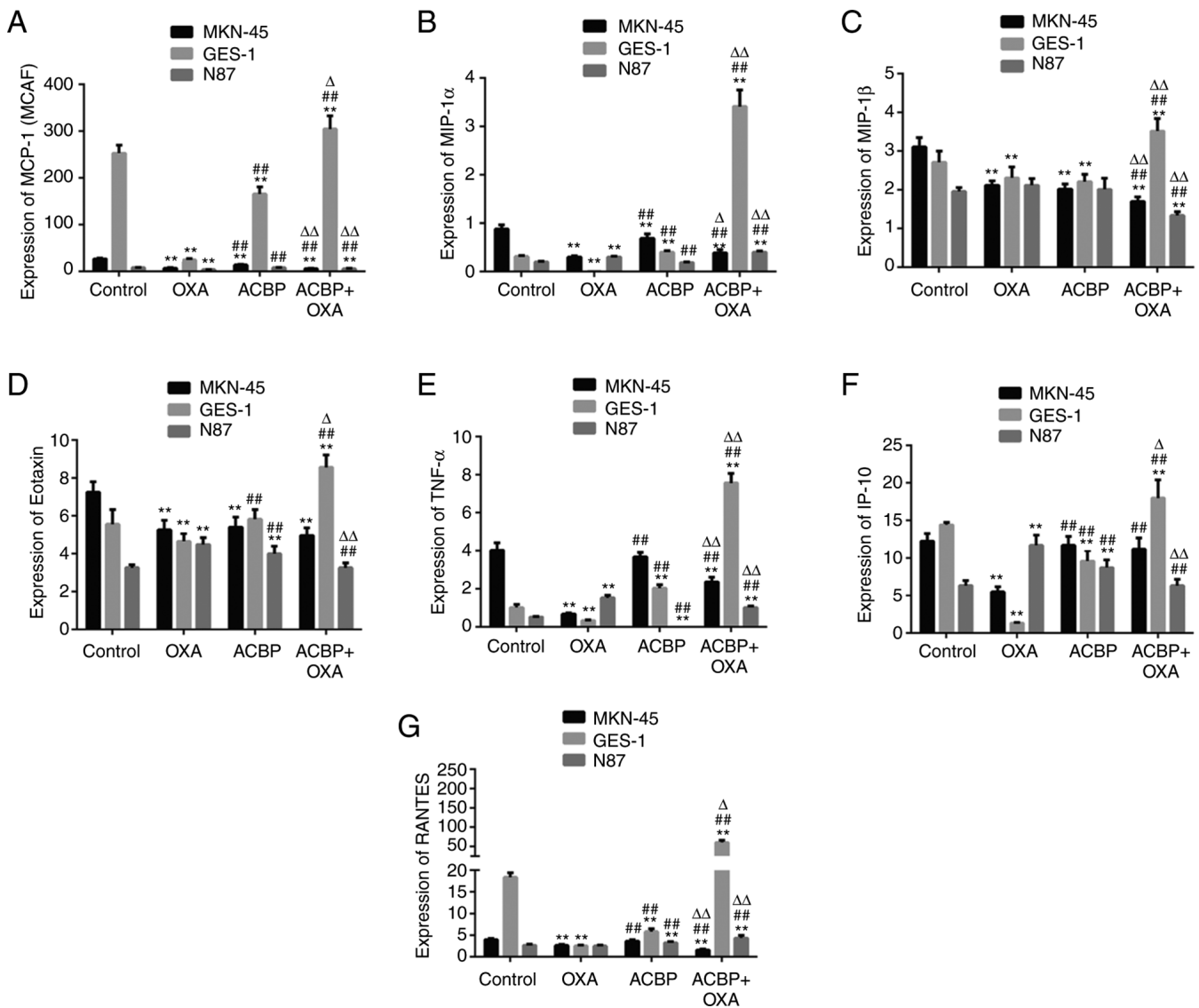


Figure 3. Expression of chemotactic factors. The levels of (A) MCP-1, (B) MIP-1α, (C) MIP-1β, (D) Eotaxin, (E) TNF-α, (F) IP-10 and (G) RANTES in the supernatants of GES-1, MKN-45 and N87 gastric cancer cells were decreased after treatment with ACBP or ACBP combined with OXA. \*\*P<0.01 vs. control group; ##P<0.01 vs. OXA group, ΔP<0.05, ΔΔP<0.01 vs. ACBP group. MIP, macrophage inflammatory protein; OXA, oxaliplatin; ACBP, anticancer bioactive peptide; MCP, monocyte chemoattractant protein; IP-10, interferon-γ-induced peptide 10; RANTES, regulated upon activation, normal T cell expressed and presumably secreted.

after ACBP treatment. Furthermore, the MCP-1 (MCAF), MIP-1β, IP-10 and RANTES levels were significantly decreased, while the MIP-1α, Eotaxin and TNF-α levels were significantly decreased in the supernatants of GES-1 cells after ACBP treatment (P<0.01).

Compared with ACBP, the levels of MCP-1 (MCAF), MIP-1α, MIP-1β, TNF-α and RANTES were shows significantly difference in the supernatants of GES-1, MKN-45 and N87 cells after ACBP combined with OXA treatment (P<0.01). The MCP-1 (MCAF), MIP-1β, Eotaxin levels were significantly decreased in the supernatants of MKN-45 and N87 cells after ACBP combined with OXA treatment (P<0.01). The MIP-1α, TNF-α and RANTES levels were significantly increased in the supernatants of N87 cells after combination treatment (P<0.01). However, the levels of IP-10 and Eotaxin showed no significant difference after combination treatment in MKN-45 cells.

Compared with OXA, the levels of MCP-1 (MCAF), MIP-1α, MIP-1β, TNF-α and RANTES were significantly increased significantly in the supernatants of GES-1 and decreased in the MKN-45 by ACBP combined with OXA treatment (P<0.01). Furthermore, the Eotaxin levels in the supernatants of GES-1 were significantly increased and decreased in the supernatants