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Identification of a novel aFGF-binding peptide with anti-tumor effect on breast cancer from phage display library

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ABSTRACT

It has been reported that acidic fibroblast growth factor (aFGF) is expressed in breast cancer and via interactions with fibroblast growth factor receptors (FGFRs) to promote the stage and grade of the disease. Thus, aFGF/FGFRs have been considered essential targets in breast cancer therapy. We identified a specific aFGF-binding peptide (AGNWTPI, named AP8) from a phage display heptapeptide library with aFGF after four rounds of biopanning. The peptide AP8 contained two (**TP**) amino acids identical and showed high homology to the peptides of the 182–188 (G**TP**NPTL) site of high-affinity aFGF receptor FGFR1. Functional analyses indicated that AP8 specifically competed with the corresponding phage clone A8 for binding to aFGF. In addition, AP8 could inhibit aFGF-stimulated cell proliferation, arrested the cell cycle at the G0/G1 phase by increasing PA2G4 and suppressing Cyclin D1 and PCNA, and blocked the aFGF-induced activation of Erk1/2 and Akt kinase in both breast cancer cells and vascular endothelial cells. Therefore, these results indicate that peptide AP8, acting as an aFGF antagonist, is a promising therapeutic agent for the treatment of breast cancer.

1. Introduction

Acidic fibroblast growth factor (aFGF) is a member of the fibroblast growth factor family, originally isolated as mitogens for fibroblasts from the brain and pituitary. aFGF is widely expressed in both developing and adult tissues and plays important roles in a variety of normal and pathological processes, including tissue development, tissue regeneration, angiogenesis, and neoplastic transformation [1]. aFGF executes its pleiotropic biological actions by binding, dimerizing, and activating cell surface FGF receptors (FGFRs). The extra-cellular ligand-binding portion of FGFRs is composed of three immunoglobulin-like (Ig-like) domains (D1–D3). The crystal structure of the ectodomain of the FGFR complex with FGF demonstrated that the ligand-binding domain of FGFR involves Ig-like domains II and III (D2 and D3, respectively), as well as the linker between D2 and D3 [2,3]. Due to the overexpression of aFGF and its receptors in tumors, the aFGF receptor system is associated with multiple biological activities, including cellular proliferation, differentiation, invasiveness, and motility, that demonstrate the potential to initiate and promote tumorigenesis [4,5].

Breast cancer, which originates in the inner lining of milk ducts or the lobules of breast tissue, is a major public health issue worldwide. It is the most commonly occurring cancer among women and is second only to lung cancer as the main cause of cancer death in women [6,7]. Breast cancer is a complex disease, and a variety of risk factors are involved in the etiology and development of breast cancer, such as ductal carcinoma in situ (DCIS), tumor angiogenesis, metastasis to regional lymph nodes, steroid-hormone receptor proteins, ploidy and S-phase fraction, mitotic index and thymidine labeling index, proteases, and epidermal growth factor receptor (EGFR) [8,9]. While significant progress has been achieved in developing early diagnosis and treatment, acquired resistance to current chemotherapies and failure of endocrine-targeted therapy in some patients have resulted in a great clinical need for exploring new therapeutic agents for breast cancer. It has been reported that expression and related amplification of aFGF and FGFRs were recently identified in breast cancers [10,11], and that they correlate with advanced-stage and high-grade tumors, as well as decreased patient survival [12]. Therefore, the development of antagonists targeting aFGF and its receptors has been regarded as a potential strategy for breast cancer therapy by inhibiting the cell

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proliferation and angiogenesis involved in tumor progression. Herein, we used a phage display library to obtain a novel peptide specific against the aFGF binding to its receptors, and further investigated the functions of the isolated peptide to evaluate its possible therapeutic potential in breast cancer.

2. Materials and methods

2.1. Reagents

A Ph.D.-7™ Phage Display Peptide Library Kit and *Escherichia coli* ER2738 were purchased from New England Biolabs (Beverly, MA). Recombinant human aFGF was obtained from our own lab (Biopharmaceutical Research & Development Center, Jinan University, Guangzhou, China). (HRP)-anti-M13 mAb was the product of Amersham Pharmacia Biotech (Uppsala, Sweden). Anti-phospho-Erk1/2, anti-Erk1/2, anti-phospho-Akt, anti-Akt, anti-Cyclin D1, anti-PCNA, anti-proliferation-associated protein 2G4 (PA2G4) mAb, anti-GAPDH antibodies, and goat anti-rabbit and goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) antibodies were obtained from Cell Signaling Technology (Danvers, MA).

