Am J Cancer Res 2025;15(6):2779-2793
www.ajcr.us /ISSN:2156-6976/ajcr0165990

Original Article

GDF15 promotes gallbladder cancer progression by activating the NF-κB mediated Vascular Endothelial Growth Factor A (VEGFA) expression

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Received May 12, 2025; Accepted June 23, 2025; Epub June 25, 2025; Published June 30, 2025

Abstract: Growth differentiation factor 15 (GDF15) has been found to be elevated in several different types of cancer, thus demonstrating its potential for use as a biomarker. Although its physiological and pathophysiological roles in cancer are increasingly understood, the specific functions and molecular mechanisms of GDF15 in gallbladder cancer remain unclear and require further investigation. Immunohistochemical staining was performed to evaluate the expression of GDF15 in tissue samples from 57 patients with gallbladder cancer. The biological function of GDF15 and the molecular mechanism underlying this were further elucidated through knockdown experiments in NOZ and OCUG-1 gallbladder cancer cell lines. Our results demonstrate that there was a significant correlation between high GDF15 expression and poor survival indicating a poor prognosis in individuals with gallbladder cancer. NanoString analysis results showed that VEGFA, a key angiogenic factor, was significantly upregulated in the GDF15 high-expression group. Moreover, GDF15 knockdown significantly reduced cell motility, as well as migration and invasion. Additionally, GDF15 knockdown in gallbladder cancer cells decreased VEGFA expression via the AKT/NF-κB pathway. Taken together, these results suggest that GDF15 contributes to the aggressive behavior of gallbladder cancer by promoting activation of the AKT/NF-κB pathway. These findings suggest that the GDF15 signaling pathway may represent a promising therapeutic target for gallbladder cancer treatment.

Keywords: GDF15, gallbladder cancer, VEGFA, AKT/NF-κB pathway, prognosis

Introduction

Gallbladder cancer, a pressing health problem worldwide, is less common than other forms of cancer but is one of the most aggressive malignancies, accounting for 80-95% of biliary tract cancers [1, 2]. As reported in recent studies, the 5-year relative survival rate for gallbladder cancer remains very low, less than 20% [3]. Despite the recognized importance of early intervention, the lack of early symptoms often delays diagnosis until the disease is at an advanced stage, resulting in very low 5-year survival rates [2, 4, 5]. This poor prognosis is further exacerbated by the aggressive biological behavior of gallbladder cancer, which presents challenges to effective treatment. The above findings highlight the urgent need for a deeper understanding of the molecular mechanisms underlying gallbladder cancer to enable the discovery of novel therapeutic targets and early diagnostic biomarkers. Therefore, understanding the molecular mechanisms underlying gallbladder cancer progression is crucial for developing novel biomarkers and therapeutic strategies.

Growth differentiation factor 15 (GDF15), a member of the transforming growth factor β (TGF-β) family, is a potential biomarker due to its significant upregulation in response to tissue injury, inflammation-related diseases, and cancer [6, 7]. The authors of previous studies have investigated the role of GDF15 in gastric, pancreatic, and colorectal cancers, with it being implicated in disease progression, angio-

https://doi.org/10.62347/NAZM2261

genesis, and therapeutic resistance; however, its role in gallbladder cancer remains unclear [8-13]. Therefore, investigating the expression pattern of GDF15 and its impact on gallbladder cancer progression and prognosis may reveal new insights and therapeutic approaches.

Vascular Endothelial Growth Factor A (VEGFA) also plays an important role in cancer development, promoting angiogenesis, which is essential for tumor growth and metastasis [14-18]. Pathological angiogenesis provides tumors with the blood supply they need to sustain rapid growth and promote metastatic spread [19, 20]. Considering the fact that cancer is characterized by uncontrolled cell proliferation, a robust and adaptable vascular supply is essential [18]. Angiogenesis not only supports tumor growth by providing essential oxygen and nutrients but also promotes metastasis through the development of abnormal tumor vasculature [21, 22].

The authors of previous studies have found an association between GDF15 and VEGFA expression in other types of cancer, suggesting a possible interaction that may similarly affect gallbladder cancer progression [23-25]. Therefore, given that angiogenesis inhibition is a promising therapeutic strategy for treating various types of cancer, including gallbladder cancer, targeting the GDF15-VEGFA axis may provide a novel therapeutic approach [26-29]. This strategy aims to inhibit new angiogenesis and disrupt the supply of nutrients and oxygen to the tumor, thereby hindering tumor growth and the potential for metastasis [30, 31]. Therefore, in this study, we aimed to investigate the biological role of GDF15 in gallbladder cancer progression, particularly to elucidate the molecular mechanisms such as regulation of VEGFA expression and its potential involvement in angiogenesis. By characterizing GDF15 as a core biomarker and exploring its tumorigenic functions, we sought to provide new insights into the developmental mechanisms of gallbladder cancer and identify potential therapeutic targets.

Materials and methods

Clinical specimens

Specimens were obtained from 57 patients diagnosed with gallbladder cancer at Chungnam National University Hospital (CNUH) in Daejeon, Republic of Korea. All patients underwent preoperative computed tomography (CT) and magnetic resonance imaging (MRI) to determine resectability. The collection and use of all samples in this study were approved by the Ethics Committee of Chungnam National University Hospital. Comprehensive clinicopathological characteristics were examined, including age, gender, pathological T stage, nodal metastasis, differentiation, perineural invasion, and lymphatic invasion. Patient staging was performed in accordance with the tumor node metastasis (TNM) staging system, and all specimens were subsequently subjected to pathologic analysis for confirmation. This study was conducted in accordance with the Declaration of Helsinki and principles of good clinical practice.

Tissue microarray construction

Tissue microarrays (TMAs) were constructed using formalin-fixed, paraffin-embedded tissue blocks from 57 patients with gallbladder cancer. Representative tumor areas were selected from hematoxylin and eosin (H&E)-stained sections of the donor paraffin blocks. Two cylindrical cores of 2 mm diameter from each tumor were obtained using an automated tissue microarrayer (3DHistech, Budapest, Hungary), and TMAs were constructed using the TMA Grand Master system (3DHistech) according to the manufacturer's instructions.