of N87 cells after combined treatment compared with OXA (P<0.01). The level of IP-10 in the supernatants of MKN-45 and GES-1 was significantly increased (P<0.01), while it exhibited no significant difference in N87 cells after the ACBP and OXA combination treatment, compared with the OXA group.

## Discussion

The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the proinflammatory cytokine



response. Major anti-inflammatory cytokines include IL-1RA, IL-4, IL-6, IL-10, IL-11 and IL-13.

IL-2 is an important immune modulator synthesized and released after activation by lymphocytes by specific antigen stimulation (28). IL-2 has antitumor effects and it binds to the IL-2 receptor on the cell membrane. IL-2, the first cytokine that was molecularly cloned, was shown to be a T-cell growth factor essential for the proliferation of T cells and the generation of effector and memory cells (29). IL-4 is also one of the key cytokines in tumor immunity. IL-4 causes the body to eliminate tumors. In different ways, IL-4 leads to weakened T cell-mediated immune function, promoting tumorigenesis and progression. IL-13 is a multipotent proinflammatory cytokine with a molecular weight of ~12 kDa that is mainly secreted by Th2 cells. The results of the present study indicated that the IL-13 levels in MKN-45 cells did not change significantly after ACBP treatment, but the IL-13 levels in MKN-45 cells decreased after the combination treatment. The levels of IL-13 in the N87 gastric cancer cell line were decreased after ACBP or combination treatment. The levels of IL-13 in GES-1 cells were reduced after ACBP treatment, with no significant change in the IL-13 levels in GES-1 cells after the combination treatment.

The IL-4 and IL-13 may have a significant role in the downregulation of inflammatory processes underlying RA pathology and beneficially modulate the course of the disease (30).

