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Inhibitory effect of a disulfide stabilized human double chain antibody against bFGF combined with cisplatin on ovarian cancer

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Abstract

The basic fibroblast growth factor (bFGF) is related to the cell replication, tumour cell migration and chemoresistance. Blocking the effect of bFGF is also a research focus of tumor therapy. Therefore, this study analyzed the growth inhibition of a disulfide stabilized human double chain antibody against bFGF (ds-Diabody anti bFGF) combined with cisplatin on cisplatin-resistant human ovarian cancer cells (SKOV3/DDP) and human ovarian cancer cells (SKOV3). The ds-Diabody anti bFGF combined with cisplatin can inhibit the proliferative capacity, migratory ability and invasiveness of ovarian cancer cells, and promote apoptosis. At the same time, the antitumor activity of combination therapy was better than that of cisplatin. The combination therapy can inhibit the phosphorylation of Akt and MAPK, regulate the expression of N-cadherin and E-cadherin. In addition, in the mouse tumour model of ovarian cancer, The combination therapy can effectively inhibit tumor growth. The tumor growth inhibition rate could reach 68%. The ds-Diabody anti bFGF had no side effect. This study stated that ds-Diabody anti bFGF could reduce the amount of cisplatin and decrease the side effects of cisplatin in tumour therapy.

Keywords

bFGF; ds-Diabody; cisplatin resistant; ovarian cancer.

1. Introduction

Ovarian cancer is the seventh most common cancer among women in the world, accounting for nearly 4% of all new cancer cases in women. The number of cases is still increasing, and the five-year survival rate is less than 45%[1]. At present, the conventional first-line chemotherapy for ovarian cancer is cisplatin-based combined chemotherapy, but the limitation of this treatment is that the tumor will be resistant to cisplatin[2]. The mechanism of cisplatin resistance is very complex. How reverse cisplatin resistance is still a difficult problem in many cancer treatments. Many studies have shown that bFGF is closely related to cisplatin resistance, and blocking the effect of bFGF can increase the sensitivity of cancer cells to cisplatin[3-5].

BFGF is overexpressed in a variety of malignant tumors and overexpression of bFGF often predicts poor prognosis and low survival[6, 7]. It stimulates cell proliferation and migration by binding and activating fibroblast growth factor receptors[8, 9]. BFGF can continuously activate MAPK/ERK signal transduction pathway, thereby enhancing the proliferation and invasion ability of tumor cells and enabling tumor cells to acquire the characteristics of metastasis[10, 11]. Therefore, blocking bFGF related signaling pathway is considered to be a feasible anti-tumor strategy.

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Studies have shown that bFGF blockers have significant inhibitory effects on a variety of tumors. BFGF/FGFR blockers can reverse the resistance of tumor cells to BET protein inhibitors, thus achieving the effect of inhibiting uveal melanoma[12]. Antibody P7 targeting bFGF can inhibit the growth of breast cancer[13]. The monoclonal antibody against bFGF can inhibit the proliferation of melanoma cells and the growth of melanoma in vitro by promoting the apoptosis of tumor cells[14, 15]. In this study, ds-Diabody antit bFGF was constructed in our laboratory and expressed in Pichia pastoris (GS115)[16]. Different from traditional antibody drugs, ds-Diabody anti bFGF is a small molecule antibody drug with strong tissue permeability. In addition, unlike scFv, ds-Diabody anti bFGF has two antigen binding sites and disulfide bonds between the two peptide chains, resulting in a stronger binding activity to bFGF and a longer half-life period[15, 17-19]. These characteristics make ds-Diabody anti bFGF have significant antitumor activity.

In this study, the inhibitory effect of ds-Diabody antit bFGF combined with cisplatin on the growth of SKOV3/DDP cells and SKOV3 cells were evaluated in vitro and in vivo, including the proliferation, migration, and invasion ability of SKOV3/DDP cells and SKOV3 cells in vitro, the apoptosis, and related signaling pathways and the tumor growth inhibiton of SKOV3/DDP cells and SKOV3 cells in vivo. The purpose of this study was to explore the anti-tumor activity of ds-Diabody anti bFGF on ovarian cancer and whether ds-Diabody anti bFGF combined with cisplatin could have a better inhibitory effect on ovarian cancer.

2. Materials and Methods

2.1. Cells and Animals

The SKOV3/DDP cells were bought from Wuhan prosaic Co., Ltd, China, which are resistant to diphtheria toxin, cisplatin, and adriamycin. The SKOV3 cells were from our lab. The DMEM medium added with 1% streptomycin / penicillin and 10% fetal bovine serum is the cells nutrition source and the ambient temperature is 37 ℃.

BALB/c nude mice (SPF grade, four week-old, female) were bought from Beijing Huafukang Co. Ltd, China. The mice were raised in the animal center of Jinan University, China. The mice experiments were supervised by the Laboratory Animal Management Center of Jinan University, China.