Specimen preparation and immunohistochemistry

Specimen preparation and immunohistochemistry were performed based on previously reported protocols [32]. For immunohistochemical staining, 3 μm thick sections were cut from recipient blocks. Briefly, the sections were dewaxed in xylene and then re-hydrated in graded alcohols. After rehydration, the sections were washed with water for antigen retrieval. Antigen retrieval was performed at 97°C for 20 min in 10 mM sodium citrate buffer (pH 6.0) using a Dako PTLink machine (Dako, Glostrup, Denmark). To inactive endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide for 10 min and then incubated with serum-free protein block solution (Dako) for 20 min to remove background staining. Human GDF15 (NBP1-81050, Novus

Biologicals, Littleton, MA, USA) was diluted 1:200 using a polyclonal rabbit antibody with background reducing diluent (Dako). The samples were incubated overnight in a humid chamber at 4°C. After washing with TBS-T, slides were incubated with EnVision anti-rabbit polymer (Dako) for 30 min. The reaction products were visualized using diaminobenzidine (DAB) plus substrate - chromogen solution for 5 min. The slides were then counterstained with Mayer's hematoxylin, mounted, and carefully rinsed with several changes of phosphate-buffered saline (PBS) between procedure steps. The negative controls consisted of excluding the primary antibody or using pre-immune IgG1 to assess non-specific staining.

Evaluation of immunohistochemical staining

The evaluation of immunohistochemical staining was performed according to previously reported protocols [32]. Immunohistochemical staining results were evaluated by a pathologist who was blinded to the clinicopathological characteristics of the patients. Immunohistochemical staining was classified according to intensity as 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. In the case of heterogeneous staining, if more than 50% of cells showed higher intensity, the higher score was used, and the average score was obtained by averaging the scores of two tumor cores for each patient. Patients with scores of 0-1 were classified into the GDF15 low-expression group, and patients with scores of 2-3 were classified into the GDF15 high-expression group.

Cell lines and cell culture

The human gallbladder cancer cell lines NOZ and OCUG-1 were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine se-rum (FBS) and 1% penicillin-streptomycin. Cell lines used in this study were selected based on laboratory availability. The cultures were maintained in a humidified incubator at 37°C under 5% CO_2 and 95% relative humidity.

Reagents and antibodies

Antibodies against GDF15 (HPA011191; Sigma, St. Louis, MO, USA), GAPDH (sc-25778; Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-cadherin (3195; Cell Signaling Technology, Danvers, MA, USA), Ep-cam (sc-25308; Santa Cruz Biotechnology), Vimentin (3932; Cell Signaling Technology), Slug (9585; Cell Signaling Technology), Twist (sc-81417; Santa Cruz Biotechnology), MMP2 (87809; Cell Signaling Technology), MMP9 (13667; Cell Signaling Technology), VEGFA (sc-152; Santa Cruz Biotechnology), p-AKT (9271; Cell Signaling Technology), AKT (9272; Cell Signaling Technology), p-STAT3 (9134; Cell Signaling Technology), STAT3 (9132; Cell Signaling Technology), p-JNK (9251; Cell Signaling Technology), JNK (9252; Cell Signaling Technology), p-p65 (3033; Cell Signaling Technology), and p65 (8242; Cell Signaling Technology) were used in the Western blot analysis.

GDF15 knockdown in gallbladder cancer cell lines

GDF15 knockdown in gallbladder cancer cell lines was performed according to previously reported protocols [8]. GDF15 knockdown in gallbladder cancer cells was achieved with the pLKO.1-puro lentiviral vector (Clontech, Mountain View, CA, USA) via lentiviral-mediated transduction of GDF15 siRNA. For stable transfection, the lentiviral vectors were co-transfected with virus mix (Sigma) into HEK-293T cells (Clontech) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Viruses were harvested from the supernatant and concentrated with Lenti-X-Concentrator (Clontech), and the concentrated virus was added to NOZ and OCUG-1 cells together with 5 μg/mL polybrene (Santa Cruz). After 20 h, the medium was replaced with fresh medium containing 3 μg/mL puromycin (Sigma). GDF15 knockdown expression was confirmed via Western blot and RT-PCR analysis.

Quantitative Real-Time PCR (RT-qPCR)

RT-qPCR was performed according to previously reported protocols [8]. Briefly, isolation of total RNA was performed using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. cDNA was synthesized from total RNA using cDNA qPCR RT Master Mix (Toyobo, Osaka, Japan), and this procedure was performed using the QuantiSpeed SYBR No-Ros kit

(PhileKorea, Seoul, Republic of Korea) on a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) ac-cording to the manufacturer's instructions. All experiments were performed in triplicate, and the relative expression levels of target mRNAs were calculated using the 2-ΔΔCT method normalized to GAPDH levels. The primer sequences used were as follows: gdf15 forward: 5'-CTCCAGATTCCGAGAGTTGC-3', reverse: 5'-ACCTGCACCTGCGTATCTCT-3'; gapdh forward: 5'-TTGATTTTGGAGGGATCTCG'3', reverse: 5'-GAGTCAACGGATTTGGTCGT-3'.

Western blot analysis

Western blot analysis was performed according to previously reported protocols [8]. Briefly, the cells were lysed in RIPA buffer (LPS Solution, Daejeon, Republic of Korea) supplemented with phosphatase inhibitor cocktail (Roche, Basel, Switzerland) and protease inhibitor cocktail (Sigma). Subsequently, cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Pall Corp., Port Washington, NY, USA). The membranes were blocked with ProNA™ General-block solution (Translab, Daejeon, Republic of Korea) for 1 h at room temperature and then incubated overnight at 4°C with the indicated primary antibodies. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (dilution 1:5000, catalog no. 7074; Cell Signaling Technology) and anti-mouse IgG (dilution 1:5000, catalog no. 7076; antibodies diluted in blocking solution for 1 h at RT. The presence of immunoreactive polypeptides was confirmed using an enhanced chemiluminescence substrate (Advansta, Menlo Park, CA, USA).

In vitro cell proliferation assay

The cell proliferation assay was performed according to previously reported proto-cols [8]. Briefly, 3×10^3 cells were seeded in a 96-well plate and measured using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD, USA). Cell proliferation assessment was performed at 24 h intervals for 4 days, and absorbance was measured at 450 nm using a microplate reader (Molecular Devices, San Jose, CA, USA).

Clonogenic assay

The clonogenic assay was performed according to previously reported protocols [8]. Briefly, 1×10^3 cells were seeded in 6-well plates, and when the colonies reached an appropriate size, the plates were rinsed three times with PBS and then fixed overnight in 10% formalin at 4°C. Subsequently, the colonies were stained with 0.1% crystal violet for 1 h at room temperature. To determine the relative number of colonies, crystal violet was eluted using 70% alcohol, and absorbance was measured at 595 nm using a microplate reader (Molecular Devices).

Gap closure assay

The gap closure assay was performed according to previously reported protocols [8]. Briefly, 5×10^4 cells were seeded on both sides of the chamber using culture inserts designed for live cell analysis (Ibidi, Munich, Germany). After culturing the cells for 24 h, the culture inserts were removed, the cells were cultured in fresh culture medium for 10 h, and their behavior and interactions were observed.

