



Original Article



Knockdown of growth differentiation factor-15 restrains prostate cancer through regulating MAPK/ERK signaling pathway

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Abstract



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Prostate cancer, prevalent among males, is influenced by various molecular factors, including Growth Differentiation Factor 15 (GDF15). Despite its recognized role in multiple tumor types, GDF15's specific involvement in prostate cancer remains insufficiently explored. This study investigates the regulatory function of GDF15 in prostate cancer. To explore GDF15's impact, we established GDF15 knockdown and overexpression models in prostate cancer cells. We quantified mRNA and protein levels using RT-PCR and Western blotting. Functional assays, including CCK8, Transwell, wound healing, and flow cytometry, were employed to evaluate cell proliferation, invasion, migration, and apoptosis. Additionally, the effect of GDF15 on tumor growth was assessed using a metastatic tumor model in nude mice. Elevated GDF15 expression was identified in prostate cancer tissues and cells. The knockdown of GDF15 led to the activation of the MAPK/ERK signaling pathway. C16PAF was found to counteract the inhibitory effects of sh-GDF15 on cell proliferation, invasion, migration, and apoptosis in LNCaP cells. It also reversed the sh-GDF15-induced alterations in the epithelial-mesenchymal transition (EMT) process. In vivo, C16PAF notably mitigated the sh-GDF15-induced suppression of tumor growth. The study demonstrated that sh-GDF15 inhibits cell proliferation, invasion, migration, EMT process, and tumor growth, while it promotes apoptosis. However, these effects were significantly reversed by C16PAF. The study underscores the potential of GDF15 as a target for novel therapeutic interventions in prostate cancer treatment and prevention. These findings illuminate GDF15's multifaceted role in prostate cancer pathogenesis and suggest its viability as a therapeutic target.

Keywords: GDF15, C16PAF, Prostate cancer, EMT.

1. Introduction

Prostate cancer is one of the common malignant tumors in men, which has a significant impact on their health and quality of life [1]. In the past few decades, the incidence rate of prostate cancer has continued to grow, becoming an important challenge to global male health [2]. Research shows that age is an important factor in the occurrence of prostate cancer, and the incidence rate of prostate cancer increases significantly with age [3]. In addition, family history and genetic factors are also related to the incidence rate of prostate cancer. Individuals with family history have a higher risk of prostate cancer [4]. Some studies also found that race and geographical location may also affect the incidence rate of prostate cancer [5].

Prostate cancer can be treated by various means, such as surgical resection, radiotherapy, hormone therapy, chemotherapy and targeted therapy according to different conditions [6]. For patients with early diagnosis, surgical resection is a common treatment method. For locally advanced or high-risk patients, radiotherapy can be used as an alternative or adjuvant treatment [7, 8]. Although there has been some progress in the treatment of prostate cancer, further in-depth research is still needed to improve unders-

tanding and treatment level of the disease.

Growth Differentiation Factor 15 (GDF15), also known as MIC-1 (Macrophage Inhibitory Cytokine 1) or PLAB (Placental Bone Morphogenetic Protein), is a multifunctional protein belonging to the transforming growth factor-beta (TGF- β) superfamily [9]. In recent years, extensive research has been conducted to investigate the regulatory role of GDF15 in different tumors [10]. The regulatory role of GDF15 in tumors is complicated. In certain cancer types, GDF15 has been shown to act as a tumor suppressor. It inhibits tumor growth and metastasis by inducing apoptosis and suppressing cell proliferation [11]. GDF15 can activate various signaling pathways to exert its anti-cancer effects. However, GDF15 can regulate immune responses within the tumor microenvironment. It has immunosuppressive properties, which can hinder the body's immune system from effectively attacking tumor cells [12]. By dampening immune surveillance, GDF15 creates an immunosuppressive environment that supports tumor growth and progression [13]. The regulatory function of GDF15 in prostate cancer remains unclear.

The MAPK/ERK (Mitogen-Activated Protein Kinase/ Extracellular Signal-Regulated Kinase) signaling pathway

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is a crucial intracellular pathway that plays diverse roles in different types of tumors [14]. It is a well-known pathway involved in cell proliferation, survival, differentiation, and migration. Here, we summarize the role of the MAPK/ERK signaling pathway in various types of tumors [15]. Silencing ACTG1 expression induces prostate cancer epithelial-mesenchymal transition through MAPK/ERK signaling pathway [16]. However, if GDF15 could affect prostate cancer through targeting MAPK/ERK signaling pathway has not been reported.