2.2. In vitro phage biopanning

Individual 96-well microtitre wells were coated with 15 μg/ml aFGF overnight at 4 °C in 0.1 M $NaHCO_3$ (pH 8.6), and then blocked with bovine serum albumin (BSA) for 2 h at room temperature. After the plate wells were washed six times with 0.05% Tween-20 in TBS (0.05% TBST), 10 μl of original library (2×10^{11} plaque-forming units, PFUS) were diluted in 100 μl TBST and added to the plate wells for 2 h at room temperature with gentle agitation. After washing with 0.05% TBST, the bound phages were eluted with 0.1 M glycine–HCl (pH 2.2) and neutralized with 1 M Tris–HCl (pH 9.1). The eluted phages were amplified by infecting them with liquid *E. coli* ER2738 culture, purified and concentrated with PEG/NaCl and titrated as described in the standard protocol (NEB), and these amplified phages were used for additional rounds of biopanning.

2.3. ELISA assay for positive phages

The aFGF were coated onto 96-well plates at 4 °C overnight. After washing with 0.05% TBST, the phage clones (10^{10} pfu/well) were added to the wells and incubated for 1 h at room temperature. The (HRP)-anti-M13 mAb (1:5000) was added and incubated for 1 h. Subsequently, the plates were washed six times and the substrate (50 μl/well of 3, 3′, 5, 5′-tetramethyl-benzidine; TMB) was added and kept at room temperature in the dark for 20 min. The reaction was terminated by adding 50 μl/well of 2 M H_2SO_4, and the absorbance was measured at 370 nm.

2.4. DNA sequencing and peptide synthesis

DNA sequences of the positive phage clones were analyzed at Invitrogen Corporation (Shanghai, China). Peptide AP8 (AGNWTPI, translated from the selected A8 phage clone DNA sequence) and an irrelevant control BP3 peptide (SWRSLRH, which was obtained from another screening strategy) were synthesized by China Peptides Co., Ltd. (Shanghai, China).

2.5. Competitive inhibition assay

The 96-well plates were coated overnight at 4 °C with aFGF. After the plates were blocked with BSA for 2 h at 4 °C, the AP8 peptide, diluted in TBST from 0 to 500 μM, was added to the coated wells at room temperature for 2 h. The A8 phage clones were added at 10^{10} pfu to the wells and incubated for another hour at room temperature. The absorbance was measured as with ELISA, described above. The competitive inhibition of binding of other phage clones (clone A1 and A13) to aFGF by the AP8 peptide was conducted using the same method.

2.6. Cell proliferation assay

Cells were seeded in 96-well plates (3×10^3 cells/well) in DMEM with 10% FBS at 37 °C for 24 h. Then, the cells were starved for 24 h and treated with aFGF alone or aFGF plus serially diluted peptides for 48 h. Cell viability was determined using the methylthiazole tetrazolium (MTT) method; 20 μl of MTT were added to each well and incubated for 4 h. The absorbance was measured at 490 nm.

2.7. Flow cytometric analysis of cell cycle

Cells were seeded in 6-well plates (2×10^5 cells/well) for 24 h, starved for another 24 h, and treated with aFGF alone or aFGF plus serially diluted peptides for 48 h. After fixing in 70% ice-cold ethanol for 30 min at 4 °C, the cells were stained with propidium iodide (PI) in the dark, at room temperature, for 30 min. The percentages of cells at various phases of the cell cycle were analyzed with the FlowJo analysis program.

2.8. Western blot analysis of mitogen-activated protein kinase (MAPK), Akt activation, and expression of PA2G4, PCNA, and Cyclin D1

Cells were seeded in 6-well plates (3.5×10^6 cells/well) for 24 h, starved for another 24 h, and treated with serially diluted peptides for 30 min prior to stimulation with aFGF for 3 h. The membrane was incubated with the primary antibody (an anti-phospho-Erk1/2 rabbit mAb or an anti-phospho-Akt rabbit mAb) overnight at 4 °C, followed by a goat anti-rabbit IgG, HRP-linked antibody (1:1000 dilution) at room temperature for 1 h. Western blot analysis of PA2G4, Cyclin D1, and PCNA expression were performed using the same method, with the following exceptions: to detect PA2G4 and PCNA expression, the starved cells were treated with 40 ng/ml aFGF alone or 40 ng/ml aFGF plus 4 μM AP8 peptides for 48 h, and to detect cyclin D1 expression, the starved cells were treated with serially diluted peptides for 30 min prior to stimulation with aFGF for 6 h.