Migration and invasion assays

Migration and invasion assays were performed according to previously reported protocols [8]. Briefly, the migration and invasion assays were conducted using 8 μm pore Transwell chambers (Corning, Inc., NY, USA). For the migration assay, the lower part of the chamber was coated with 0.1% gelatin (Sigma Aldrich, Merck KGaA), and for the invasion assay, the upper part of the chamber was coated with 25 μg/mL of Matrigel (BD Biosciences, San Jose, CA, USA). The upper chamber was supplied with serum-free medium, and 1×10^5 cells were seeded in each well; the lower chamber was filled with culture medium containing 10% FBS. For the migration assay, the chambers were incubated for 24 h, and for the invasion assay, they were incubated for 48 h at 37°C. After incubation, cells present on the upper surface of the Transwell membrane were removed using a cot-ton swab, fixed with 10% formalin, and stained with 0.1% crystal violet. For each analysis, the number of cells in five randomly selected fields was counted to determine the extent of migration and invasion and the number of cells passing through the chamber was

counted under a microscope (IX71; Olympus, Tokyo, Japan).

Transmission Electron Microscope (TEM)

Cultured cells were washed with PBS and fixed in 3% glutaraldehyde in PBS. The cells were then collected using a scraper and centrifuged at 4000 rpm for 10 s to allow them to clump together. The supernatant was carefully discarded, and the fixative was added slowly. The samples were then sealed with Parafilm. Lastly, the samples were prepared for transmission electron microscopy (TEM) analysis at the Korea Basic Science Institute.

Gene expression omnibus (GEO Database)

Data from the experiments are expressed as means ± the standard error of the mean (SEM). Differences between groups were analyzed using the Student's *t*-test. *P*-value < 0.05 was considered to indicate statistical significance. Each mean was calculated from at least three independent experiments.

Statistical analyses

GEO data retrieval: Two GDF15 expression profiling datasets (GSE100363 and GSE132223) associated with gallbladder cancer were retrieved from the GEO data portal (https://www.ncbi.nlm.nih.gov/geo/accessed on August 31, 2023.). The first dataset, GSE100363, was analyzed using next-generation sequencing to study circ-RNA expression [33]. The second dataset, GSE132223, consisted of expression profiles of gallbladder cancer liver metastatic tumors, primary tumors, and adjacent non-tumor tissues [34]. These datasets included GDF15 expression data from gallbladder cancer patients and normal tissues.

NanoString nCounter assay

PhileKorea Technology conducted the NanoString nCounter PanCancer Progression gene expression analysis, which resulted in 770 mRNAs being identified. Differential gene expression analysis was conducted on data obtained from nCounter analysis using nSolver version 2.0 (NanoString Technologies, Seattle, WA, USA). According to the nSolver 2.0 Analysis Software User Manual, all reporter code count files from the nCounter instrument passed the default quality control settings. Raw gene expression data were normalized using positive control and housekeeping genes. Fold-change and false discovery rate (FDR)-adjusted *P*-values were calculated by averaging the normalized lanes in the normal control group.

Bioinformatics analysis

Gene expression levels were correlated with Spearman coefficients using DAVID (version 6.8; https://david.ncifcrf.gov/accessed on 30 September 2023). KEGG pathway enrichment analysis was performed using a reference gene set for humans. Only significantly enriched paths with an FDR < 0.05 were selected.

Statistical analysis

Statistical analyses were performed using Prism software (version 5.01, GraphPad Software, Inc., San Diego, CA, USA). An unpaired two-tailed Student's *t*-test was used to evaluate differences between two groups. For comparisons involving multiple datasets, one-way analysis of variance and Dunnett's test were used. Each experiment was performed at least three times, and data are expressed as the mean ± standard deviation (SD) or ± standard error of the mean (SEM). Statistical significance is indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and NS: not significant.

Ethics statement

Human biospecimens used in this study were provided by the Biobank of Chungnam National University Hospital, a member of the Korea Biobank Network. The study was approved by the Institutional Review Board of Chungnam National University Hospital (IRB No. CNUH 2021-03-090), and informed consent was waived due to the use of fully anonymized samples.

Results

GDF15 is upregulated in human gallbladder cancer tissue compared to normal tissue

To investigate the role of GDF15 in gallbladder cancer, we analyzed GDF15 expression in tumor specimens obtained from 57 gallbladder cancer patients. Histological and immunohisto-