In this study, we constructed knockdown and overexpression models of GDF15 in LNCaP cells. Knockdown of GDF15 significantly suppressed cell proliferation, invasion, migration, EMT processing, and tumor growth, while promoting cell apoptosis. However, the impact of GDF15 was markedly reversed by C16PAF. We firstly demonstrated that knockdown of GDF15 could inhibit prostate cancer through regulating MAPK/ERK signaling pathway. Our findings may provide a novel therapeutic strategy for the prevention and treatment of prostate cancer through targeting MAPK/ERK signaling pathway.

2. Materials and methods

2.1. Cell culture

In our research, we employed various cell lines, namely RWPE-1, PC-3, DU145, and LNCaP, all acquired from ATCC, USA. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, #12491015, Langley, OK, USA), enriched with 5% Fetal Bovine Serum (FBS, Gibco, #10099141C, Langley, OK, USA), 40 µg/ml streptomycin, and 40 IU/ml penicillin, within an environment containing 5% CO₂ at 37°C. For experimental purposes, cells were treated with C16PAF at a concentration of 20 µM

2.2. Cell transfection

The study involved the use of sh-GDF15 and pcDNA-GDF15 vectors, along with their respective controls, sourced from Realgene. Transfections were conducted employing Lipofectamine 2000 (Invitrogen, #11668019, Carlsbad, CA, USA), as per the manufacturer's guidelines. To evaluate the efficacy of transfection, GDF15 mRNA levels were quantified using RT-PCR 48 hours post-transfection.

2.3. Immunofluorescence staining

For immunofluorescence staining, cells were initially fixed using 4% formaldehyde at room temperature for 25 minutes. This was followed by three PBS washes, each lasting 10 minutes. Cells were then treated with 0.2% Triton X-100 for 3 minutes and blocked using 5% BSA for 20 minutes. The application of the primary antibody occurred overnight at 4°C. Post-primary antibody application, the cells were exposed to the secondary antibody for 2 hours at room temperature and subsequently washed thrice with PBS. The stained cells were finally examined under a fluorescence microscope.

2.4. Transwell assay

The Transwell assay incorporated Matrigel gel (#356255, 1:5 diluted, Corning, Corning, NY, USA), refrigerated overnight at 4°C. The gel was diluted with serum-free DMEM, and 100 µL was applied to the surface of a PET membrane in a 24-well plate. Cells in the logarithmic

growth phase, at a concentration of 2.5×10^5 /ml, were washed with PBS, trypsinized, and resuspended in serum-free medium. The lower chamber was filled with 500 µL of DMEM containing 10% FBS, and the upper chamber received 500 µL of cell suspension. After a 24-hour incubation, the medium was discarded, cells fixed with 75% methanol for 40 minutes, and stained with 0.1% crystal violet (Sigma-Aldrich, #C0775, St. Louis, MO, USA) for 20 minutes. Non-invading cells were removed, and the remaining cells were counted under a microscope.

2.5. Western blotting

Protein lysates were prepared using RIPA buffer (Beyotime, #P0013B, Shanghai, China) containing a protein phosphatase inhibitor. Protein concentration was determined by the BCA method (Beyotime, #P0009, Shanghai, China). Equal protein amounts underwent 10% SDS-PAGE and were transferred to a PVDF membrane (Millipore, #IPVH00010, Billerica, MA, USA). The membrane, blocked with TBST containing 5% non-fat milk, was incubated with primary antibodies overnight at 4°C and with secondary antibodies for 2 hours at room temperature. Target proteins were detected using an enhanced chemiluminescence kit (Thermo Fisher, #32132, Waltham, MA, USA), with band analysis via ImageJ software.

2.6. Immunohistochemical staining

Tissue sections for immunohistochemical staining were microwaved for 3 minutes and treated with 5% hydrogen peroxide for 1 minute. Blocking occurred with 5% non-fat milk followed by primary antibody incubation (Ki67, ab15580, Abcam, Cambridge, MA, USA). Post three PBS washes, the secondary antibody (Goat anti-rabbit IgG, ab150077, Abcam, Cambridge, MA, USA) was applied for 3 hours. The sections, post-DAB chromogenic treatment, dehydration, and mounting, were visualized using an Olympus BX41 microscope.

