P7 peptides targeting bFGF sensitize colorectal cancer cells to CPT-11

WU LUO1*, YONGLIN YU1*, RUIXUE WANG1, DAN HE1, CONG WANG1,2, XIANGFENG ZENG1, XILEI CHEN1, XIANGPENG TAN1, TAO HUANG1 and XIAOPING WU1,2

1Institute of Tissue Transplantation and Immunology, Jinan University, Guangzhou, Guangdong; 2School of Pharmaceutical Science, Wenzhou Medical College, Wenzhou, Zhejiang, P.R. China

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Abstract. The low survival rate of patients with colorectal cancer (CRC) is mainly due to the drug resistance of tumor cells to chemotherapeutic agents. It has been reported that basic fibroblast growth factor (bFGF) is an essential factor involved in the epigenetic mechanisms of drug resistance, which provides a novel potential target for improving the sensitivity of tumor cells to chemotherapeutic agents. In this study, we first demonstrate that a novel bFGF antagonist, peptide P7, previously isolated by phage display technology, reversed bFGF-induced resistance to irinotecan hydrochloride (CPT-11), and counteracted the anti-apoptotic effects of bFGF on CPT-11-treated HT-29 cells. Further experiments indicated that the inhibition of Akt activation, the suppression of bFGF internalization, the increase in the Bax to Bcl-2 ratio and the downregulation of cytokeratin 8 (CK8) by P7 may contribute to the counteracting of the anti-apoptotic effects of bFGF, and further reversal of bFGF-induced resistance to CPT-11. Our results suggest that peptide P7 may have therapeutic potential in CRC as a sensitizer to chemotherapeutic agents by targeting bFGF.

Introduction

Colorectal cancer (CRC) is the fourth most common malignant tumor worldwide, leading to approximately 200,000 deaths per year in Europe and the US (1). Although improved surgical techniques combined with multi-disciplinary approaches have been applied in therapy, the 5-year survival rate for patients with CRC remains poor, mainly due to tumor cells acquiring multidrug resistance properties. The overexpression of drug efflux proteins (2-4) is considered a common mechanism of multidrug resistance, as shown in preclinical studies. However, no significant improvement in the chemotherapeutic effectiveness has been observed in clinical practice by the inhibition of drug efflux proteins (5,6), suggesting the existence of other chemoresistance mechanisms.

Irinotecan hydrochloride (CPT-11) is a water-soluble derivative of camptothecin (7), presenting a wide spectrum of antitumor activity by preventing DNA religation, resulting in DNA double-strand breaks and eventually leading to apoptosis (8). The cytotoxic activity of CPT-11 has been reported in several malignant tumors, including breast, lung, ovarian, and colon cancer (9-16). CPT-11 serving as a DNA topoisomerase I inhibitor, combined with 5-fluorouracil (5-FU) and leucovorin has been accepted as a first-line treatment for patients with advanced CRC. However, this combination only offers a 2-month median survival advantage over previous chemotherapeutic agents (8). Any treatment conferring a modest survival benefit for CRC will have significant meaning. Understanding the mechanisms of drug resistance will greatly contribute to the development of more effective treatments for improving survival in patients with CRC.

Basic fibroblast growth factor (bFGF) is a pleiotropic factor involved in the processes of cell proliferation, differentiation and anti-apoptosis in a wide variety of cells derived from the mesoderm and neuroectoderm. It has been well documented that bFGF plays an essential role in tumor growth and progression by stimulating the proliferation of tumor cells and promoting angiogenesis (17-19). During chemotherapy in CRC, CPT-11 enhances the levels of intratumoral bFGF, and upregulates bFGF levels in recurrent tumors following chemotherapy, suggesting that elevated levels of extracellular bFGF may mediate an epigenetic mechanism of multidrug resistance. bFGF serving as a key factor involved in an epigenetic mechanism by which cancer cells become resistant to chemotherapeutic agents may provide a novel potential target for designing strategies to sensitize tumor cells to chemotherapeutic drugs.