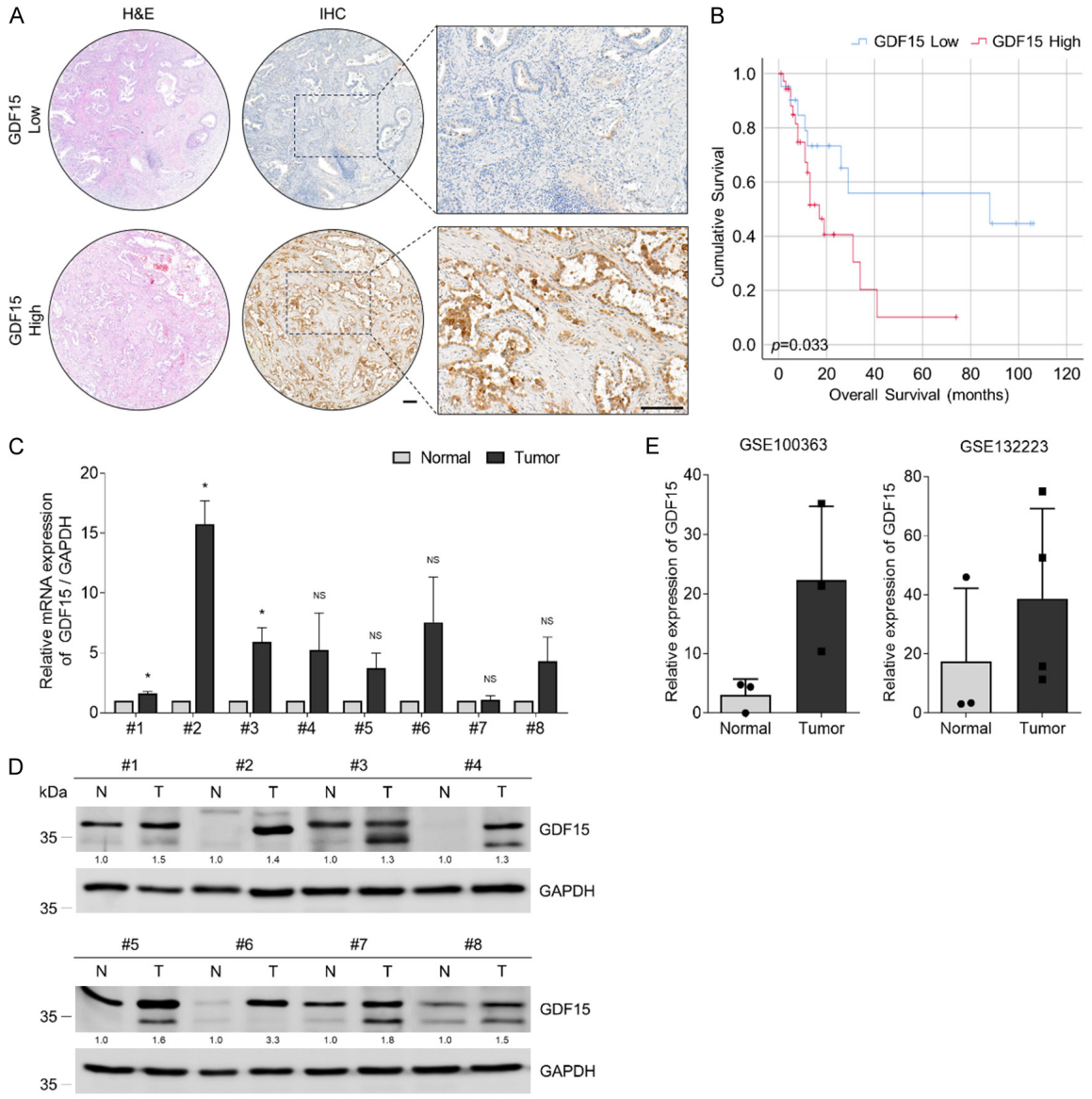

Figure 1. GDF15 expression in normal human gastric tissue and gallbladder cancer. A. Expression of GDF15 in human gallbladder cancer tissues. Representative H&E staining and immunohistochemical staining with anti-GDF15 (magnification: × 100). Scale bar, 100 um. Upper, low expression; lower, high expression. B. Survival analysis based on the Kaplan-Meier plot showed that high expression of GDF15 was associated with lower overall survival compared to low expression in gallbladder cancer patients ($P = 0.033$). C. The expression of GDF15 was confirmed using RT-qPCR in adjacent normal (light gray) and tumor (dark gray) gallbladder tissues. D. GDF15 expression was confirmed in adjacent normal and tumor gallbladder tissues. E. Boxplots showing the differential expression of GDF15 in tumor (dark gray) compared to paired normal tissues (light gray) in the GSE100363 and GSE132223 datasets. Data are presented as the mean ± SD and were evaluated using Student's *t*-test. NS: not significant. *$P < 0.05$.

chemical analysis results showed that GDF15 was present in the cytoplasm and/or membrane with variable staining intensity. Based on the immunohistochemical analysis results, these patients were classified into two groups: the GDF15 low-expression group (21 cases) and the GDF15 high-expression group (36 cases) (**Figure 1A**). Additionally, an analysis was performed to evaluate the relationship between GDF15 expression and several clinicopathological factors that could potentially affect the prognosis of patients with gallbladder cancer. Kaplan-Meier analysis results showed that elevated GDF15 levels were sig-

Table 1. Relationship of GDF15 expression and clinicopathological characteristics of gallbladder carcinoma

	GDF15 expression			
	Total (*n = 57*)	Low (*n = 21*)	High (*n = 36*)	*P*-value
Age, years				0.198*
< 65	16	8 (38.1%)	8 (22.2%)	
≥ 65	41	13 (61.9%)	28 (77.8%)	
Gender				0.707*
Male	28	11 (52.4%)	17 (47.2%)	
Female	29	10 (47.6%)	19 (52.8%)	
Pathologic T stage				0.063†
1	6	4 (19.0%)	2 (5.6%)	
2	31	12 (57.1%)	19 (52.8%)	
3	19	5 (23.8%)	11 (38.9%)	
4	1	0 (0.0%)	1 (2.8%)	
Nodal metastasis				0.169*
Absent	43	18 (85.7%)	25 (69.4%)	
Present	14	3 (14.3%)	11 (30.6%)	
Differentiation				0.062†
G1	4	3 (14.3%)	1 (2.8%)	
G2	32	14 (66.7%)	18 (50.0%)	
G3	16	2 (9.5%)	14 (38.9%)	
G4	5	2 (9.5%)	3 (8.3%)	
Perineural invasion				0.059*
Absent	26	13 (61.9%)	13 (36.1%)	
Present	31	8 (38.1%)	23 (63.9%)	
Lymphatic invasion				0.008*
Absent	20	12 (57.1%)	8 (22.2%)	
Present	37	9 (42.9%)	28 (77.8%)	

**P*-values were calculated by pairwise comparisons from χ^2 test. †*P*-values were calculated by comparisons of four groups from linear-by-linear associations.

nificantly correlated with poor survival in these patients (**Figure 1B**). We also assessed the expression of GDF15 in eight pairs of gallbladder cancer tissues and adjacent normal tissues from Chungnam National University Hospital (CNUH) using RT-qPCR (**Figure 1C**). Expression levels of GDF15 were normalized to GAPDH, and the results were expressed as fold-change in tumor tissue compared to matched adjacent normal tissue. The analysis showed increased GDF15 expression in seven out of eight gallbladder cancer tissue samples compared to paired adjacent normal tissues. We also used Western blot analysis to determine the expression level of GDF15 (**Figure 1D**). Compared with normal tissues, we found that tumor tissues exhibited higher levels of GDF15 expression. Furthermore, we analyzed a dataset from the Gene Expression Omnibus (GEO). Specifically, we evaluated GDF15 expression in gallbladder cancer tissue and adjacent non-tumor tissue using datasets GSE100363 and GSE132223 (**Figure 1E**). Additionally, analysis of clinicopathological characteristics revealed a significant association between high GDF15 expression and lymphovascular invasion (P = 0.008) (**Table 1**). Tumors with high GDF15 expression tended to show higher T stage, nodal metastasis, higher grade, and perineural invasion compared to GDF15 low-expression tumors. These data suggest that GDF15 expression is associated with gallbladder cancer progression.