2.7. Wound healing assay

In this assay, cells were prepared by trypsinization, followed by centrifugation at 1200 rpm for 5 minutes, and subsequently seeded at a density of 1×10^6 cells per well in a 6-well plate. After 24 hours of incubation to achieve approximately 90% confluence, a 200 µL pipette tip was utilized to create a linear scratch across the cell monolayer. Post-scratch, the cells were rinsed twice with PBS to clear non-adherent cells, thereby delineating the wound area. Fresh serum-free culture medium was added, and the wound gap was measured at 0 and 24 hours, facilitating analysis of cell migration.

2.8. Flow cytometry

For flow cytometry, cells in their logarithmic growth phase were washed with PBS (#10010023, Gibco, Langley, OK, USA), trypsinized (#108444, Sigma-Aldrich, St. Louis, MO, USA), and suspended in serum-free medium to achieve a concentration of 1×10^7 cells/ml. A 100 µL aliquot of this cell suspension was transferred to a microcentrifuge tube and mixed with Annexin V (Beyotime, #C1062L, Shanghai, China) and propidium iodide. This mixture was incubated at room temperature in the dark for 20 minutes and subsequently analyzed using the Guava® Muse® Cell Analyzer.

2.9. Reverse transcription polymerase chain reaction (RT-PCR)

RNA extraction from tissues was performed using TRIzol (#R0016, Beyotime, Shanghai, China), with RNA purity assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The Takara PrimeScript RT reagent kit with gDNA (#RR047A) eraser was employed for reverse transcription. RT-PCR was conducted on the Bio-Rad CFX96 system (Hercules, CA, USA), and relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method.

2.10. CCK8 assay

In the CCK8 assay (#CA1210, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), cells in their logarithmic phase were washed, trypsinized, and resuspended in serum-free medium to a density of 1×10^5 cells/ml. These cells were plated in a 96-well plate and incubated for 24 hours. Cell proliferation was assessed post 30-minute incubation with CCK8 reagent.

2.11. Bioinformatics analysis

To analyze the role of GDF15 in the survival and prognosis of tumor patients, we utilized GEPIA (<http://gepia.cancer-pku.cn/>) and TCGA (<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>) databases. These databases were mainly used to analyze the prognosis and expression of GDF15 in tumor tissues.

2.12. Statistical analysis

Data are presented as mean \pm standard deviation. Statistical significance was determined using Statistic Package for Social Science (SPSS) (Version 18, Chicago, IL, USA), with a p-value of less than 0.05 indicating significance. T-tests and ANOVA were utilized for statistical comparisons.

3. Results

3.1. Elevated Expression of GDF15 in Prostate Cancer

The GEPIA database analysis indicated differential GDF15 expression across various tumor types (Figures 1A-B), with a significant elevation observed in prostate cancer tissues and cells, particularly in PC-3, DU145, and LNCaP lines (Figures 1C-D). Moreover, higher GDF15 levels correlated with poorer prognosis (Figure 1E), highlighting its potential role in prostate cancer progression.

3.2. GDF15 knockdown activates MAPK/ERK signaling

We successfully developed and applied sh-GDF15 and pcDNA-GDF15 vectors in LNCaP cells (Figures 2A-B). Investigation into the impact of these constructs on the MAPK/ERK signaling pathway revealed a substantial activation by sh-GDF15, whereas pcDNA-GDF15 notably suppressed it (Figures 2C-D). Further exploration was conducted to ascertain if C16PAF could negate the sh-GDF15-induced activation of this pathway in LNCaP cells.

3.3. C16PAF counters sh-GDF15-induced cellular inhibition

Treatment with sh-GDF15 led to a marked decrease in Ki67 expression and cell proliferation (Figures 3A-B),

but these effects were significantly reversed by C16PAF, which enhanced both Ki67 expression and proliferation. Additionally, C16PAF effectively counteracted the sh-GDF15-induced reduction in cell invasion and migration (Figures 3C-D).