3. Results

3.1. Selection of phage clones to bind specifically to aFGF

In this study, a Ph.D.-7™ Phage Display Peptide Library was used to isolate high-affinity phages that could specifically bind to aFGF. As shown in Table 1, the recovery efficiency was 140-fold higher (from 6.0×10^{-5} to 8.4×10^{-3}) after four rounds of panning

Table 1
Enrichment of aFGF binding phages for each panning round.

Round	aFGF (μg)	Concentration of Tween20 (v/v)	Input phage (pfu)	Output phage (pfu)	Recovery (output/input)
1	15	0.05	2.0×10^{11}	1.2×10^{6}	6.0×10^{-5}
2	10	1	3.1×10^{11}	8.2×10^{7}	2.65×10^{-4}
3	5	0.3	4.0×10^{11}	2.6×10^{7}	6.5×10^{-4}
4	2.5	0.5	5.0×10^{11}	4.2×10^{8}	8.4×10^{-3}

than after the first round, suggesting that the phages specifically bound to aFGF were successfully enriched.

In order to identify the binding ability of the selected phage clones, 24 independent phage clones were randomly selected and detected by ELISA after four rounds of selection. As shown in Fig. 1A, ten clones showed a high binding ability to aFGF (clones 1, 6, 8, 13, 16, 17, 18, 20, 23, 24) when compared to the control blocking buffer, and the NO.8 clone was the highest, suggesting that it might have a higher affinity for aFGF than the other clones. We then selected the ten clones for sequencing.

aFGF executes its pleiotropic biological actions by binding to the cell surfaces of FGFR1, FGFR2, FGFR3, and FGFR4. Among the four receptors, the affinity of aFGF binding to FGFR1 is the highest [13]. The crystal structure of the aFGF complex with FGFR showed that the ligand-binding domains of FGFR involve the highly conserved Ig-like D2 and D3, and the linker region between D2 and D3 [3]. Therefore, the amino acid sequences of the selected peptides were compared with the motif (151–355 aa) located at D2–D3 of FGFR1. As shown in Table 2, the phage clones A8, A16, A17, A18, A20, A23, and A24 (the peptide was named AP8) showed the highest sequence similarity to FGFR1 (0.004878, PAM250 Matrix). The AP8 peptide (**AGN**W**TP**I) contains two (**TP**) and three (**AGN**) amino acids identical to the peptides of the 182–188 (**GTP**NPTL) and 343–349 (**AGN**SIGL) sites, respectively, of FGFR1 (Genebank ID AAH15035.1). In the physiological condition, AP8, FGFR1182–188, and FGFR1 all carry the negative charges. Moreover, the hydrophobic profile of AP8 was similar to the FGFR1182–188 peptide. Taken together, these data suggest that the AP8 peptide, with amino acids identical to FGFR1, may bind aFGF via electrostatic interactions and may interrupt aFGF binding to FGFR1. Thus, peptide AP8 was chosen for further study.

Table 2
Properties of peptides displayed by specific aFGF-binding phages.

Clone	Peptide	Sequence (N-C)	Similarities to FGFR1	Theoretical PI	GRAVY
A1	AP1	RP**T**SHQL	0.0012195	9.76	−1.500
A6	AP6	QW**TP**SHP	0.0024390	6.74	−1.757
A8/16/17/18/20/23/24	AP8	**AGN**W**TP**I	0.004878	5.57	−0.114
A13	AP13	APD**T**KTQ	0.0012195	5.88	−1.729
	$FGFR1_{182-188}$	G**TP**NPTL		5.52	−0.671
	$FGFR1_{343-349}$	**AGN**SIGL		5.57	0.714
	FGFR1			5.82	−0.372

3.2. Competitive inhibition assay

To determine whether the synthetic peptide AP8 and the corresponding phage clone A8 competed for the same binding site, a competitive inhibition assay was performed. The synthetic peptide AP8 was used as the inhibitor of phage clone A8, and peptide BP3 (SWRSLRH) was used as a control peptide. Phage clones A1 and A13, which appeared rather different from clone A8, were chosen to test whether the other clones could be blocked by AP8. As shown in Fig. 1C, with an increase in the concentration of AP8, the inhibition gradually increased. The control peptide BP3 had no effect on the binding of the phage A8, even at 500 μM. In addition, no significant competitive inhibition was observed for AP8 to phage clones A1 and A13 (Fig. 1D). These results indicated that the synthetic peptide AP8 and the corresponding phage clone A8 thus compete for the same binding site, and the binding of phage A8 to aFGF was mediated by the peptide AP8.