GDF15 reduces gallbladder cancer cell proliferation and migration

To confirm the functional role of GDF15 in gallbladder cancer progression, we con-ducted GDF15 knockdown experiments in NOZ and OCUG-1 cells. The expression level of GDF15 was evaluated through Western blot analysis in GDF15 knockdown cells (**Figure 2A**). Expression levels of GDF15 were assessed by Western blot analysis in GDF15 knock-down cells, detecting mainly ~35 kDa pro-GDF15, the unprocessed precursor form of the protein. Furthermore, GDF15 knockdown resulted in reduced cell growth, as shown by the results of the CCK-8 assay (**Figure 2B**) and colony formation assay (**Figure 2C**). Using a wound-healing assay to assess cell motility, the results showed that GDF15 inhibition resulted in decreased cell motility in GDF15 knockdown cells (**Figure 2D**). Additionally, compared to the controls, cell migration and invasion capabilities were significantly reduced in GDF15 knockdown cells (**Figure 2E**, **2F**). GDF15 knockdown also altered the ex-pression levels of epithelial and mesenchymal markers, indicating regulation of epithelial-to-mesenchymal transition (EMT). Specifically, the protein expression of epithelial markers, including E-cadherin and Ep-cam, was upregulated in GDF15 knockdown cells compared to the control cells. Conversely, the

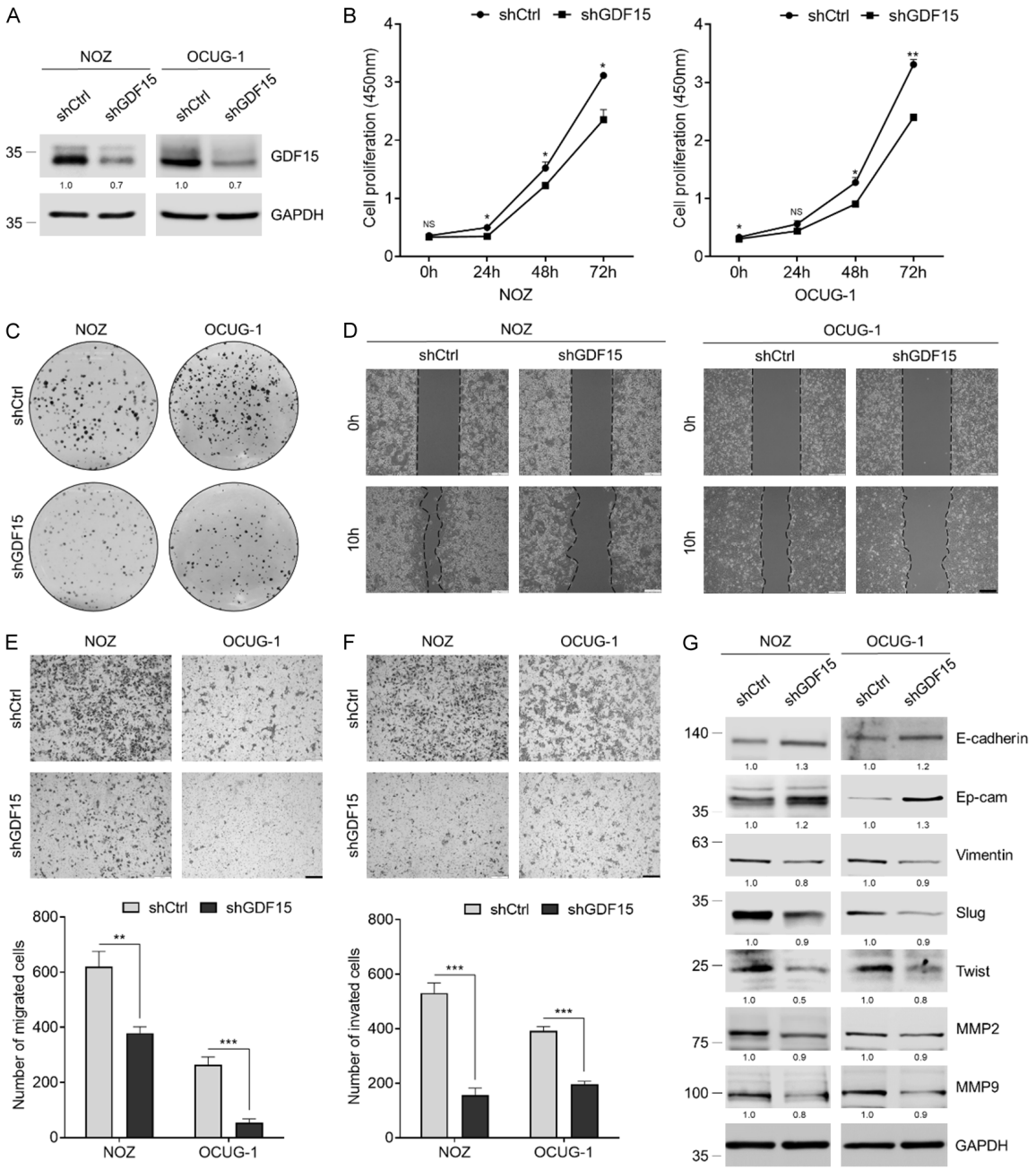

Figure 2. GDF15 knockdown decreases cell proliferation, migration, and invasion. A. Western blot analysis of GDF15 in GDF15 knockdown cells and shCtrl cells. B. The Cell proliferation rate was determined by absorbance measurement using GDF15 knockdown and shCtrl cells. Absorbance was measured using a spectrophotometer at 450 nm (n = 3). C. Colony assays were performed in 6-well culture plates using GDF15 knockdown and shCtrl cells. The cells stained with crystal violet were dissolved in 70% alcohol, and absorbance was measured using a spectrophotometer at 595 nm (n = 3). D. Wound-healing assays were performed in GDF15 knockdown cells (light gray) and shCtrl cells (dark gray) (n = 3). Scale bars, 100 μm. E, F. Transwell migration and invasion assays in GDF15 knockdown cells and shCtrl cells (n = 3). Five randomly selected fields were quantified, and representative images are provided. Scale bar, 50 μm. G. Western blot analysis of epithelial cell markers, including E-cadherin and Ep-cam, and mesenchymal cell markers, including Vimentin, Slug, and Twist and MMP2 and MMP9 in GDF15 knockdown and shCtrl cells. Data are reported as the mean ± SD and were analyzed using Student's *t*-test. NS: not significant. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

expression of mesenchymal markers, including Vimentin, Slug, and Twist, was downregulated in GDF15 knockdown cells com-pared to the controls (**Figure 2G**). These results suggest that

GDF15 promotes EMT in gallbladder cancer cells, thereby contributing to their migratory and invasive potential. In summary, these results highlight the important role of GDF15 in gallbladder cancer cell progression, with it impacting cell growth, motility, migration, and invasion while also regulating the expression of epithelial and mesenchymal markers.

PanCancer progression analysis reveals the relationship between GDF15 expression and VEGFA signaling in gallbladder cancer

We conducted nCounter PanCancer Progression gene expression assays using gallbladder cancer tissues (n = 10) and gallbladder normal tissues (n = 2). Gallbladder cancer tissues were classified into two groups based on GDF15 expression levels: the GDF15 low (n = 5) and GDF15 high (n = 5) (**Figure 3A**). The normalized data was then adjusted to generate a heatmap using unsupervised clustering of the GDF15 high and low groups (**Figure 3B**). Volcano plot analysis revealed the significant differentially expressed genes (DEGs) with fold-change > 1.5 and *P*-value < 0.05 (**Figure 3B**). Gene ontology analysis of DEGs indicates the upregulation of VEGF related pathways (**Figure 3C**). Subsequently, we investigated the association between previously identified VEGF pathway-related genes and GDF15, validating a high correlation between the expression of GDF15 and VEGFA (**Figure 3D**). These findings indicate that elevated levels of GDF15 correlate with the VEGFA signaling pathway in human gallbladder cancer.