In our previous studies, using phage display technology, we obtained a novel bFGF antagonist peptide (named P7) with strong inhibitory activity against bFGF-induced cell proliferation and angiogenesis (20,21). Our results suggested that

*Correspondence to: Dr Xiaoping Wu, Institute of Tissue Transplantation and Immunology, Jinan University, 601 West Huangpu Avenue, Tianhe, Guangzhou, Guangdong 510632, P.R. China E-mail: twxp@jnu.edu.cn

*Contributed equally

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P7 peptides may bind to bFGF and block the biological activities of bFGF. In this study, we aimed to investigate the effects and mechanisms of action of our previously isolated P7 peptide on the bFGF-induced resistance of CRC cells to CPT-11.

Materials and methods

Materials. The CRC cell line, HT-29, was kept in our laboratory. Recombinant human bFGF was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). P7 peptides (PLLQATLGGGS) with a purity of >98% were synthesized at SBS Genetech Co., Ltd. (Beijing, China). Dynabeads® M-280 Streptavidin, Dynamag-2 magnet, RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). Anti-phospho-Akt, anti-Akt, anti-Bcl-2, anti-Bcl-xL, anti-Bax and anti-GAPDH antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Billerica, MA, USA). The enhanced chemiluminescence (ECL) detection kit was the product of Pierce (Rockford, IL, USA). The Alexa Fluor® 488 Annexin V/PI kit was from Invitrogen. The SYBR-Green qPCR Master Mix was the product of Transeau Biotech (Beijing, China). The materials for 2-dimensional gel electrophoresis, including immobilized pH gradient (IPG) strips (pH 3-10 non-linear), CHAPS, Bio-Lyte 3-10 Ampholyte 40% solution, acrylamide, methylene-bisacryamide, sodium dodecyl sulfate (SDS), tetramethylthylene diamine (TEMED) and iodoracetamide were the products of Bio-Rad (Hercules, CA, USA). Glycerol and ammonium persulfate were purchased from Sigma (St. Louis, MO, USA). Dithiothreitol (DTT) and urea were obtained from Promega (Madison, WI, USA).

Cell viability assay. The cells were seeded into 96-well culture plates at a density of 5x10^3 cells/well and cultured overnight. After being starved in RPMI-1640 medium with 0.4% FBS for 24 h, the cells were treated with serially diluted CPT-11 (15, 30, 60, 120, 180 µM) for 48 h. The IC50 of CPT-11 was determined by MTT colorimetric assay.

After determining the IC50 of CPT-11 as 60 µM, the cells were further treated as follows: with 60 µM CPT-11 alone, 60 µM CPT-11 plus 20 ng/ml bFGF, or 60 µM CPT-11 plus 20 ng/ml bFGF combined with 4 µM P7. The viability of the cells was determined by MTT colorimetric assay following incubation for 48 h.

Apoptosis assay. The cells were seeded in a 6-well plate at a density of 4x10^3 cells/well, starved in RPMI-1640 medium with 0.4% FBS overnight, and then treated with 60 µM CPT-11 alone, 60 µM CPT-11 plus 20 ng/ml bFGF, or 60 µM CPT-11 plus 20 ng/ml bFGF combined with 4 µM P7 for 48 h. After being washed with cold PBS twice, the cells were resuspended in 1X Annexin-binding buffer, incubated with Alexa Fluor 488 Annexin V and PI for 15 min in the dark at room temperature, and subjected to flow cytometric analysis. FCS Express version 3 software was applied to analyze the apoptotic ratio of the cells.

Akt activation assay. The cells were seeded in a 12-well plate at a density of 5x10^3 cells/well and cultured overnight. After being starved for 24 h, the cells were pre-treated with 20 ng/ml bFGF, or 20 ng/ml bFGF combined with 4 µM P7 for 4 h prior to treatment with 60 µM CPT-11. After being washed twice with cold PBS, the cells were harvested and centrifuged at 12,000 x g for 10 min at 4°C to remove the insoluble components. The resultant protein samples were separated by 10% SDS-PAGE gel and were then transferred onto a PVDF membrane. In order to block non-specific binding, the membrane was incubated with 5% non-fat milk in TBST (25 mM Tris, pH 7.4, 150 mM NaCl and 0.1% Tween-20) buffer for 1 h. After being washed with TBST 3 times, the membrane was incubated with primary antibody [an anti-phospho-Akt rabbit monoclonal antibody (mAb) or an anti-Akt rabbit mAb] overnight followed by probing with goat anti-rabbit IgG, HRP-linked antibody for 1 h at room temperature. The ECL detection kit was used to detect the proteins on the blots. The relative ratio was determined by Quantity One software.