2.2. The expression of ds-Diabody anti bFGF in shake flask

The pichia pastoris which expressed ds-Diabody anti bFGF were constructed and kept in our lab[16]. The recombinant yeasts were cultivated in YPG medium (containing 1% glycerol, 1% yeast extract, and 2% tryptone) and cultured in a constant temperature shaking table at 28 ℃ and 240 rpm/min for 48 hours. Then the old YPG medium was replaced with a YPM medium (containing 1% methanol, 1% yeast extract, and 2% tryptone). Every 24 h, 1% methanol was added to the medium and the fermentation was conducted for 96 h.

2.3. Protein purification

The fermentation supernatant was precipitated by saturated ammonium sulfate, dialyzed with phosphate buffer, and purified with a nickel column. The elution was performed using 20, 50, 150, and 500 mM imidazole, respectively.

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2.4. Western blot analysis

After electrophoresis, the protein was shifted to the PVDF membranes. The membranes were incubated in 5% skim milk at 37 ℃ for two hours. The PVDF membranes were incubated in the liquid of anti-myc/c-myc mouse monoclonal antibody (1:2000) at 4 ℃ for 12 h, and in the solution of horseradish peroxidase goat anti-mouse IgG antibody with HRP label (1:4000) at 37 ℃ for 40 min. The PVDF membranes were stained with ECL luminescent solution and visualized using a gel imaging system.

2.5. Cell proliferation test

The functions of cisplatin and ds-Diabody anti bFGF on ovarian cancer cells proliferation was tested by Cell Counting Kit-8 (Biosharp, China) experiment. The ovarian cancer cells (3000 cells per well) were added in 96 well cell culture dish for 12 h. Then the cells were starved for 12 h (0.5% FBS), and the tumour cells were cultured in medium added with 10 ng/mL bFGF. These cells were treated in different experimental groups for 48 h. According to the instructions, the proliferation of tumour cells was analysed by CCK-8 kit, and the OD450 value was detected with microplate reader (BioTek Instruments Co., Ltd, USA), Experimental groups: (1) ds-Ddiabody against bFGF group alone (6.25, 12.5, 25, 50, 100 μg/mL); (2) cisplatin group alone (1, 2, 4, 8, 16 μg/mL); (3) ds-Diabody anti bFGF (6.25, 12.5, 25, 50, 100 μg/mL) separately combined with cisplatin (1, 2, 4, 8, 16 μg/mL); (4) negative control group (equal volume of PBS); (5) blank control group (no inoculated cells).

2.6. Clone formation experiment

The proliferation influence of ds-Diabody anti bFGF on ovarian cancer cells was evaluated by clone formation experiment. SKOV3/DDP cells and SKOV3 cells (1000 cells per well) were cultured in medium and these cells were treated in different experimental groups for 7 days. Then these cells were soaked in 4% paraformaldehyde, dyed by crystal violet, photographed, and counted with Image J software. Experimental groups: (1) ds-Ddiabody against bFGF group alone (final concentration: 100 μg/mL); (2) cisplatin group alone (final concentration: 2 μg/mL); (3) combination therapy (100 μg/mL ds-Diabody anti bFGF combined with 2 μg/mL cisplatin); (4) negative control group (equal volume of PBS).

2.7. Migration and invasion experiment

Ovarian tumour cells were starved for 12 h. The transwell chamber with an 8 μm filter was placed in a 24-well plate. The cells (12000 cells per well) were inoculated into the chamber, and cultured in a medium without fetal bovine serum added with 10 ng/mL bFGF. These cells were treated in different experimental groups for 24h. Then these cells were soaked in 4% paraformaldehyde, dyed by crystal violet, photographed with a microscope, and counted by

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Image J software. The experimental groups were designed to be the same as the clone formation experiment.

For the invasion experiment, the Matrigel matrix diluted 30 times by DMEM medium was added to the Transwell chamber with an 8 μm filter. The cells (20000 cells per well) were inoculated into the chamber, and other experimental procedures were designed to be the same as the migration assay.

2.8. Apoptosis assay

Ovarian cancer cells (200000 cells per well) were treated in different experimental groups for 24 h. According to the instructions of the apoptosis kit (Dojindo, Japan), the apoptosis of tumor cells were analysed by annexin V and FITC/PI fluorescence. The experimental groups were designed to be the same as the clone formation assay.

2.9. Mice model of transplanted tumor

BALB/C nude mice （n=5） were stochastically separate into four groups (1) ds-Ddiabody against bFGF group (5 mg/kg ds-Ddiabody against bFGF was injected through a caudal vein); (2) cisplatin group (2 mg/kg cisplatin was injected intraperitoneally); (3) ds-Diabody anti bFGF combined with cisplatin group; (4) negative control group (normal saline). SKOV3/DDP cells and SKOV3 cells (3×10^6 cells per mouse) were injected into the dorsum of mice. After the tumour grew, the mice were injected according to the groups for 6 times at an interval of 3 days. At the end of the treatment, euthanasia was performed on all experimental animals. The tissue sections of the organs of the mice were stained with hematoxylin-eosin (HE stain) to detect the pathological changes in the organs.

2.10. Statistical analysis

One way ANOVA was used to compare the differences between the experimental groups. $P<0.05$ was considered statistically significant.