GDF15 regulation of VEGFA through the AKT/NF-κB pathway in gallbladder cancer

We investigated the correlation between GDF15 and VEGFA levels in clinical gallbladder cancer samples using immunohistochemical staining (**Figure 4A**). In gallbladder cancer tissues, patients with high GDF15 expression were observed to have significantly increased VEGFA expression compared to patients with low GDF15 expression (**Figure 4B**). Furthermore, a positive correlation was observed between the expression of GDF15 and VEGFA in clinical tissue samples obtained from gallbladder cancer patients (**Figure 4C**). In addition, the association between GDF15 and VEGFA expression in gallbladder cancer cell lines was confirmed through Western blot analysis (**Figure 4D**). Several signaling pathways are involved in the regulation of VEGFA expression, including AKT, STAT3, JNK and NF-κB [35-37]. We found that GDF15 knockdown significantly decreased the phosphorylation of AKT and NF-κB in gallbladder cancer cell lines (**Figure 4E**). These results indicate that GDF15 positively regulates VEGFA through the AKT/NF-κB pathway in gallbladder cancer.

Discussion

The lack of early diagnostic markers and poor prognosis at intermediate and advanced stages of disease remain important challenges in the treatment of gallbladder cancer [38, 39]. These limitations have a significant negative impact on the effectiveness of treatment and the quality of patient outcomes. It is therefore important to elucidate the molecular mechanisms that promote tumor progression in gallbladder cancer and find new biomarkers for early diagnosis. Our clinical data showed a strong positive correlation between GDF15 levels and lymphovascular invasion, highlighting its possible association with the aggressive behavior of gallbladder cancer [40, 41]. Given that GDF15 is a secreted protein detectable in the circulation, its correlation with lymphovascular invasion high-lights its potential as a noninvasive biomarker for aggressive gallbladder cancer [42, 43]. Additionally, gallbladder cancer tends to metastasize to surrounding tissues through blood vessels, lymph nodes, and the liver, which is considered another key challenge in developing optimal treatment strategies [44, 45]. Understanding the functional role of this substance in gallbladder cancer may therefore provide new insights and open avenues for the development of targeted therapeutic approaches.

GDF15 is a protein highly expressed in a variety of human cancers and has been the subject of extensive research due to its involvement in the regulation of several biological processes [35, 36, 46]. Moreover, GDF15 secretion tends to increase in response to cellular stress or damage, and consequently secreted GDF15 protein can be identified in both the extracellular matrix and in the human bloodstream [30, 35, 37]. For this reason, the results of recent studies have highlighted the potential of GDF15 as a predictive biomarker that can pre-

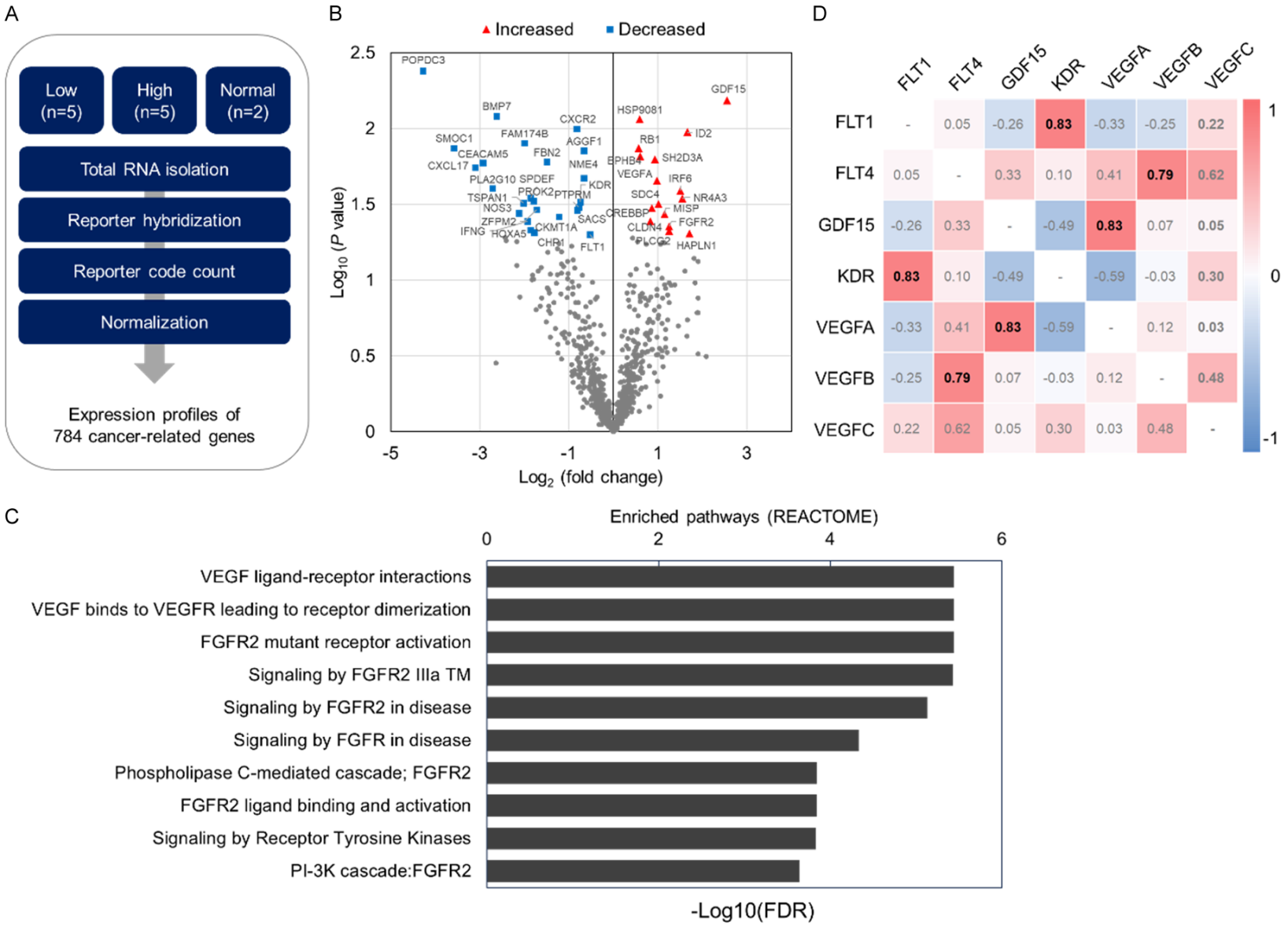

Figure 3. Analysis of cancer progression reveals the association between VEGF and GDF15 expression in GBC patients. A. Schematic diagram of the cancer progression analysis performed in this study. B. Volcano plots representing 770 genes associated with cancer progression show log2-fold-change plotted against the nega-

tive log10-adjusted *P*-value for the GDF15 high-expression group compared to the GDF15 low-expression group. Red dots indicate upregulated genes and blue dots indicate downregulated genes in the GDF15 high-expression group compared to the GDF15 low-expression group. Data for genes that were not classified as differentially expressed are plotted in gray. C. REACTOME path-way enrichment analysis of DEGs resulted in 10 statistically significantly enriched path-ways in the GDF15 high-expression group compared to the GDF15 low-expression group. D. Correlation matrix between GDF15- and VEGFA-related genetic variables from the GBC patients. Positive and negative correlations are shown in red and blue, respectively.