bFGF internalization assay. The biotinylation of bFGF was performed on cysteine residues according to the manufacturer's instructions (Vector Laboratories, Inc., Burlingame, CA, USA). The modification of bFGF at cysteine residues has no effect on the biological activity of the growth factor (22). After being starved for 24 h, the HT-29 cells were pre-treated with 4 µM peptides for 5 min prior to stimulation with 20 ng/ml biotinylated bFGF (biot-bFGF) for 4 h. The cells were harvested by centrifugation at 250 x g for 5 min at 4°C, and washed twice in cold PBS. The Nuclear and Cytoplasmic Protein Extraction kit was used to extract the nuclear and the cytoplasmic proteins according to the manufacturer's instructions (KeyGen Biotech. Co. Ltd., Nanjing, China). The protein concentrations were determined using the Bradford method, as previously described (23). An equal amount of protein sample was incubated in the presence of streptavidin beads for 30 min at room temperature with gentle rotation. After extensive washing with PBS containing 0.1% BSA 5 times, the bound proteins were directly resuspended in 0.1% SDS by boiling the beads for 5 min and they were subsequently analyzed by western blot analysis using anti-bFGF antibody.

Analysis of the expression of apoptosis-related proteins. The cells were seeded in a 12-well plate at a density of 5x10^3 cells/well and cultured overnight. After being starved for 24 h, the cells were pre-treated with 20 ng/ml bFGF, or 20 ng/ml bFGF combined with 4 µM P7 for 4 h prior to treatment with 60 µM CPT-11 for 48 h. The cells were harvested and lysed in 1X SDS-PAGE loading buffer. The resultant protein samples were separated by 10% SDS-PAGE gel and were then transferred onto a PVDF membrane, which was incubated in 5% non-fat milk in TBST buffer for 1 h, and subsequently with polyclonal antibodies against Bcl-2, Bax, Bcl-xL and GAPDH overnight, and then with HRP-conjugated secondary antibody for 1 h. The ECL detection kit was used to visualize the blots. The results were analyzed using Quantity One software to determine the relative ratio.

2-Dimensional gel electrophoresis (2-DE) and mass spectrometric analysis. The HT-29 cells (1x10^6 cells) were grown in cell culture flasks (75 cm²) overnight. After being starved for 24 h, the cells were treated with 60 µM CPT-11 alone, 60 µM CPT-11 plus 20 ng/ml bFGF, or 60 µM CPT-11 plus 20 ng/ml bFGF combined with 4 µM P7 for 4 h prior to treatment with 60 µM CPT-11. After being washed twice with cold PBS, the cells were harvested and centrifuged at 12,000 x g for 10 min at 4°C to remove the insoluble components. The resultant protein samples were separated by 10% SDS-PAGE gel and were then transferred onto a PVDF membrane. In order to block non-specific binding, the membrane was incubated with 5% non-fat milk in TBST (25 mM Tris, pH 7.4, 150 mM NaCl and 0.1% Tween-20) buffer for 1 h. After being washed with TBST 3 times, the membrane was incubated with primary antibody [an anti-phospho-Akt rabbit monoclonal antibody (mAb) or an anti-Akt rabbit mAb] overnight followed by probing with goat anti-rabbit IgG, HRP-linked antibody for 1 h at room temperature. The ECL detection kit was used to detect the proteins on the blots. The relative ratio was determined by Quantity One software.