3. Results

3.1. The expression and purification of ds-Diabody anti bFGF

The biomass and target protein production were accumulated continuously with the process of the shake flask experiment. At the 96th hour of the shake flask, the cell OD600 value was 15.59, and the cell wet weight reached 116.67 g/L (Fig. 1A). The yield of the ds-Diabody anti bFGF (with a relative molecular mass of about 35 kDa) reached 22.5 mg/L (Fig. 1B). The Expression supernatant was precipitated by ammonium sulfate, dialyzed, and further purified by nickel ion affinity chromatography column. It was shown that the target protein was eluted under the condition of 150 mm imidazole, and the purity of the purified target product was over 90% (Fig. 1C).

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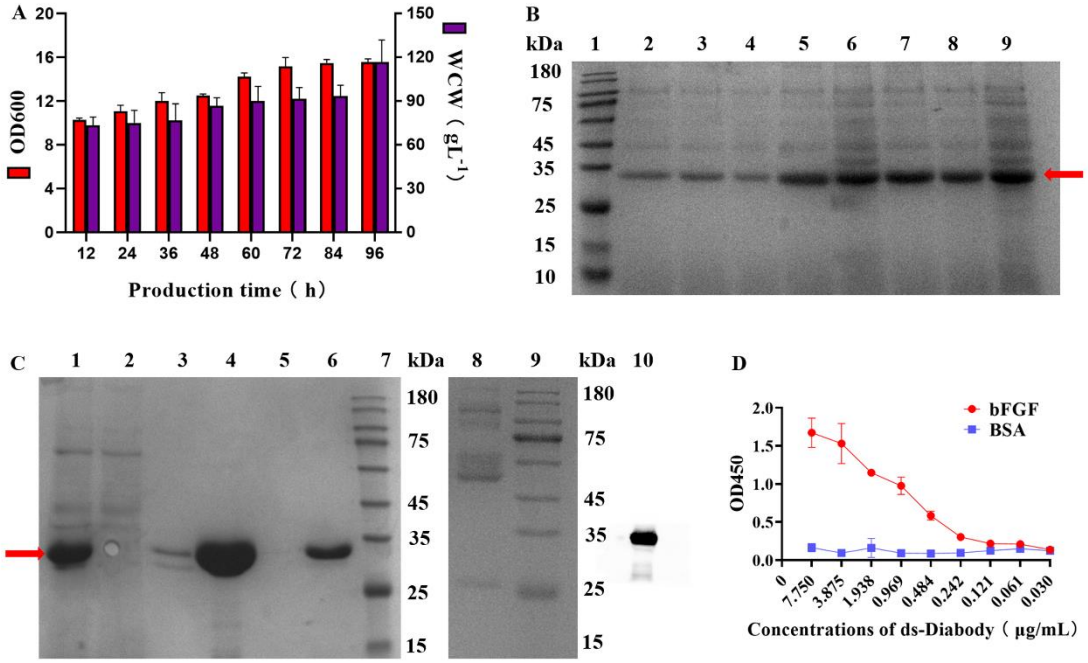

Fig. 1 The expression, purity, binding specificity and activity of target protein. (A) The cell density OD600 value and wet cell weight (WCW) increased with the extension of induction time in shake flask fermentation. (B) SDS-PAGE of the target protein expression during fermentation. Lane1, protein standard; lanes 2-9, the expression of ds-Diabody anti bFGF at 12, 24, 36, 48, 60, 72, 84, 96 h, respectively. The target protein was about 35 kDa and indicated by red arrows. (C) SDS-PAGE of the target protein purification by nickel column. Lane1, proteins precipitated by saturated ammonium sulfate; lanes 2-5, proteins eluted with 20, 50, 150, 500 mM imidazole, respectively; lane 6, the purified ds-Diabody under reduction condition; lane 7, molecular weight marker; lane 8, the purified ds-Diabody anti bFGF with non-reduction condition; lane 9, molecular weight marker; lane 10, Western blot analysis of the purified target protein. (D) ELISA of the bFGF binding specificity and activity of ds-Diabody. BSA served as the negative control.

3.2. Analysis of antigen conjugating specificity and activity of ds-Diabody anti bFGF

The results of ELISA showed that the binding activity between ds-Ddiabody and BSA was very low. When the dilution ratio of ds-Diabody was 1:32000 (0.969 μg/mL), OD450 was 1 (Fig. 1D). The experiments stated that the ds-Diabody showed high conjugating specificity and activity with bFGF.

3.3. ds-Diabody anti bFGF combined with cisplatin control the proliferation of ovarian tomour cells

The effects of ds-Diabody anti bFGF, cisplatin, and the combination on SKOV3/DDP and SKOV3 cell proliferation in vitro were analyzed by CCK-8 experiment. The experimentation stated that cisplatin had inhibitory effects on ovarian tomour cells (Fig. 2A). However, the inhibition rate of cisplatin on SKOV3 cells is higher than that of SKOV3/DDP cells because SKOV3/DDP cells was resistant to cisplatin. Besides, ds-Diabody anti bFGF also had inhibitory effects on ovarian tomour cells (Fig. 2B). It is worth noting that the inhibitory effect of combination therapy was more obvious than that of cisplatin (Fig. 2C, D). The experiments stated that combination therapy can reduce the dosage of cisplatin, and then reduce the side effects of cisplatin.