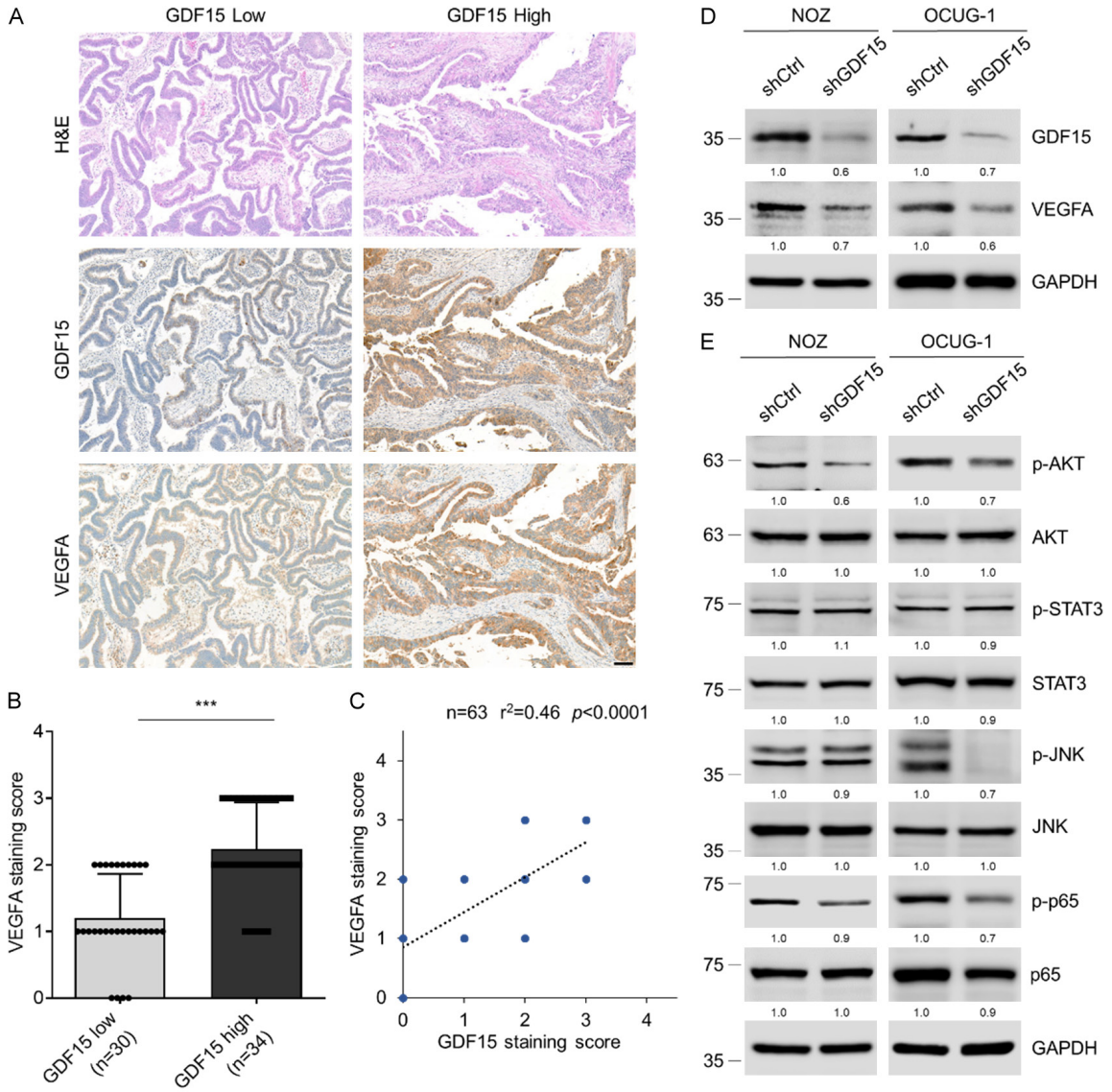

Figure 4. GDF15 promotes VEGFA expression through the AKT/NF-κB signaling pathway. A. Representative images of VEGFA immunohistochemical staining in gallbladder cancer tissues from 63 clinical gallbladder cancer patients. Scale bar, 50 μm. B. Comparison of the expression of VEGFA shown for patients classified into GDF15 low-expression (light gray) and GDF15 high-expression (dark gray) groups. C. Correlation analysis of the staining index of GDF15 protein expression levels in human gallbladder specimens. D. Western blot analysis of GDF15 and VEGFA in GDF15 knockdown cells and shCtrl cells. E. Western blot analysis of p-AKT, p-STAT3, p-JNK, and p-p65 in GDF15 knockdown cells and shCtrl cells. ***$P < 0.001$.

dict survival and recurrence across a variety of cancers [47, 48]. GDF15 continues to be the subject of intense research as its complex functions in cancer biology are further uncov-

ered. In this study, we detail the molecular mechanism of GDF15, revealing a significant correlation between GDF15 expression and activation of the AKT/VEGFA signaling pathway in gallbladder cancer. Furthermore, the results of our in vitro experiments demonstrated that GDF15 promotes cell growth, migration, and invasion, highlighting its potential role as a key molecule in gallbladder cancer progression, particularly associated with VEGFA. In light of these findings, we focused on the relationship between GDF15 and angiogenesis in gallbladder cancer. However, it should be noted that in this study, only the precursor form (~35 kDa) of GDF15 was evaluated using Western blotting due to the specificity of the antibody used. The mature, biologically active form (~15 kDa) was not directly evaluated. Because the functional effects of GDF15 may vary depending on its active form, further studies are needed to assess its expression in cancer tissues and cell supernatants using antibodies that can detect both forms.