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bFGF combined with 4 μM P7 for 48 h. The samples for 2-DE were prepared as follows: 1 mg of protein sample was resuspended in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% pH 3-10 ampholyte, and 0.001% bromophenol blue) to a total volume of 350 μl. The resuspended proteins were loaded on a 17-cm immobilized pH gradient strip and separated first according to the isoelectric point of the proteins. After reduction for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCL, pH 8.8 and 2% DTT) for 15 min and alkylation for an additional 15 min in the same solution (except that 2% DTT was substituted with 2.5% iodoacetamide), the proteins were then separated in 12% SDS-PAGE gels, followed by staining with Coomassie brilliant blue G-250.

After being analyzed using PDQuest 8.0 software, the protein spots were excised and subjected to in-gel trypsin digestion as previously described (21). The digested samples were dried and analyzed by tandem time-of-flight (TOF) mass spectrometry (ABI 4800 TOF/TOF). Mascot protein identification software was used for peptide detection in the IPI human database.

Real-time PCR (qPCR). Total RNA was isolated from the HT-29 cells using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). First-strand cDNA was synthesized from the isolated total RNA using random primers. Relative qPCR was performed with SYBR-Green PCR Master Mix using a MiniOpticon real-time PCR detector. The sequences of the primers used for PCR were as follows: cytokeratin 8 (CK8) forward, 5'-ATCAGCTCCTCCTGAGCTTCTC-3' and reverse, 5'-TCCAGGAAACCGTACCTTGTC-3'; and GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-AAGATGGTGATGGGATTTC-3'.

Statistical analysis. Data are presented as the means ± standard deviations (SD) from at least 3 independent experiments and statistical analysis was performed using GradPad Prism software version 5.0. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

P7 peptides reverse bFGF-induced resistance to CPT-11. The effects of P7 peptides on bFGF-induced CPT-11 resistance were evaluated by MTT assay. The results are presented in Fig. 1. CPT-11 inhibited cell growth in a dose-dependent manner, with an IC₅₀ of 60 μM. At the fixed CPT-11 concentration (IC₅₀), bFGF induced resistance to CPT-11, as indicated by the increase in the survival rate of the HT-29 cells treated with CPT-11 plus bFGF. In the presence of bFGF, the addition of P7 peptides reversed the bFGF-induced resistance to CPT-11.

P7 attenuates the anti-apoptotic effects of bFGF on CPT-11-treated cells. It has been known that the failure to activate the apoptotic programme is an essential mode of drug resistance in tumor cells. bFGF has anti-apoptotic potential, and is involved in the induction of the resistance of tumor cells to chemotherapeutic drugs. Therefore, in order to investigate whether the P7 peptide reversed bFGF-induced resistance to CPT-11 by attenuating the anti-apoptotic effects of bFGF, dual staining with Alexa Fluor 488 Annexin V and PI followed by flow cytometric analysis was carried out to determine the effects of peptide P7 on apoptosis. As illustrated in Fig. 2, CPT-11 markedly enhanced the apoptotic rate, with the late-stage apoptotic rate increasing from 25.64±1.33% to 38.01±0.12%. Treatment with bFGF decreased the late-stage apoptotic rate

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to 34.30±0.27%; this was then increased to 44.09±0.29% by the addition of P7 peptides, suggesting that P7 peptides may reverse bFGF-induced CPT-11 resistance by counteracting the anti-apoptotic effects of bFGF on CPT-11-treated cells.

**P7 blocks bFGF-induced Akt activation.** Since the PI3K/Akt signaling pathway decisively contributes to drug resistance by mediating anti-apoptotic signals, we further examined the effects of P7 peptides on the activation of the PI3K/Akt signaling pathway by western blot analysis. As shown in Fig. 3, CPT-11 decreased the activation of Akt; however, the addition of bFGF enhanced the phosphorylation of Akt which was downregulated by CPT-11, whereas P7 peptides attenuated the activation of Akt induced by bFGF, revealing that the PI3K/Akt signaling pathway is involved in mediating the reversal effects of P7 peptides on bFGF-induced apoptosis and resistance to CPT-11.