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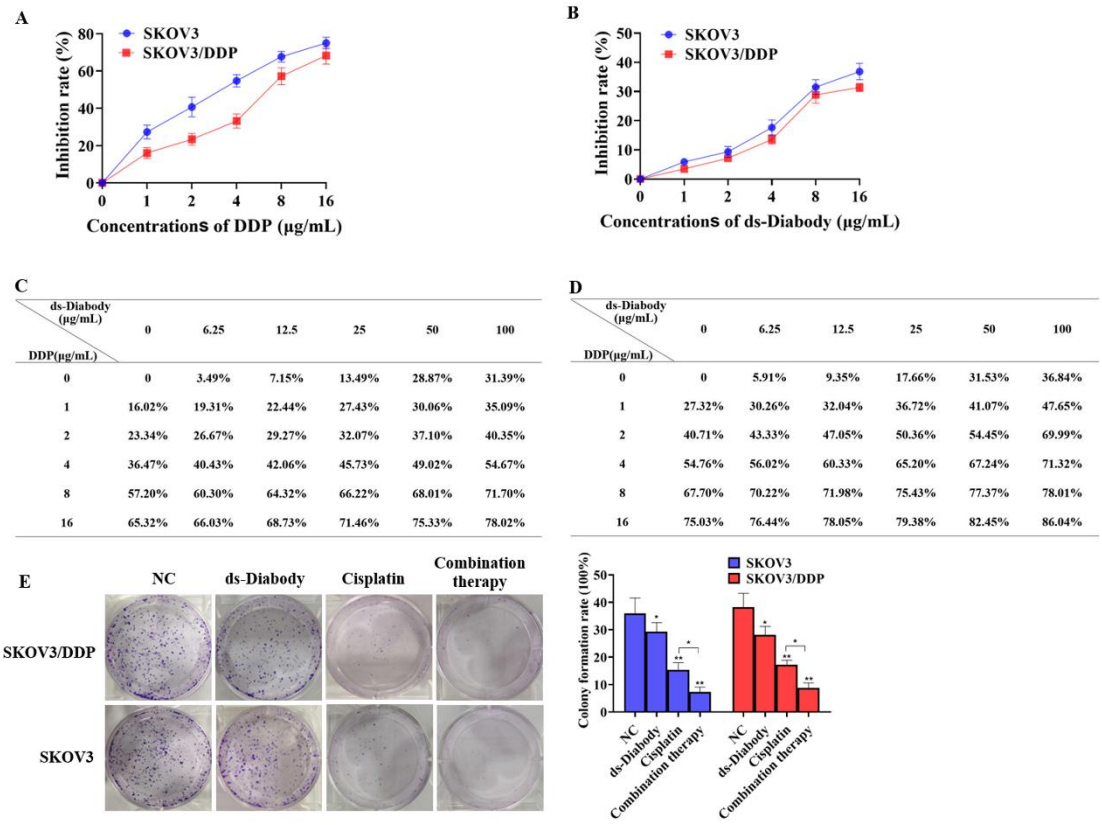

Fig. 2 The inhibiting functions of ds-Diabody anti bFGF on the proliferation of ovarian tumour cells. (A) The effect of cisplatin on ovarian tumour cells. (B) The inhibiting functions of ds-Diabody anti bFGF on ovarian tumour cells. (C) The inhibition of combination therapy on SKOV3/DDP cells. (D) The inhibition of combination therapy on SKOV3 cells. (E) Clone formation test of combination therapy on ovarian tumour cells. *P < 0.05, **P < 0.01.

3.4. ds-Diabody anti bFGF combined with cisplatin inhibited the clonogenicity of ovarian cancer cells

Cell clonogenicity assay was used to detect the influences of ds-Diabody anti bFGF combined with cisplatin on SKOV3/DDP and SKOV3 proliferation ability. The results of tests indicated thatovarian tomour cells could form clonal clusters after the treatment of cisplatin, ds-Diabody, and combination therapy. The clone formation rates of SKOV3/DDP cells were 38.2% in the negative control group, 28.1% in the ds-Diabody group, 17.2% in the cisplatin group and 8.8% in the combination therapy group. The clone formation rates of SKOV3 cells were 35.9% in the negative control group, 29.3% in the ds-Diabody group, 15.3% in the cisplatin group and 7.3% in the combination therapy group (Fig. 2E). Cisplatin had a strong inhibition function on the clone formation of ovarian tomour cells. Although SKOV3/DDP has a certain resistance to cisplatin, cisplatin can still inhibit the clonal formation of SKOV3/DDP in vitro. The ds-Diabody anti bFGF also had inhibition functions of clonal formation on ovarian tumour cells. We also found that the inhibition functions of combination therapy on ovarian cancer cells was more obvious than that of cisplatin. The experimentation stated that combination therapy could reduce the cloning rate of ovarian tumour cells and reduce the dosage of cisplatin.