IL-6 is a prototypical cytokine for maintaining homeostasis (31). The IL-6 cooperative factor granulocyte colony-stimulating factor (G-CSF) can alter neutrophil function before neutrophils enter the tumor microenvironment. Neutrophils regulated by G-CSF/IL-6 promote tumor angiogenesis before entering the tumor microenvironment, so IL-6 can promote tumorigenesis and progression (32). IL-10 is an important immunosuppressor in the process of tumor development. It can exert its immunosuppressive role by suppressing the functions of lymphocytes, macrophages, dendritic cells and other immune cells so that tumors can develop through immune escape (33). The cytokine IL-10 is a key anti-inflammatory mediator ensuring protection of a host from over-exuberant responses to pathogens and microbiota, while having important roles in other settings such as sterile wound healing, autoimmunity, cancer and homeostasis (34).

IL-7 is a cytokine that is mainly secreted by thymocytes, bone marrow stromal cells, small intestinal epithelial cells and skin keratinocytes during the normal development of the human immune system. IL-7 has an important role in maintaining human immune system homeostasis (35). In addition, it can induce the growth and proliferation of hematopoietic cells and hematological malignant tumor cells (such as leukemia and lymphoma).

As a cytokine and immunological modulatory factor, IL-12 has a direct antagonistic effect on tumors and has an important role in the immune response of both primary and secondary tumors (36). The results of the present study showed that the levels of IL-12 were significantly increased after treatment of MKN-45 cells with ACBP, but IL-12 levels decreased after the combination treatment. The anticancer biological activity and the levels of IL-12 in the supernatant were decreased in the N87 gastric cancer cell line.

GM-CSF was originally identified as a growth factor due to its ability to promote the proliferation and differentiation of bone marrow progenitor cells into granulocytes and macrophages *in vitro* (37). It can not only stimulate the generation of blood cells such as macrophages but also induce humoral and cellular immunity, thus increasing the bactericidal activity of effector cells in natural immunity. GM-CSF, as an immune adjuvant for melanoma vaccines, was more durable and more effectively enhanced the immune effect of tumor vaccines relative to other cytokines (IL-4, IL-6, etc.). Considering the well-characterized antitumor effects of this cytokine, many clinical trials and immunotherapy approaches have been designed to enhance IFN- $\gamma$ -mediated immunity for different types of cancer (38). The results of this experiment showed that the GM-CSF and IFN- $\gamma$  levels were decreased in MKN-45 and N87 cells after ACBP and OXA combined treatment.

Cancer development and its response to therapy are regulated by inflammation, which either promotes or suppresses tumor progression, potentially displaying opposing effects on therapeutic outcomes. Chronic inflammation facilitates tumor progression and treatment resistance, whereas induction of acute inflammatory reactions often stimulates the maturation of dendritic cells and antigen presentation, leading to anti-tumor immune responses (39). IL-1 $\beta$  is a strong inhibitor of gastric acid secretion that contributes to *H. pylori* invasion, leading to more severe gastritis, and it may have a role in gastric atrophy as well as gastric adenocarcinoma progression (40). The results of the present study showed that the levels of IL-1 $\beta$  in the supernatants of the MKN-45 and N87 gastric cancer cell lines were decreased. However, the levels in the GES-1 cells were increased after treatment with the combination of ACBP and OXA. IL-1RA is able to inhibit inflammation and gastric cancer development (41). Research has indicated that stomach-specific expression of human IL-1 $\beta$  in transgenic mice leads to spontaneous gastric inflammation and cancer that correlate with early recruitment of myeloid-derived suppressor cells to the stomach. The same study showed that the levels of IL-1RA were decreased in the MKN-45 and increased in the N87 gastric cancer cell lines. Therefore, ACBP may not inhibit the growth of cancer cells by affecting the expression level of this factor. In the present study, the levels of IL-1RA in GES-1 cells increased significantly after treatment with a combination of ACBP and OXA.

High expression of IL-8 in precancerous lesions and gastric cancer is not only associated with the occurrence, development and division of gastric cancer but also related to the progression of gastric cancer. The expression of IL-8 is 10 times higher in gastric cancer than in normal tissues and is not related to the pathological histotype of gastric cancer. Meta-analysis results provided evidence that the IL-8-251A>T polymorphism is significantly associated with an increased risk of gastric carcinogenesis in Asian populations (42). The results of the present study showed that the levels of IL-8 in the supernatants of the MKN-45 gastric cancer cell line were significantly increased after ACBP treatment, and these levels were higher than those observed after the MKN-45 cell line was treated with the drug combination. IL-8 levels were increased in N87 cells treated with the drug combination, while there were no significant changes after treatment with ACBP alone. The IL-8 levels in GES-1 cells were decreased

after ACBP treatment and the IL-8 levels were significantly increased after treatment with the combination.