3.4. C16PAF mitigates sh-GDF15 effects on apoptosis and EMT

sh-GDF15 transfection resulted in increased cell apop-

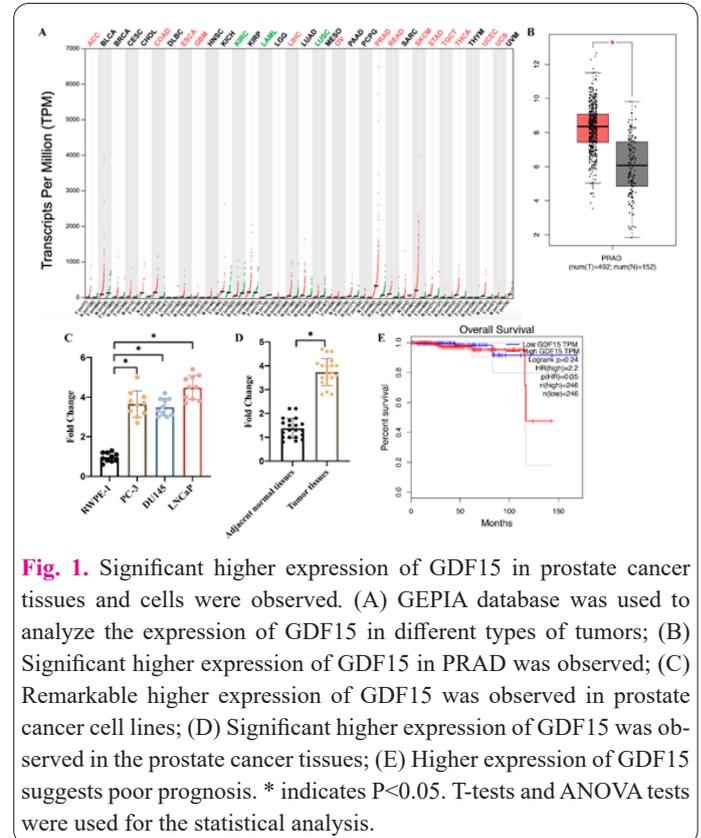


Fig. 1. Significant higher expression of GDF15 in prostate cancer tissues and cells were observed. (A) GEPIA database was used to analyze the expression of GDF15 in different types of tumors; (B) Significant higher expression of GDF15 in PRAD was observed; (C) Remarkable higher expression of GDF15 was observed in prostate cancer cell lines; (D) Significant higher expression of GDF15 was observed in the prostate cancer tissues; (E) Higher expression of GDF15 suggests poor prognosis. * indicates $P < 0.05$. T-tests and ANOVA tests were used for the statistical analysis.

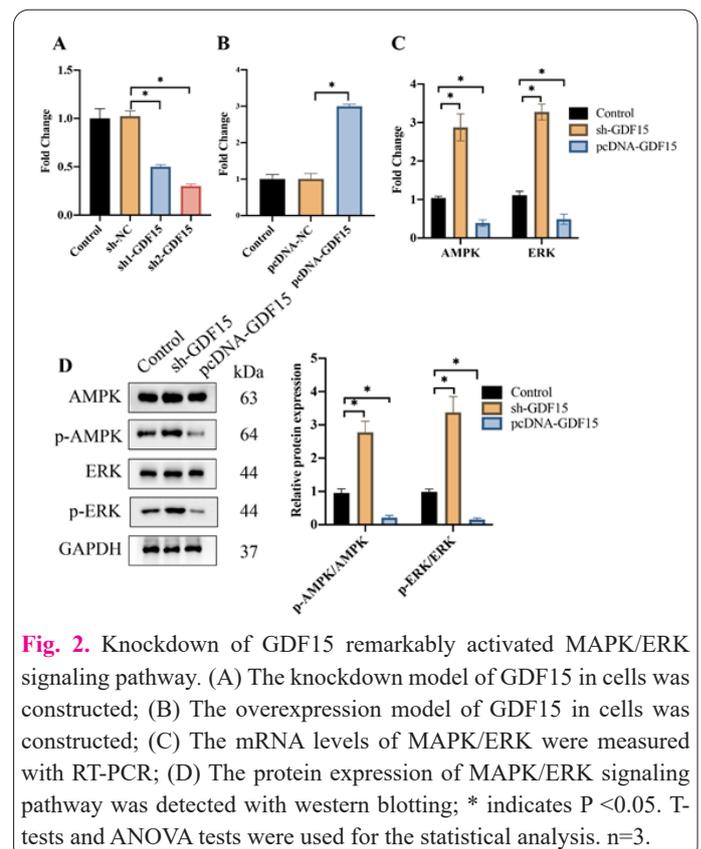


Fig. 2. Knockdown of GDF15 remarkably activated MAPK/ERK signaling pathway. (A) The knockdown model of GDF15 in cells was constructed; (B) The overexpression model of GDF15 in cells was constructed; (C) The mRNA levels of MAPK/ERK were measured with RT-PCR; (D) The protein expression of MAPK/ERK signaling pathway was detected with western blotting; * indicates $P < 0.05$. T-tests and ANOVA tests were used for the statistical analysis. $n=3$.