3.5. ds-Diabody anti bFGF combined with cisplatin controled the migration of tumor cells

The migration experiment results showed that ovarian tumour cells migrated to different degrees after treatment with different drugs (Fig. 3A). The migration rate of SKOV3/DDP cells was 64.09% in the ds-Diabody group, 54.70% in cisplatin group and 49.72% in combination

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therapy group （The negative control group was set to 100%）. The migration rate of SKOV3 cells was 79.14% in the ds-Diabody group, 37.42% in the cisplatin group, and 28.22% in a combination therapy group. The results indicated that combination therapy could control the migration of ovarian tumour cells, and the Inhibition function of combination therapy was stronger than that of cisplatin.

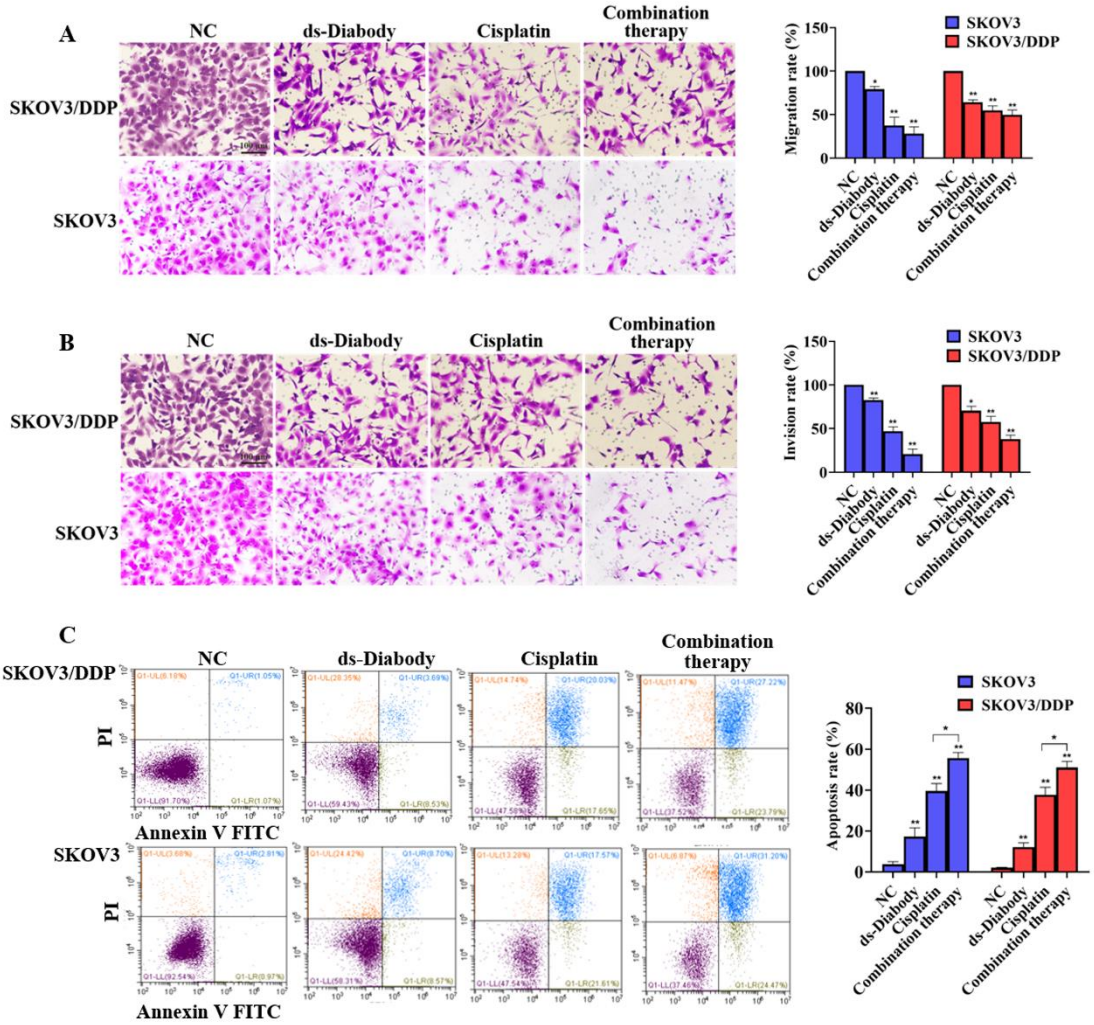

Fig. 3 The migration inhibition, invasion inhibition and apoptosis promotion of ds-Diabody anti bFGF on ovarian tumour cells. (A) The migration inhibiting functions of ds-Diabody anti bFGF on SKOV3/DDP cells and SKOV3 cells. Scale: 100 μm. (B) The invasion inhibiting functions of ds-Diabody anti bFGF on SKOV3/DDP cells and SKOV3 cells. Scale: 100 μm. (C) The apoptosis promoting functions of ds-Diabody anti bFGF in ovarian tumour cells. *P < 0.05, **P < 0.01.