IL-9 is involved in mediating the association between tumor cells and nonmalignant inflammatory infiltrating cells (43). The results of the present study showed that the levels of IL-9 in the supernatants of the MKN-45 and N87 gastric cancer cell lines were decreased after treatment with ACBP alone or the combination. Treatment of GES-1 cells with ACBP caused no significant change in IL-9 expression, while IL-9 expression increased after the combination treatment of GES-1 cells.

IL-15 is an important cytokine that promotes the proliferation, activation and survival of NK and CD8<sup>+</sup> T cells (44). The IL-15 domain activates NK and CD8<sup>+</sup> T cells and is thus used for the treatment of tumors. The results of the present study showed that the levels of IL-15 in MKN-45, GES-1 and N87 cells were decreased after ACBP and the combination treatment.

IL-17 is known as a Th17-cell-derived proinflammatory cytokine (45). Increased expression of IL-17 in tumor tissues can have an oncogenic role by promoting the generation of blood vessels in tumor tissues (46). How IL-17 inhibits tumors and the specific mechanisms of the antitumor effects of IL-17 remain to be fully elucidated. It may also promote the formation of blood vessels in tumor tissues and have a tumor-promoting role. The results of the present study showed that the levels of IL-17 were decreased after anticancer bioactive peptides were administered to MKN-45 and N87 cells, and the levels of IL-17 in MKN-45 and N87 cells were significantly decreased after the combination treatment. Treatment of the GES-1 cells decreased IL-13 expression, while IL-13 expression was increased in GES-1 cells upon combination treatment. bFGF is a pleiotropic growth factor that promotes growth of mesenchymal and epithelial cells, and stimulates angiogenesis and neuroprotection (47). bFGF is a polypeptide factor that, in addition to its pro-proliferative effect on fibroblasts and vascular endothelial cells, is involved in tumor development, particularly tumor invasion and metastasis. bFGF promotes the growth and development of tumor blood vessels and accelerates tumor growth. Studies have shown that PDGF-BB promotes endothelial cell proliferation and neovascularization, promoting tumor growth (48). It has an important role in promoting the emergence of new lymphatic vessels and the lymphatic metastasis of tumors. The present results showed that the expression levels of PDGF-BB were decreased in MKN-45 and M87 cells after treatment with ACBP combined with OXA. Furthermore, the levels of bFGF were decreased after the ACBP and the combination treatment were applied to MKN-45 and N87 cells. The levels of bFGF were decreased after treatment of the normal gastric cell line GES-1 with ACBP, whereas the bFGF levels were significantly increased in GES-1 cells after combination treatment.

Molecular biological studies have confirmed that Eotaxin-1 can promote epithelial-mesenchymal transition by activating the downstream MAPK kinase 1, ERK1/2 and transducer and activator of transcription 3 signaling pathways, thus increasing the invasion and metastasis abilities of tumor cells (49). The results showed that the Eotaxin-1 levels in MKN-45 cells were decreased after ACBP or combination treatment.

Studies have reported that the diagnostic accuracy of IP-10 is on par with that of IFN- $\gamma$  (50). IP-10 promotes the