**P7 inhibits the internalization of bFGF.** The binding of bFGF with its receptors unleashes its full biological activity, including its anti-apoptotic properties, by activating signal transduction pathways and enabling its internalization into cells (24). Therefore, the effects of P7 peptides on bFGF internalization were further analyzed by anti-bFGF antibody probing exogenous biot-bFGF in the cytoplasmic and nuclear fractions. The intense signals of biot-bFGF were detected in both the cytoplasmic and nuclear proteins extracted from the biot-bFGF treated cells (Fig. 4, lanes 3 and 4), whereas pre-treatment of the cells with P7 peptides (4 µM) for 5 min prior to stimulation with biot-bFGF weakened the signals detected in the cytoplasmic and nuclear fractions (Fig. 4, lanes 5 and 6), indicating that P7 peptides significantly inhibited bFGF internalization, which may partly contribute to the counteracting effects of P7 peptides, preventing the blockade of apoptosis by bFGF in the cells treated with CPT-11, and further reducing bFGF-induced resistance to CPT-11.

**P7 counteracts the regulatory effects of bFGF on the expression of apoptosis-related proteins in CPT-11-treated cells.** Given that P7 peptides attenuated the anti-apoptotic effects of bFGF in CPT-11-treated cells, and Bcl-2 family proteins play essential roles in modulating the process of apoptosis, western blot analysis was carried out to elucidate the involvement of Bcl-2 family proteins in the effects of P7 peptides on the apoptosis of HT-29 cells. As shown in Fig. 5, when the HT-29 cells were exposed to 60 µM CPT-11, the ratio of Bax to Bcl-2 increased. Pre-treatment with bFGF for 4 h prior to the administration of CPT-11 downregulated the ratio of Bax to Bcl-2, which was rescued by the addition of P7 peptides. Compared with the control, no significant change in Bcl-xL expression was observed in all the groups. These results suggest that the downregulation of the anti-apoptotic protein, Bcl-2, and the upregulation of the pro-apoptotic protein, Bax, may correlate with the attenuation of the anti-apoptotic effects of bFGF by P7 peptides in CPT-11-treated cells.

**Identification of proteins associated with the effects of P7 peptides on bFGF-induced resistance to CPT-11.** A proteomic approach based on 2-DE coupled with mass spectrometry was applied to identify proteins participating in the effects of P7 peptides on bFGF-induced resistance to CPT-11 in HT-29 cells. The protein profiles presented in Fig. 6 were first compared between CPT-11-treated and bFGF-stimulated HT-29 cells to select the specific proteins involved in the bFGF regulation of resistance to CPT-11, and then compared the intensities...
of the selected protein spots between bFGF-stimulated and P7 peptide-treated groups to determine the proteins associated with resistance to CPT-11 which were affected by P7 peptides targeting bFGF (Fig. 7). The selected differentially expressed protein spots were excised from the gels and then subjected to trypsin digestion; they were then analyzed by matrix-assisted laser desorption/ionization (MALDI)-TOF-TOF mass spectrometry. As shown in Table I, the selected proteins were confidently identified by searching the data collected from MALDI-TOF-TOF against the IPI human database with the Mascot online search tool. The expression of the identified protein, CK8, which was increased by bFGF stimulation, was downregulated by the addition of the P7 peptides.

qPCR was performed to validate the expression levels of CK8 identified by proteomic analysis. CK8 expression was upregulated by bFGF stimulation and decreased by the addition of the P7 peptides (Fig. 8). The result coincided with that obtained from proteomic analysis.
Table I. Differentially expressed proteins identified by MALDI-TOF-TOF mass spectrometry.

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Discussion

CRC is a type of lethal gastrointestinal malignancy. The main reason for its high mortality rate is the frequent relapse due to resistance to chemotherapeutic drugs. The development of novel targeted therapies will open up new opportunities to combat drug resistance. It was recently reported that bFGF plays an essential role in mediating chemoresistance, and thus may be served as a potential target for the development of new therapeutic strategies to combat the relapse of cancer (25,26).