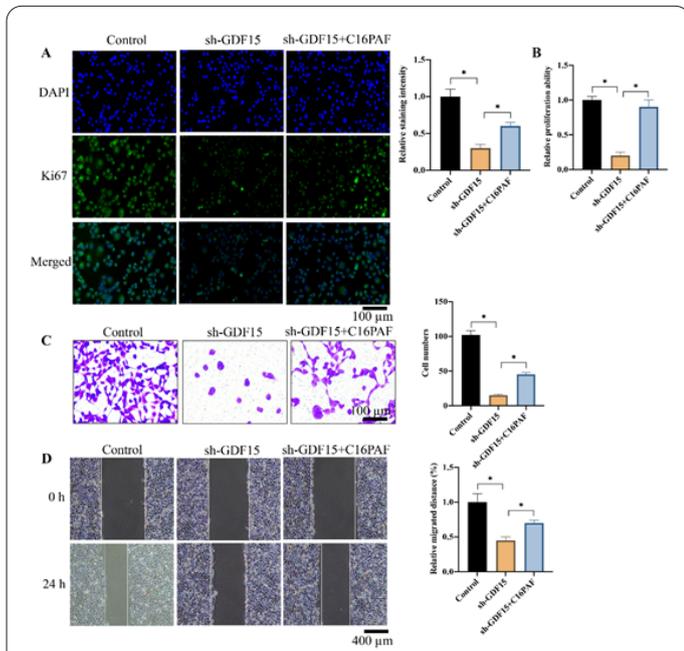


Fig. 3. C16PAF reversed the inhibition of cell proliferation, invasion, and migration of LNCaP cells caused by sh-GDF15. (A) The Ki67 expression in cells was measured with IHC staining (Magnification: 100×); (B) The cell proliferation ability was detected with CCK8 assay; (C) The cell invasion ability was evaluated with Transwell assay (Magnification: 100×); (D) The cell migration ability was evaluated with wound healing assay (Magnification: 40×). * indicates $P < 0.05$. ANOVA tests was used for the statistical analysis. $n = 3$.

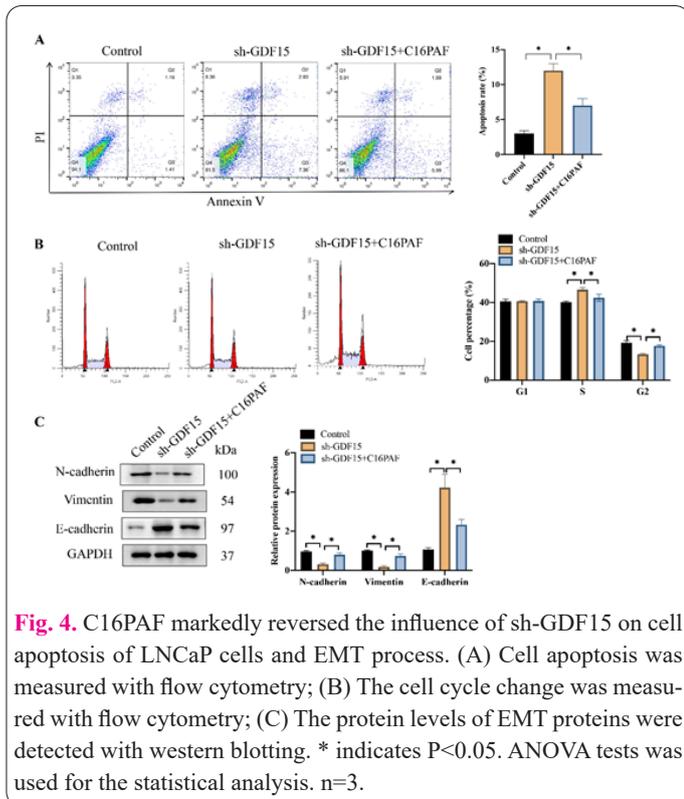


Fig. 4. C16PAF markedly reversed the influence of sh-GDF15 on cell apoptosis of LNCaP cells and EMT process. (A) Cell apoptosis was measured with flow cytometry; (B) The cell cycle change was measured with flow cytometry; (C) The protein levels of EMT proteins were detected with western blotting. * indicates $P < 0.05$. ANOVA tests was used for the statistical analysis. $n = 3$.