production of IL-17 and IFN- $\gamma$  and promotes the migration and differentiation of uterine decidual T cells into Th1 cells and Th17 cells (25). As a chemokine, IP-10 is a protein whose expression is induced by interferon stimulation, and it may be inferred that IP-10 secretion is closely related to surgical trauma. With postoperative injury repair, IP-10 levels gradually decrease but are still higher than normal levels, suggesting that IL-8 and IP-10 participate in local inflammatory infiltration and tissue damage in HCC. MCP-1, also known as C-C-motif chemokine ligand 2, is a member of the family of CC chemokines (51). Because *in vitro*-cultured tumor cells often produce significant amounts of MCP-1, tumor cells are considered to be the main source of MCP-1. MCP-1 production in tumors is a consequence of complex interactions between tumor cells and non-tumor cells (52). MIP-1 $\alpha$  is a member of the chemokine family and was originally determined to be a soluble factor secreted by activated macrophages (53). MIP-1 $\alpha$  has a strong chemotactic capacity for monocytes, giant warm cells and lymphocytes, and higher expression of adhesion molecules and additional related cytokines may inhibit the growth of tumor cells through activation of the release of lysozyme. The present results showed that the levels of MIP-1 $\alpha$  were decreased in MKN-45 cells after ACBP and combination drug treatment.

TNF- $\alpha$ , IL-1 $\alpha$  and IL-1 $\beta$  have pleiotropic properties. Both cytokines are now known to be potent inducers of a number of cell-selective chemotactic cytokines, which belong to a novel superfamily of structurally related low-molecular-weight proteins. One of the most prominent members is termed IL-8 and represents a neutrophil-selective attractant, whereas another one called MCP-1 is a monocyte-selective chemotaxin (54). The expression of chemotactic cytokines, i.e., MCP-1, MIP-1 $\beta$ , RANTES and TNF- $\alpha$ , is closely linked with tumor progression and metastasis due to their overexpression and the subsequent induction of chemoresistance (55). MIP-1 $\beta$ , an inflammatory cytokine, has a cancer-promoting effect, but high concentrations can induce tumor apoptosis. The expression of RANTES is significantly enhanced in numerous malignant tumor tissues (56). RANTES directly acts on tumor cells through paracrine mechanisms and it promotes tumor development, enhances the tumor invasion ability, promotes tumor blood vessel formation, suppresses the cellular immune responses and promotes tumor growth (55). TNF- $\alpha$  is mainly secreted by activated macrophages and can also be produced by tumor cells, and TNF- $\alpha$  exerts direct antitumor effects (57). Inhibition of VEGF has been confirmed to be an efficacious antiangiogenetic approach for cancer treatment (58). Its main effect is to promote neovascularization in the tumor body and the immune suppression of the body.

The present results showed that the levels of MIP-1 $\beta$ , RANTES, VEGF and TNF- $\alpha$  in MKN-45 cells were decreased after treatment with ACBP combined with OXA. However, the use of only one assay is a limitation of the present study. However, the anti-cancer effects of these 25 cytokines are challenging to explain/discuss due to the lack of tests dedicated to proliferation and cell interactions, which is also a limitation of the present study. Future studies should include apoptosis, signaling pathway analysis or the behavior of immune cells (e.g., in co-culture).



In conclusion, in the present study, the levels of growth factors, immune regulatory factors and chemokines that were secreted after treatment with ACBP or ACBP+OXA were quantified. An analysis of immune regulatory factors (IL-2, IL-4, IL-7, IL-10, IL-12, IL-13, IFN- $\gamma$ , VEGF and GM-CSF), growth factors (IL-1RA, IL-1 $\beta$ , IL-8, IL-9, IL-15, IL-17, bFGF and PDGF-BB) and chemokines [MCP-1 (MCAF), IP-10, Eotaxin, MIP-1 $\alpha$ , MIP-1 $\beta$ , TNF- $\alpha$  and RANTES] was performed and cytokines and growth factors from different gastric cancer cells that can induce humoral and cellular immunity and have anticancer biological activity were identified. These findings support the use of the combination of ACBP and OXA for gastric cancer treatment to inhibit tumorigenesis and progression via the cytokines MCP-1, MIP-1 $\beta$  and IL-13. Therefore, the mechanism by which gastric cancer cell proliferation and neovascularization are promoted and tumor growth is driven will allow us to understand and improve clinical outcomes.

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### Availability of data and materials

All data generated or analysed during this study are included in this published article.

### Authors' contributions

XLS and XL conceived and designed the study. JQP conducted experiments and contributed new reagents or analytical tools. XL analyzed data and wrote the manuscript. XLS and JQP checked and approved the authenticity of the raw data. All authors have read and approved the manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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