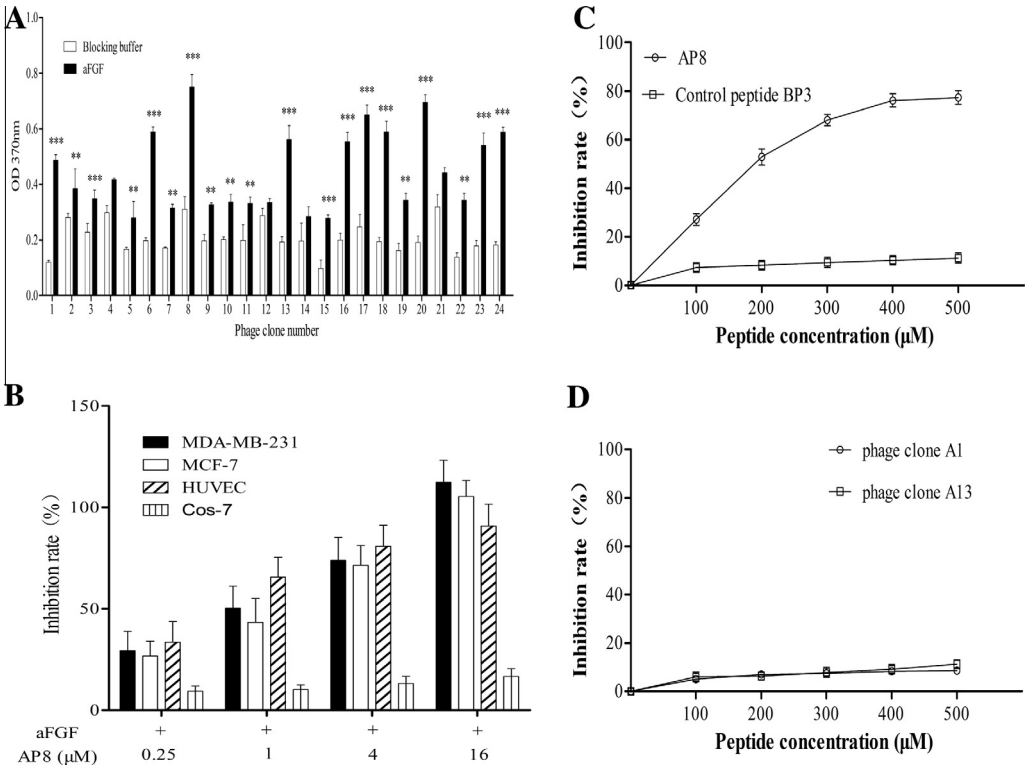

Fig. 1. (A) ELISA appraisal of binding ability of phage clones to aFGF. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus blocking buffer sample. (B) Inhibition of aFGF-stimulated proliferation of cells by AP8. (C) Concentration-dependent inhibition of AP8 and control peptide BP3 to phage clone A8. (D) Concentration-dependent inhibition of AP8 to phage clones A1 and A13. Data presented are the mean OD values (±SD) of triplicate samples.

3.3. AP8 inhibits aFGF-stimulated cell proliferation

Cell lines expressing high level of FGFRs including MDA-MB-231, MCF-7 breast cancer cells, and human umbilical vein endothelial cells (HUVECs), and Cos-7 cells that do not express FGFRs were used in the study [14–17]. The effect of AP8 on the proliferation of aFGF-stimulated MDA-MB-231, MCF-7, HUVEC and Cos-7 cells was determined by an MTT assay. As shown in Fig. 1B, the proliferation of MDA-MB-231, MCF-7 and HUVEC cells stimulated with aFGF could be inhibited by AP8 in a dose-dependent manner, while AP8 had little inhibitory effect on Cos-7 cells that do not express aFGF receptors.

3.4. AP8 arrests aFGF-induced cells at the G0/G1 phase via Cyclin D1

To determine the effect of AP8 on the cell cycle progression of MDA-MB-231, MCF-7 and HUVEC cells induced by aFGF, a flow cytometry analysis was performed. As shown in Fig. 2(A–F), cells treated with aFGF plus AP8 had a higher G0/G1 phase percentage and lower S phase percentage than those treated with aFGF alone.