tosis and altered cell cycle dynamics, specifically causing arrest in the S phase and a decrease in the G2 phase population. C16PAF significantly reversed these effects (Figure 4B). Moreover, sh-GDF15 inhibited the epithelial-mesenchymal transition (EMT) process, an effect that was counteracted by C16PAF, evidenced by changes in N-cadherin, vimentin, and E-cadherin expression (Figure 4C).

3.5. C16PAF reverses sh-GDF15-induced tumor growth inhibition *in vivo*

In vivo, sh-GDF15 knockdown considerably reduced tumor weight and volume (Figures 5A-C) and suppressed Ki67 expression in tumor tissues. C16PAF treatment reversed these inhibitory effects of sh-GDF15 on tumor growth and Ki67 expression (Figures 5A-D).

3.6. C16PAF reverses sh-GDF15 effects on EMT and apoptosis proteins *in vivo*

Following sh-GDF15 transfection, EMT-related proteins (N-cadherin and vimentin) showed reduced expression, while E-cadherin levels increased (Figures 6A-C). sh-GDF15 also influenced apoptotic protein expression, elevating pro-apoptotic and reducing anti-apoptotic pro-

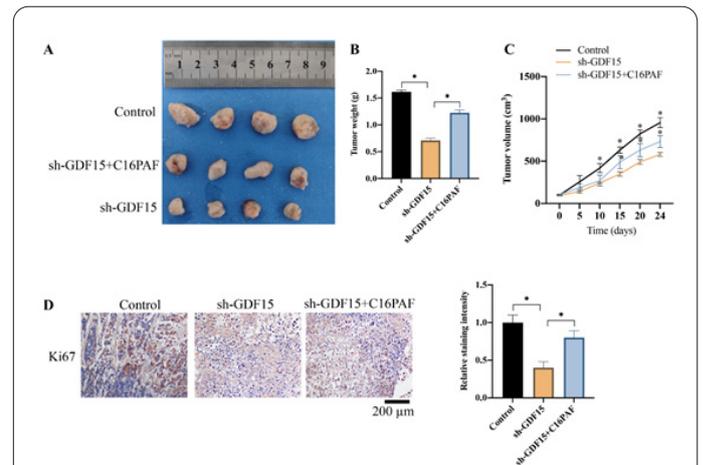


Fig. 5. C16PAF remarkably reversed the inhibition of tumor growth *in vivo* caused by sh-GDF15. (A) Metastatic tumor experiment was performed; (B) The tumor weight was recorded; (C) The tumor volume was analyzed; (D) The ki67 expression in the tumor tissue was evaluated (Magnification: 100×). * indicates $P < 0.05$. ANOVA tests was used for the statistical analysis. $n = 3$.