We previously obtained a novel bFGF antagonist peptide, P7, using phage display technology (20,21). Our results indicated that P7 exerts strong inhibitory effects on bFGF-induced cell proliferation and angiogenesis by specifically inhibiting the binding of bFGF to its receptors. We speculated that the previously isolated P7 peptides targeting bFGF may also have potent inhibitory effects on bFGF-induced chemoresistance. Therefore, in this study, MTT assay was first carried out to investigate the effects of P7 peptides on bFGF-induced resistance to CPT-11. The results revealed that bFGF increased the survival rate of CPT-11-treated HT-29 cells; however, the survival rate decreased by the addition of P7 peptides, suggesting that P7 peptides reversed bFGF-induced resistance to CPT-11. Further analysis of the effects of P7 peptides on apoptosis, which is closely related to drug resistance in tumor cells, indicated that P7 peptides counteracted the anti-apoptotic effects of bFGF in CPT-11-treated cells.

The mechanisms responsible for the P7 peptides counteracting the anti-apoptotic effects of bFGF in CPT-11-treated cells were further explored by analyzing the effects of P7 peptides on the anti-apoptotic PI3K/Akt signaling pathway, bFGF internalization, and the expression of Bel-2 family members involved in regulating the intrinsic pathway for the activation of apoptotic effectors. The role of Bel-2 family members in regulating the response to chemotherapy has been extensively investigated. It has been demonstrated that the overexpression of the anti-apoptotic Bel-2 family protein, Bcl-2, or the loss of the pro-apoptotic Bcl-2 family member, Bax, expression decreases sensitivity to chemotherapeutic agents (27-30), whereas the downregulation of Bcl-2 or the overexpression of Bax sensitizes cancer cells to chemotherapeutic agents (29). The ratio of Bax to Bcl-2 is usually applied to determine the susceptibility of cancer cells to chemotherapeutic-mediated apoptosis (31). Our results demonstrated that decrease in Akt activation, the suppression of bFGF internalization and the increase in the Bax to Bcl-2 ratio induced by P7 peptides contributed to the counteracting of the anti-apoptotic effects of bFGF, and the reversal of bFGF-induced resistance to CPT-11.

Proteomic analysis has been proven to be an efficient strategy for the identification of proteins involved in various pathologies. Therefore, we applied this approach to identify proteins associated with the effects of P7 peptides on bFGF-induced resistance of HT-29 cells to CPT-11. The differentially expressed protein spots identified by 2-DE were subjected to analysis by MALDI-TOF-TOF mass spectrometry, and were confidently identified by searching the data collected from MALDI-TOF-TOF against the IPI human database with the Mascot online search tool (Table I). The identified protein, CK8, whose expression level was further confirmed by qPCR, belongs to the keratin family of intermediate filament proteins, located on the cell surface of a variety of tumor cells, including colon cancer, breast cancer and liver cancer cells. Wang et al reported that the suppression of CK8 enhanced the sensitivity of the cells to cisplatin, while the overexpression of CK8 provided resistance to cisplatin-mediated apoptosis in human nasopharyngeal carcinoma cells (32). Jaquemar et al demonstrated that CK8-deficient embryos were exquisitely sensitive to apoptosis (33). Previous studies have implicated CK8 in the resistance to tumor necrosis factor family receptor- and Fas-induced apoptosis (34,35). Moreover, the activation of Akt increases CK8 expression at the post-transcriptional level, and the knockdown of Akt decreases CK8 protein levels (36). Our proteomic analysis revealed that P7 peptides reversed the upregulation of CK8 expression which was induced by bFGF. Combined with the results that P7 peptides decreased Akt activation induced by bFGF, it is reasonable to speculate that the downregulation of Akt activation by P7 peptides may result in the decreased expression of CK8, which would partly counteract the anti-apoptotic effects of bFGF, and would thus attenuate the resistance of HT-29 cells to CPT-11 induced by bFGF.

It has been previously demonstrated that P7 peptides inhibit tumor growth and progression through the suppression of cell proliferation and angiogenesis stimulated by bFGF (20,21,37,38). In this study, we demonstrated that P7 peptides have the potential to reverse bFGF-induced chemoresistance. It seems reasonable to speculate that P7 peptides combined with chemotherapeutic agents, such as CPT-11, may not only exert inhibitory effects on cell proliferation and angiogenesis, but may also enhance the antitumor efficacy of chemotherapeutic agents, resulting in greater therapeutic benefits in cancers characterized by bFGF-induced chemoresistance.

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