3.6. ds-Diabody anti bFGF combined with cisplatin controled the invasion of tumor cells

The invasion experiment results stated that ovarian tumour cells both had different degrees of invasion after the treatment with ds-Diabody anti bFGF and cisplatin (Fig. 3B). The rate of invasion in SKOV3/DPP cells was 70.47% in ds-Diabody group, 57.51% in cisplatin group and 37.82% in a combination therapy group. The rate of invasion in SKOV3 cells was 82.51% in the ds-Diabody, 46.99% the in the cisplatin group, and 20.77% in a combination therapy group. Similarly, combination therapy could control the invasiveness of ovarian tumour cells, and the inhibitory effect of combination therapy was stronger than that of cisplatin alone.

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3.7. ds-Diabody anti bFGF combined with cisplatin promoted apoptosis of tumor cells

The apoptosis effect of combination therapy on SKOV3/DDP cells and SKOV3 cells was tested with flow cytometry using Annexin/PI double-stained method. The results showed that ovarian cancer cells had different degrees of apoptosis after the treatment with ds-Diabody anti bFGF and cisplatin (Fig. 3C). The rate of apoptosis in SKOV3/DDP cells was 2.12% in the negative control group, 12.22% in ds-Diabody group, 37.68% in cisplatin group and 51.01% an in combination therapy group. The rate of apoptosis in SKOV3 cells was 3.78% in the negative control group, 17.27% in the ds-Diabody group, 39.18% in the cisplatin group, and 55.67% in a combination therapy group. The combination therapy could promote the apoptosis of ovarian tumour cells, but combination therapy could significantly promote apoptosis than that of cisplatin.

3.8. Western blot assay of related signal pathways

To further explore the inhibition functions of ds-Diabody on the bFGF signal, the expression of p-MAPK and p-Akt was detected with western blot. Experimentation stated that combination therapy could inhibit the expression of p-MAPK and p-Akt on ovarian cancer cells (Fig. 4A, B).

Epithelial-mesenchymal transition (EMT) is the initial cause of tumor metastasis, while N-cadherin and E-cadherin are closely related[[20]]. The results of western blot displayed that combination therapy could reduce the expression of N-cadherin protein and raise the expression of E-cadherin protein in ovarian cancer cells, which suggested that ds-Diabody anti bFGF combined with cisplatin might inhibit the EMT process of tumor cells (Fig. 4C, D).

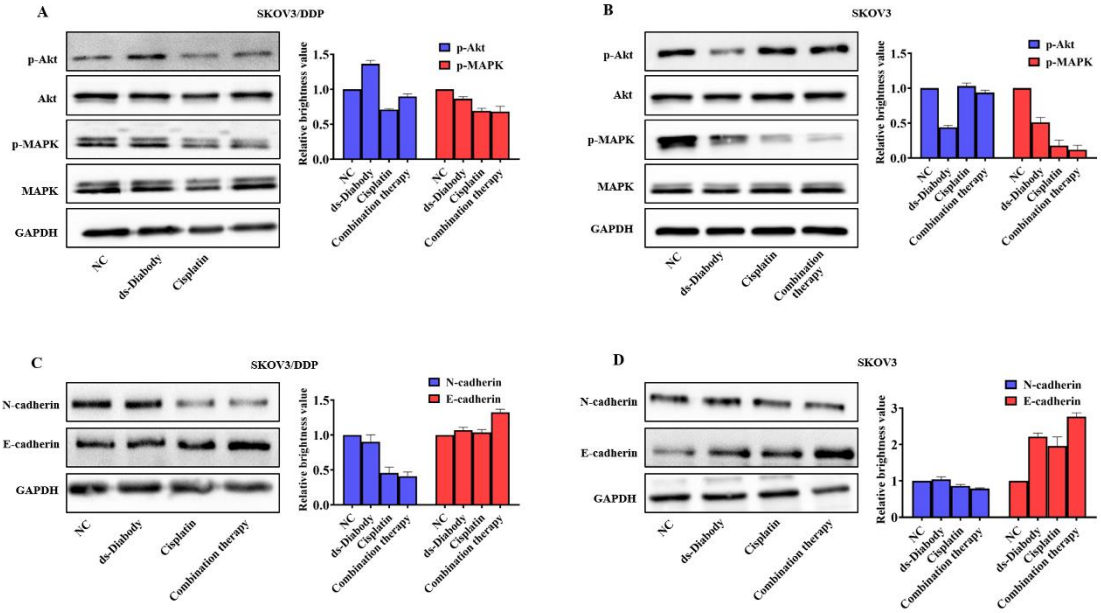

Fig. 4 The influences of ds-Diabody anti bFGF on the signaling pathways and EMT on ovarian tumour cells. (A) The expression of p-MAPK and p-Akt on SKOV3/DDP cells. (B) The expression of p-MAPK and p-Akt on SKOV3 cells. (C) The level of N-cadherin and E-cadherin in SKOV3/DDP cells. (D) The level of N-cadherin and E-cadherin in SKOV3 cells.