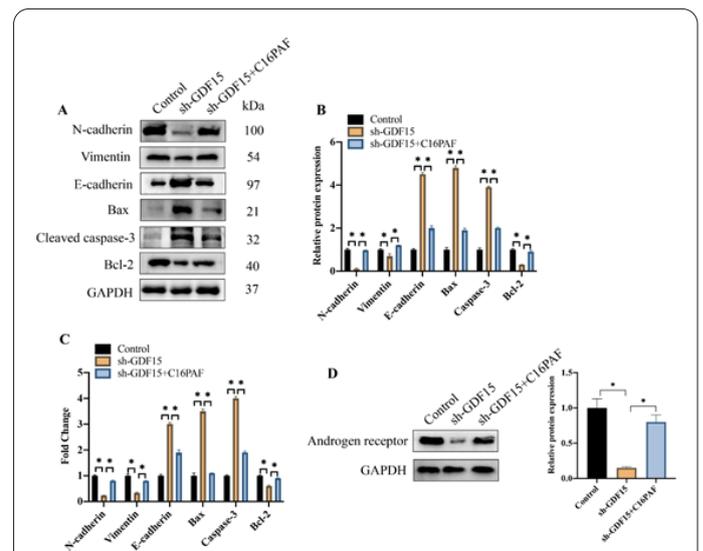


Fig. 6. C16PAF significantly reversed the inhibition of EMT process, and apoptosis-related protein expression caused by sh-GDF15 *in vivo*. (A) The EMT and apoptosis-related proteins were measured with western blotting; (B) The protein expression was analyzed; (C) The mRNA expression of EMT and apoptosis-related proteins was measured with RT-PCR; (D) The protein expression of androgen receptor was measured. * indicates $P < 0.05$. ANOVA tests were used for the statistical analysis. $n = 3$.

teins. These alterations were reversed by C16PAF treatment (Figures 6A-C). Additionally, androgen receptor levels, crucial in tumor progression, were modulated by GDF15 knockdown and C16PAF treatment (Figure 6D).

4. Discussion

It is important to note that GDF15's regulatory role in different tumors can vary depending on the cancer type, stage, and microenvironment [17]. In some cancer types, increased GDF15 expression has been linked to resistance to certain cancer therapies, such as chemotherapy and radiation [18]. High GDF15 levels can promote cancer cell survival and reduce the effectiveness of treatment, leading to treatment resistance [19]. As a result, the precise mechanisms by which GDF15 influences tumorigenesis and tumor progression are still being extensively studied [20]. Understanding the complexities of GDF15's functions in various cancers may provide valuable insights for developing targeted therapies and precision medicine approaches to combat cancer effectively [21]. In this research, we found that knockdown of GDF15 could suppress the development of prostate cancer.

MAPK/ERK signaling pathway has been proven to be closely related to the initiation and progression of prostate cancer [22]. The MAPK/ERK pathway can modulate androgen receptor activity in prostate cancer cells [23]. ERK activation can enhance androgen receptor signaling, leading to increased transcription of androgen-responsive genes that inhibit cell survival and growth [24]. In this research, sh-GDF15 greatly inhibited MAPK/ERK signaling pathway and androgen receptor expression, which might be the potential mechanism inhibiting prostate cancer.

EMT is a process by which epithelial cells acquire mesenchymal characteristics, contributing to cancer cell migration and invasion [25]. MAPK/ERK signaling has been implicated in promoting EMT in prostate cancer, thereby enhancing the invasive and metastatic potential of cancer cells [26]. We also found that sh-GDF15 greatly inhibited the EMT process. Activation of the MAPK/ERK pathway is associated with resistance to various therapies in prostate cancer, including androgen deprivation therapy and chemotherapy. Targeting GDF15/MAPK/ERK axis in combination with standard therapies may overcome resistance and improve treatment outcomes.

The androgen receptor signaling pathway plays a key role in the pathogenesis of prostate cancer. Androgen deprivation has always been the main treatment for advanced prostate cancer [27]. However, it has been found in clinical practice that after a period of androgen deprivation treatment, androgen resistance can lead to disease progression and failure of endocrine therapy, suggesting that there may be other factors involved in the pathogenesis of the disease [28]. We demonstrated that C16PAF treatment significantly reversed the inhibition of androgen receptor expression caused by sh-GDF15, which might be the potential function mechanism. There are several limitations in this research. (1) More clinical data need to be integrated to validate our conclusions. (2) How GDF regulate the prostate cancer was not clarified.

5. Conclusion

The knockdown of GDF15 had significant suppressive effects on cell proliferation, invasion, migration, EMT process, and tumor growth, while it accelerated cell apoptosis.

However, these effects of sh-GDF15 were remarkably reversed by C16PAF. We first demonstrated that knockdown of GDF15 could inhibit prostate cancer through regulating MAPK/ERK signaling pathway. These findings highlight the potential of targeting GDF15 as a novel therapeutic strategy for the prevention and treatment of prostate cancer.

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Availability of data and material

The data and material used to support the findings of this study are included in the manuscript and supplementary files.

Ethical approval and consent to participate

The experimental protocol was approved by Zhangzhou Affiliated Hospital of Fujian Medical University (2020LWB143).

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no conflicts of interest.

Authors' contributions

MY and ZX conceived and designed the experiments; MY, MG, CS, WH performed the experiments; ZX wrote the paper.

Abbreviations

growth differentiation factor 15 (GDF15); MAPK/ERK (Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase).

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