Cyclin D1, which is a G1/S-specific regulating protein, has become particularly well known for its prominent role in driving tumorigenesis and controlling cell cycle progress [18]. As shown in Fig. 2(G–L), aFGF significantly increased the expression of Cyclin D1 in MDA-MB-231, MCF-7 and HUVEC cells, whereas treating cells with aFGF plus AP8 led to a significant decrease in the expression of Cyclin D1, suggesting that AP8 peptides arrest cells at G0/G1 phase partly via decreasing the expression of G1/S-specific Cyclin D1.

3.5. Effect of AP8 on aFGF-induced Akt and MAP kinase activation

The effects of AP8 on aFGF-triggered signal transduction were determined by measuring its capacity to inhibit the activation of Akt and MAP kinases in MDA-MB-231, MCF-7 and HUVEC cells after stimulation by aFGF. As shown in Fig. 3, aFGF significantly stimulated the phosphorylation of Erk1/2 and Akt signal molecules, while the pretreatment of cells with AP8 for 30 min prior to aFGF stimulation resulted in significant blockage of the activation of the both signal molecules in a dose-dependent manner.

3.6. AP8 counteracts the regulatory effect of aFGF on PA2G4 and PCNA expression

In order to determine whether AP8 inhibited aFGF-stimulated cell proliferation by influencing PA2G4 and PCNA signal transduction, a Western blot analysis was performed. As shown in Fig. 4, the expression of PA2G4 was downregulated by aFGF stimulation and enhanced by AP8 treatment. In addition, aFGF significantly increased the expression of PCNA, whereas treating cells with aFGF plus AP8 led to a significant decrease in the expression of PCNA. These results suggested that PA2G4 and PCNA play an important role in AP8 counteracting the aFGF-stimulated proliferation in MDA-MB-231, MCF-7 and HUVEC cells.

4. Discussion

Conventional cancer therapies are limited by drug-resistant metastatic tumors and side effects such as irradiation and