3.9. ds-Diabody anti bFGF combined with cisplatin inhibited the progression of ovarian cancer cell in vivo

The mice model of transplanted ovarian tumour was established to analyse the antitumor activity of ds-Diabody anti bFGF and cisplatin. The tumor growth (SKOV3/DDP) was inhibited by ds-Diabody anti bFGF, cisplatin, and combination therapy (Fig. 5A). The growth inhibition

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rate of SKOV3/DDP xenografts was 43.44% in ds-Diabody group, 58.69% in cisplatin group and 68.34% in the combination group. Similarly, the tumor growth (SKOV3) was inhibited by ds-Diabody anti bFGF, cisplatin, and combination therapy (Fig. 6A). The growth inhibition rate of SKOV3 xenografts was 38.34% in the ds-Diabody group, 47.92% in cisplatin group and 54.31% in the combination group. The experiments proved that combination therapy could inhibit the growth of ovarian tumour, and the growth inhibition rate was much higher than that of cisplatin. Therefore, combination therapy could reduce the dosage of cisplatin in vivo, and then reduce the side effects of cisplatin in the treatment for tumour.

Further, HE staining was performed on the organs of nude mice to evaluate their biological safety. The tissue sections of the organs were evenly stained and the morphology was normal in ds-Diabody anti bFGF, which showed that ds-Diabody had no obvious side effects in nude mice. A group of ds-Diabody anti bFGF combined with cisplatin, showed some side effects. (Fig. 5E). For SKOV3 cells, the results of HE staining were similar to those in SKOV3/DDP cells (Fig. 6E). The tests indicated that cisplatin has strong side effects, so we are more convinced that ds-Diabody anti bFGF combined with cisplatin has a good prospect in ovarian tumour treatment.

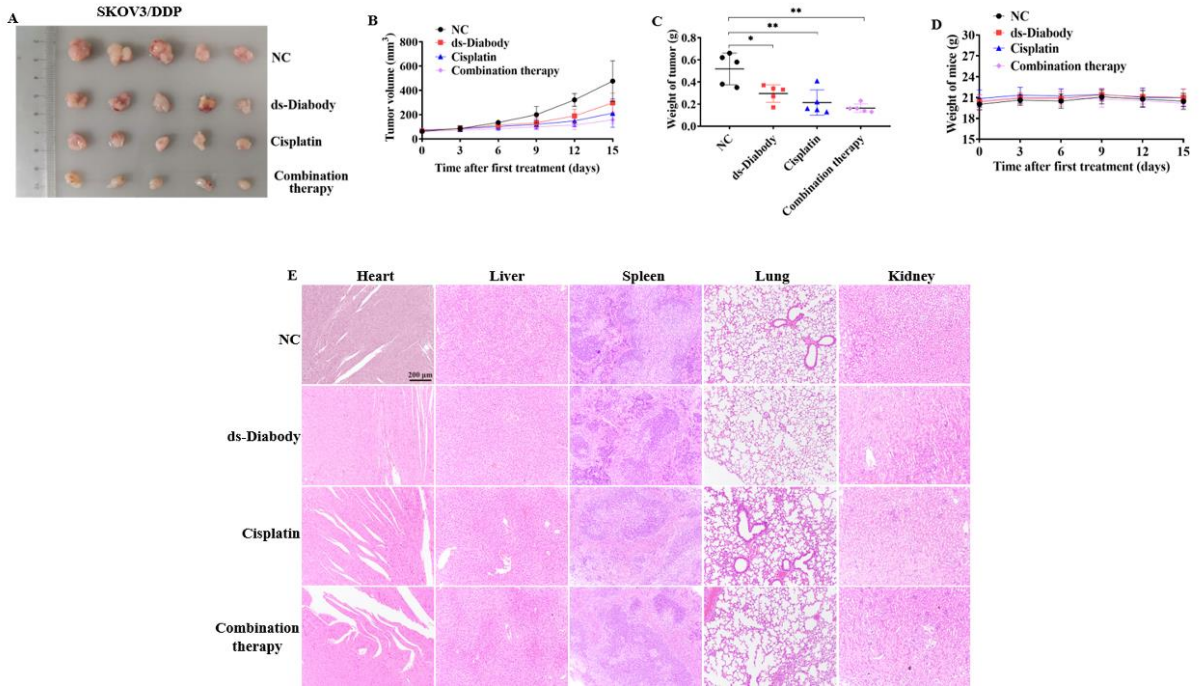

Fig. 5 The inhibiting functions of ds-Diabody anti bFGF on cisplatin resistant ovarian cancer. (A) The tumors dissected from mice (SKOV3/DDP). (B) The curved lines of tumour volume (SKOV3/DDP). (C) The curved lines of tumour weight (SKOV3/DDP). (D) The curved lines of mice weight (SKOV3/DDP). (E) HE staining of mice organs. Scale: 200 μm. *P < 0.05, **P < 0.01.