Angiogenesis is an essential process for tumor growth and metastasis because it ensures the supply of oxygen and nutrients necessary for the rapid proliferation of cancer [49-51]. We performed gene expression profiling studies using human gallbladder samples. Our findings showed a significant correlation between GDF15 expression and the expression of several genes associated with angiogenesis, including FLT1, FLT4, KDR, VEGFA, VEGFB, and VEGFC (**Figure 3D**), when analyzed using the NanoString nCounter Assay [52-55]. In particular, among these genes, VEGFA showed the strongest positive correlation with GDF15 expression in the human gallbladder samples. Additional analysis using NanoString nCounter assays and immunohistochemical staining of tumor tis-sues showed that patients with high GDF15 expression had significantly higher VEGFA levels compared to patients with low GDF15 expression. Additionally, GDF15 knockdown resulted in a significant decrease in VEGFA expression compared to the control. These findings imply that GDF15 may increase angiogenesis in gallbladder cancer by upregulating the expression of VEGFA.

VEGFA is known to have the ability to stimulate both physiological and pathological angiogenesis by activating various signaling pathways that stimulate cell growth, migration, and differentiation [26, 56, 57]. Previous studies have indicated a correlation between GDF15 and VEGFA expression in glioblastoma and colorectal cancer, further demonstrating the regulatory role of GDF15 in angiogenesis [23, 25, 58]. Additionally, the results of other studies show that multiple signaling pathways, such as ERK, JNK, AKT, NF-κB, and STAT3, are involved in the regulation of VEGFA expression [24, 59]. Therefore, we investigated in greater depth the activity of signaling pathways downstream of GDF15 in gallbladder cancer cells. Our results showed that GDF15 knockdown significantly reduced the phosphorylation levels of NF-κB and AKT, indicating that GDF15 may affect tumor progression through these important pathways. These findings suggest that GDF15 increases VEGFA expression through the AKT/NF-κB/VEGFA signaling axis during the progression of gallbladder cancer. Given its regulatory role in VEGFA expression, GDF15 may also influence the response to antiangiogenic therapy in gallbladder cancer. This conclusion is further supported by an overexpression model in which GDF15 induces VEGFA expression and activates downstream signaling molecules such as p-AKT and p-NF-κB (Supplementary Figure 1). Therefore, targeting the GDF15-VEGFA axis may represent a promising therapeutic approach in gallbladder cancer. Although this possibility requires further investigation, it highlights the potential of GDF15 not only as a biomarker but also as a therapeutic target in precision oncology. Although our study has a limitation in that we did not perform functional angiogenesis assays, our in vitro data and clinical correlation analyses strongly suggest that GDF15 promotes gallbladder cancer progression by upregulating VEGFA. Future studies are needed to evaluate the direct role of GDF15 in tumor angiogenesis using in vitro and in vivo functional models.

Furthermore, this study was limited to in vitro models, and we did not perform in vivo validation or tissue-level analysis such as IHC or IF for p-AKT or p-NF-κB. In addition, our Western blot analysis was restricted to the precursor form (~35 kDa) of GDF15 due to antibody specificity, and the mature, biologically active form (~15 kDa) was not evaluated. Future studies incorporating in vivo experiments and detection of the mature form of GDF15 will be neces-

sary to further confirm the translational relevance of the proposed mechanism. Although functional assays were performed using a single shRNA due to sample limitations, we confirmed consistent knockdown of GDF15 and VEGFA suppression with a second independent shRNA, supporting specificity. This limitation has been acknowledged and will be addressed in future studies.

In summary, our findings demonstrate an important role of GDF15 in gallbladder cancer progression via the AKT/VEGFA signaling pathway. Using both clinical and gene expression data, we established a strong association between GDF15 and gallbladder cancer progression via AKT/VEGFA. Taken together, these results suggest that GDF15 may be a promising therapeutic target for the treatment of gallbladder cancer.

Acknowledgements

This study was presented at the ASCO Breakthrough Meeting 2023 (Yokohama, Japan; Aug 3-5) and published as abstract no. 167 in JCO Global Oncology 9, 167 (2023). And this research was supported by the National Research Council of Science & Technology (NST) grant by the Korea government (MSIT) (No. GTL24021-000) and research fund of Chungnam National University.

Patient consent was waived because the study was conducted on routine diagnostic FFPE samples.

Disclosure of conflict of interest

None.

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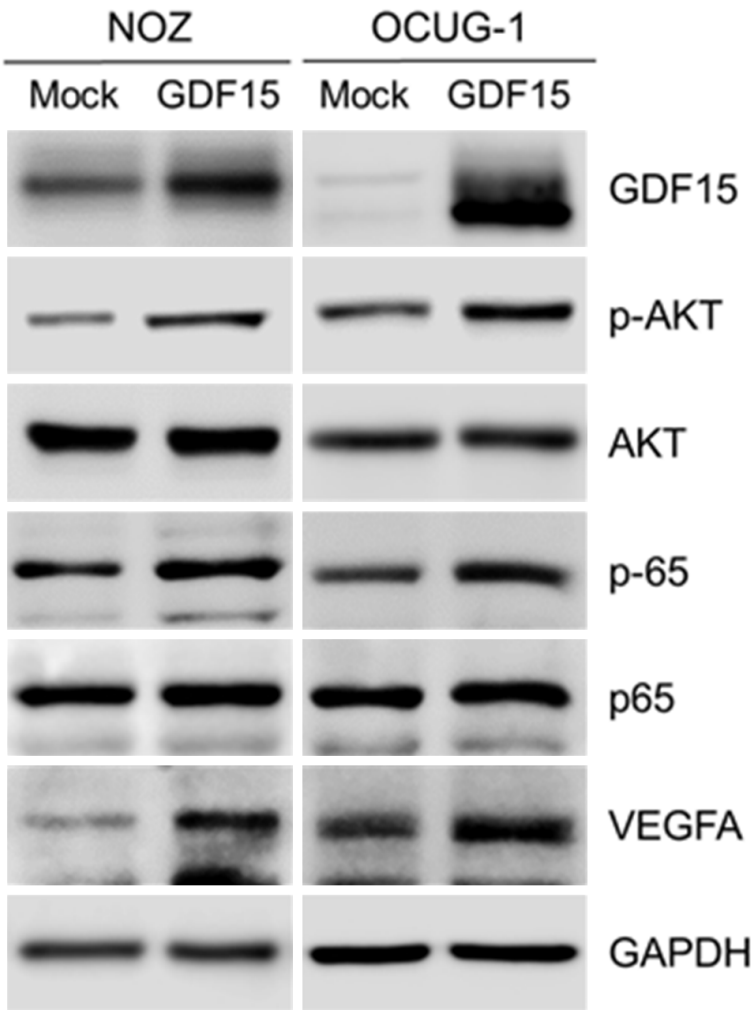

Supplementary Figure 1. Relationship between GDF15 and VEGFA expression in gallbladder cancer cells and analysis of related signaling pathways by Western blot analysis.