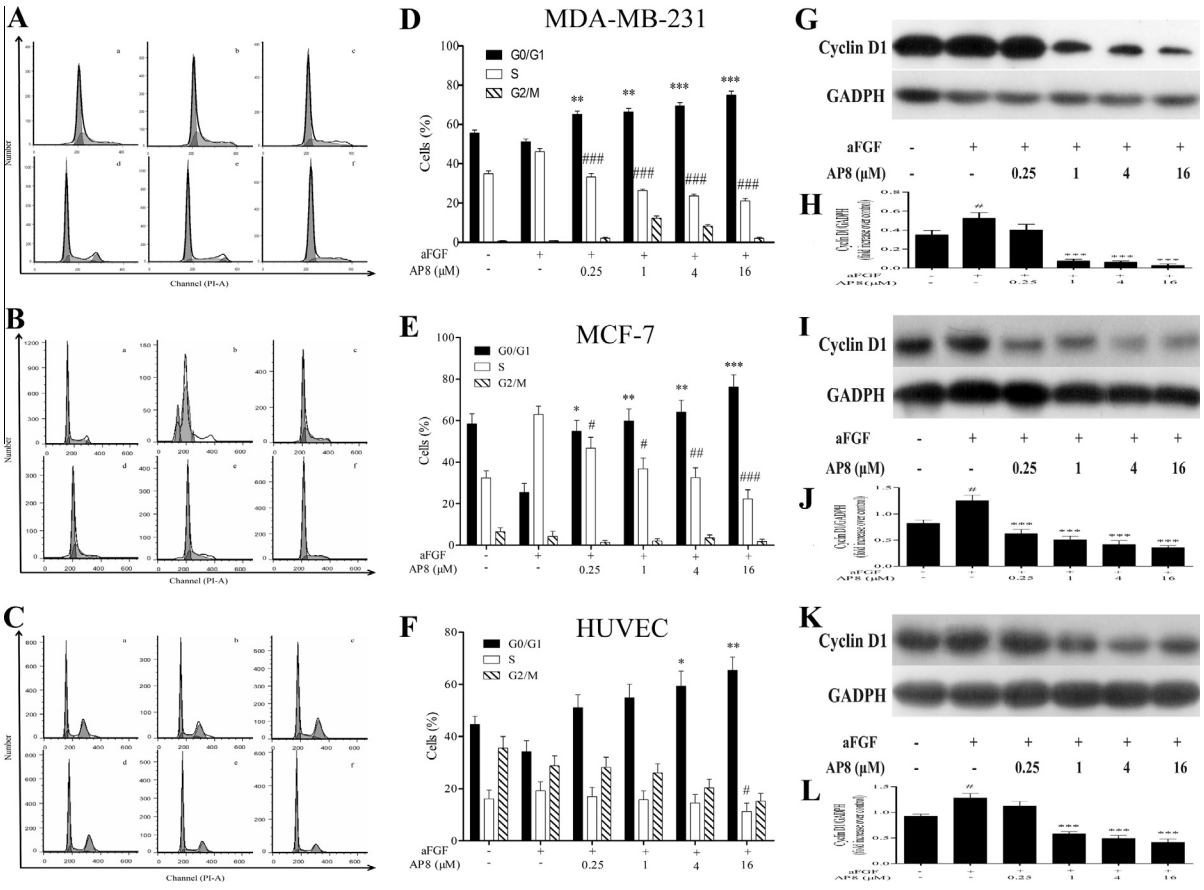

Fig. 2. (A–C) Flow cytometric analysis of cell cycle. MDA-MB-231, MCF-7 and HUVEC cells were treated with 40 ng/ml aFGF (b) or 40 ng/ml aFGF plus various concentrations of AP8 (c–f: 0.25, 1, 4, and 16 μM). (a) Control cells without aFGF or AP8 treatment. (D–F) Cell cycle distribution. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus aFGF group in G1/G0 phase; $^{\#}P < 0.005$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus aFGF group at S phase. (G, I, and K) AP8 counteracted the regulatory effect of aFGF on Cyclin D1. (H, J and L) Density ratios of Cyclin D1 proteins to GADPH protein. $^{\#}P < 0.05$ versus control group; $^{***}P < 0.001$ versus aFGF group. Data presented are the mean OD values (±SD) of triplicate samples.