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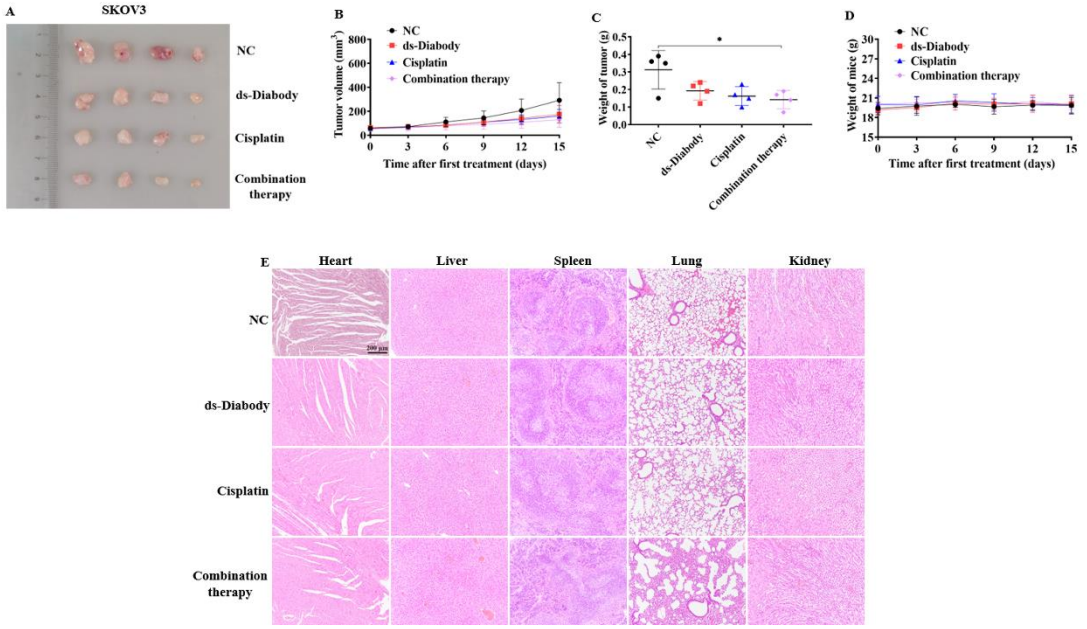

Fig. 6 The inhibiting functions of ds-Diabody anti bFGF on normal ovarian cancer. (A) The tumors dissected from nude mice (SKOV3). (B) The curved lines of tumour volume (SKOV3). (C) The curved lines of tumour weight (SKOV3). (D) The curved lines of mice weight (SKOV3). (E) HE staining of mice organs. Scale: 200 μm. *P < 0.05, **P < 0.01.

4. Discussion

The high expression of bFGF in the tumor is related to tumor development, and chemotherapy resistance[21]. Therefore, targeting bFGF is a promising anti-tumor strategy. The bFGF/FGFR blockers can reverse the resistance of uveal melanoma to BET inhibitors[12]. Anti- bFGF antibodies can inhibit the growth of U-87 glioma cells and tumor angiogenesis[22, 23]. These studies show that blocking the effect of bFGF can inhibit the growth of many tumors. Our study indicated that ds-Diabody anti bFGF and combination therapy could control the proliferative capacity and migratory ability in ovarian tumour cells in vitro, promote tumor cell apoptosis, and control tumor development of the transplanted tumor in vivo. In addition, the bFGF signals are closely related to MAPK and Akt pathways, which are targets of tumor therapy. RAS/MAPK and PI3K/Akt pathways are involved in the growth and migration of a variety of tumor cells[24]. To determine the mechanism of ds-Diabody anti bFGF inhibiting the proliferative capacity of ovarian cancer cells, the related proteins pathways were analysed with the western blot experiment. The experiments indicated that ds-Diabody anti bFGF could reduce the expression of p-MAPK and p-Akt and regulate the level of E-cadherin and N-cadherin on ovarian tumour cells. Therefore, we believe that ds-Diabody anti bFGF controls the proliferative capacity of ovarian cancer cells by inhibiting the phosphorylation of MAPK and Akt, and inhibits the migratory ability and invasiveness of tumour cells by inhibiting the level of N-cadherin. Furthermore, in all experiments, the anti-tumor effect of the combination therapy group was always better than that of the cisplatin group.

Although bFGF is linked to cisplatin tolerance, we are not sure whether targeting bFGF improve the sensitivities of ovarian tumour for DDP. It is certain that compared with the cisplatin group, the combination therapy does enhance the anti-tumor effect. Combination therapy can reduce the use of cisplatin and reduce the side effects. However, the inhibitory mechanism of ds-Diabody anti bFGF on cisplatin-resistant ovarian cancer still needs further study.

The ds-Diabody anti bFGF was expressed in pichia pastoris eukaryotic system, and its production and purification process was very simple. Different from traditional antibody drugs, ds-Diabody anti bFGF is a small molecule antibody drug with strong tissue permeability.

Moreover, unlike single-chain antibodies, ds-Diabody anti bFGF has two antigen-binding sites and disulfide bonds between two peptide chains, resulting in stronger binding activity and longer half-life for bFGF. These characteristics make the ds-Diabody anti bFGF have significant anti-tumor activity. More importantly, ds-Diabody anti bFGF combined with cisplatin has a good anti-tumor effect. Therefore, ds-Diabody anti bFGF combined with cisplatin may be a new idea in ovarian tumour treatment.

Acknowledgments

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Conflict of interest

All authors state no competing interests.

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