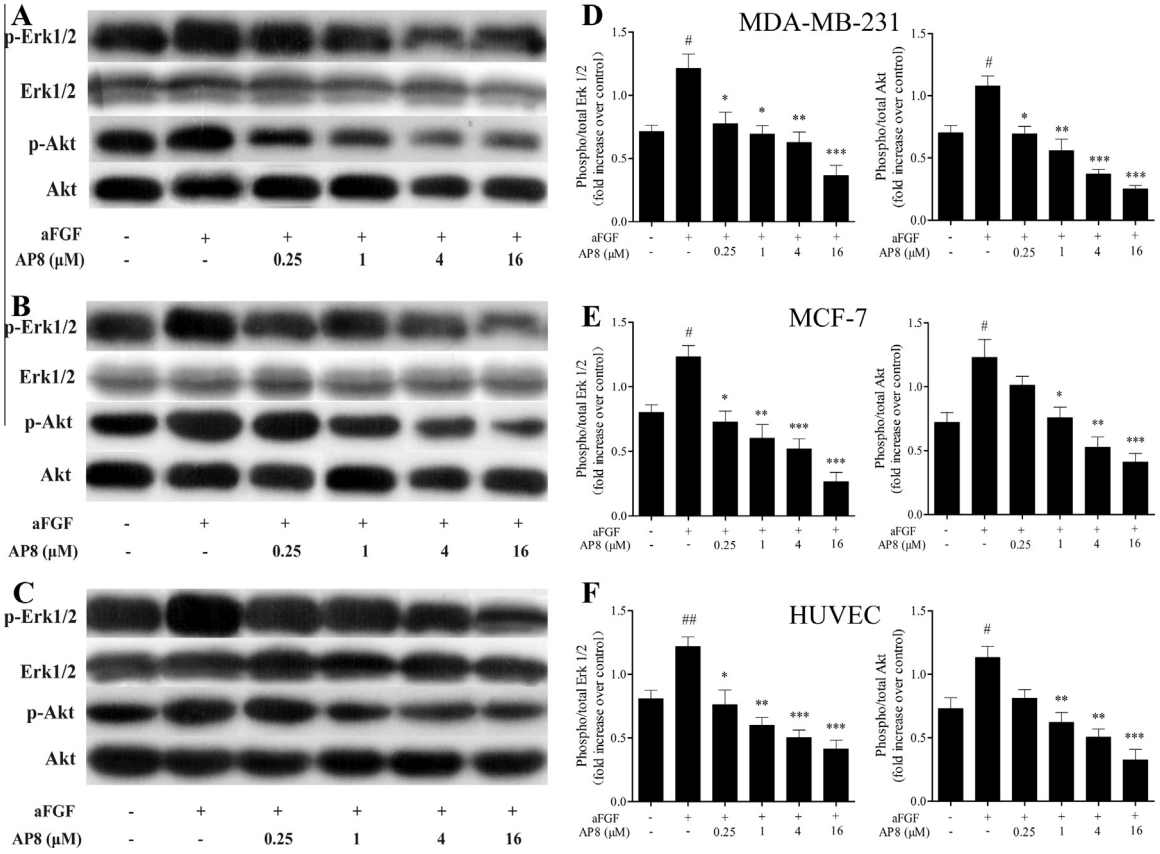

Fig. 3. AP8 peptides inhibited aFGF-induced Erk 1/2 and Akt activation. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus aFGF group. Data presented are the mean OD values (±SD) of triplicate samples.

chemotherapy [19]; therefore, it is necessary to search for novel tumor-targeting agents. Phage display technology, a highly efficient tool for finding and identifying small molecules, such as antibodies and peptides, that bind to specific targets, has been investigated in recent years for use in the clinical diagnosis and treatment of various types of cancers [20]. These small molecules, which are characterized from phage display libraries, exhibit great efficiency in penetrating into the targeted sites with low immunogenicity [21] and high concentration and validity [22].

Several studies have shown that the FGF/FGFR complexes that act as driving oncogenes in certain cancers maintain the malignant properties of tumor cells, and that the "anti-growth factor" approach to blocking the FGF–FGFR interaction represents a well-established treatment strategy for cancer [23,24]. Angiogenesis corresponds to the formation of new blood vessels and is involved in different physiological and pathological conditions including ovulation, embryogenesis, tumor growth, wound repair and rheumatoid arthritis [16]. Because aFGF can accelerate tumor growth and angiogenesis, and is upregulated in breast cancer, it is considered a potential target in breast cancer therapy.

In the present study, we used a phage-displayed heptapeptide library to isolate aFGF antagonists, and the conditions of the selection were strictly limited to enrichment specific aFGF-binding phages. After four rounds of panning, one of the 24 selected candidate phage clones showed a significantly positive signal and exhibited the strongest binding according to ELISA. Alignment of the selected peptide sequences with the aFGF high-affinity receptor, FGFR1, revealed high sequence homology between AP8 and FGFR1 D2–D3, and specifically competed the same site for phage clone A8 binding to aFGF. Thus, it is reasonable to speculate that AP8 may have the capability to bind aFGF and block the biological activity of aFGF by interrupting its interactions with FGFR1.

It has been reported that PA2G4 inhibits the proliferation and induces the differentiation of human breast cancer cells [25]. The results of an MTT assay showed that AP8 was effective in inhibiting MDA-MB-231, MCF-7 and HUVEC cells proliferation in a dose-dependent manner, while AP8 hardly suppressed the growth of Cos-7 cells that do not express FGFRs. The Western blot analysis also showed that AP8 increased PA2G4 expression in MDA-MB-231, MCF-7 and HUVEC cells. These data indicate that AP8 may inhibit the aFGF-stimulated cell proliferation by increasing PA2G4 expression.

Furthermore, AP8 has been shown to mediate the activation of Cyclin D1 and PCNA, both of which modulate cell cycle progression. Cell proliferation is regulated during the G0/G1 phase in the cell cycle, and CDKs interacting with the cyclin D family of proteins drive the G0/G1 phase into the DNA synthesizing S-phase [26]. The active Cyclin D1-CD4/6 complexes release E2F transcription factors and motivate the specific gene expressions required for G1 to S phase progression [27]. PCNA, a member of the DNA sliding clamp family of proteins that assist in DNA replication and repair, can also form complexes with Cyclin-CDK complexes, and stimulate their phosphorylation to control cell cycle progression [28]. Our results revealed that AP8 arrested aFGF-stimulated cells at the G0/G1 phase and downregulated the expression of Cyclin D1 and PCNA induced by aFGF. These findings suggest that the mechanisms by which AP8 restricts aFGF-induced G1 to S phase progression might partly be the downregulation of the expression of G1/S-specific proteins cyclin D1 and PCNA.

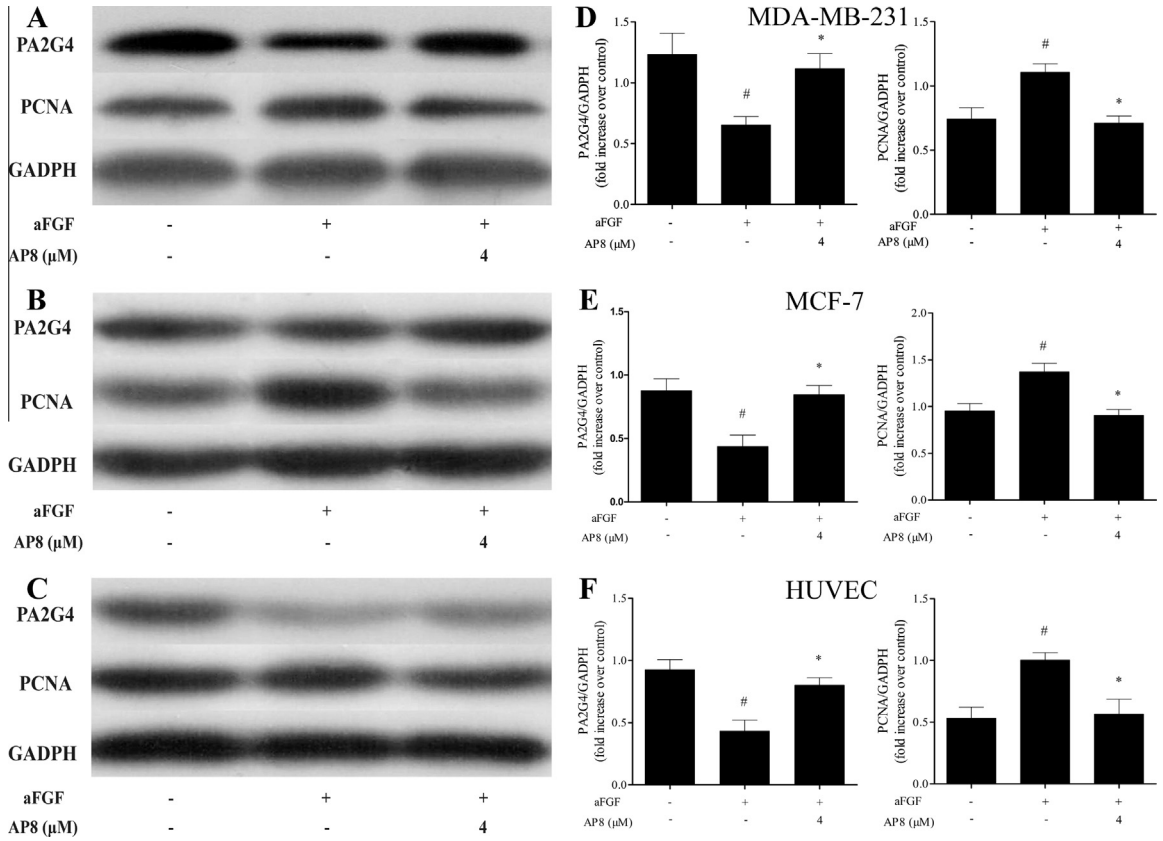

Fig. 4. AP8 counteracted the regulatory effect of aFGF on proliferation-associated protein 2G4 (PA2G4) and proliferation-associated protein PCNA expression. $^{\#}P < 0.05$ versus control group; $^{*}P < 0.05$ versus aFGF group. Data presented are the mean OD values (±SD) of triplicate samples.

The tyrosine kinase-activated Ras/MEK/Erk pathway and Ras/PI3K pathway play a pivotal role in cell proliferation by regulating cyclin D1 expression during mid-G1 and driving cells past the G1-restriction point [29]. In order to explore the potential of AP8 in cancer treatment, we further investigated the effects of AP8 on MAPK and Akt signal transduction. The Western blot analysis showed that AP8 suppressed aFGF-induced Erk1/2 and Akt phosphorylation in a dose-dependent manner in MDA-MB-231, MCF-7, and HUVEC cells. It is reasonable to speculate that AP8 might have counteracted aFGF-stimulated proliferation and the cell cycle by blocking activation of the MAP kinase and Akt, increasing PA2G4 expression, and suppressing the expression of cyclin D1 and PCNA.

In summary, we successfully isolated an aFGF-binding peptide, AP8, by phage display technology, providing an effective aFGF/FGFRs antagonist that might have potential applications in the treatment of a variety of cancers, including breast cancer, characterized by the upregulation of aFGF/FGFRs.

Acknowledgments

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