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ORIGINAL ARTICLE

# The expression of growth differentiation factor 15 in gallbladder carcinoma

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## Summary

***Purpose:*** *This study initially explored the expression of GDF-15 in gallbladder carcinoma and its clinical significance, and analyzed the correlation between the expression of GDF-15 and the clinicopathological features as well as the prognosis of patients with gallbladder carcinoma.*

***Methods:*** *Enzyme-linked immunosorbent assay (ELISA) was used to determine the expression of GDF-15 in the serum of 42 patients with gallbladder cancer. The control group included 24 patients with cholecystitis and 20 healthy volunteers. The immunohistochemical method (IHC) was used to detect the expression of GDF-15 in 42 cases of gallbladder tumor tissue and 35 cases of adjacent non-tumor gallbladder tissue specimens.*

***Results:*** *The results of ELISA showed that the concentration of GDF-15 in serum was considerably higher in gallbladder cancer patients than that in gallbladder benign lesions and healthy volunteers ($p=0.006$, $p<0.001$). In the group of patients with gallbladder cancer, the consistence of GDF-15 in patients with lymph node metastasis was significantly higher than that of patients without lymph node metastasis ($p<0.001$). Immunohistochemical staining showed that the expression of GDF-15 in gallbladder carcinoma was markedly higher than that in non-tumor gallbladder tissues ($p=0.003$), and the high expression of GDF-15 was significantly correlated with the differentiation grade of gallbladder carcinoma and tumor TNM stage ($p=0.005$, $p=0.002$).*

***Conclusion:*** *GDF-15 is related to the occurrence and development of gallbladder cancer. GDF-15 in serum can be used as a potential marker for the diagnosis of gallbladder cancer and can be used to predict the lymph node metastasis of gallbladder cancer.*

***Key words:*** *enzyme-linked immunosorbent assay, gallbladder carcinoma, growth differentiation factor 15, immunohistochemistry*

## Introduction

Gallbladder carcinoma (GBC) is one of the most common malignant tumors of the digestive system. It is the most common tumor of the biliary system, accounting for about 80-95% of biliary malignant tumors 1, which has a markedly high malignant grade and its prognosis is considerably poor. Statistics showed that the overall average survival time of gallbladder cancer is only 6 months, and the number of deaths due to gallbladder cancer worldwide is about 165000 each year, accounting for 1.7% of the global cancer deaths [2]. At present, radical surgical resection is the only possible treatment that can make patients with gallbladder cancer obtain long-term survival. There is a chance of radical resection for early-stage gallbladder cancer, and in such a case the prognosis is relatively good. However, patients with advanced gallbladder cancer usually have a poor prognosis and even lose the opportunity of radical resection. However, the initial symptoms of gallbladder cancer lack

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specificity, which makes it difficult to distinguish from the beginning of lesions such as cholecystitis. The early symptoms are not obvious while distant lymph node metastasis can occur [3]. Therefore, most of the patients with gallbladder cancer are in the middle and late stages when they go to the hospital. The 5-year survival rate of early gallbladder cancer is 50%, while the 5-year survival rate of advanced gallbladder cancer is less than 5% [4]. It has been reported that the proportion of patients with gallbladder cancer who can undergo radical resection is only 10% [5]. Therefore, early diagnosis is very important for the comprehensive treatment of this disease.

Growth differentiation factor 15 (GDF-15) is a growth factor, also known as macrophage inhibition cytokine-1 (MIC-1), which is one of the members of TGF-β superfamily [6]. Relevant studies have shown that under normal physiological conditions, GDF-15 is less expressed in tissues except placenta, but under the stimulation of inflammation, hypoxia, acute injury, oncogene activation and other pathological conditions, the expression of GDF-15 in affected organs and serum is significantly increased [7].

In recent years, studies on GDF-15 have emerged in an endless stream. Authors have found that the expression of GDF-15 is closely related to the occurrence, development and prognosis of patients with liver cancer, gastric cancer, pancreatic cancer and other digestive system cancers. However, the correlation between GDF-15 and gallbladder cancer has not been reported. This study is the first study to analyze the correlation between GDF-15 and gallbladder cancer. In this study, analyzed was the expression of GDF-15 in gallbladder cancer and its significance in order to find a serum tumor marker for the prediction of early-stage gallbladder cancer that could provide evidence for monitoring its recurrence.

## Methods

*Experimental materials*

*Clinical blood samples*

In this study, blood samples were collected from 42 patients with gallbladder cancer in Henan Provincial People's Hospital from January 2017 to December 2018. Five ml of fasting elbow venous blood was collected before surgery. In addition, blood samples from 24 patients with cholecystitis and 20 healthy volunteers were collected and served as controls. After admission, 5ml fasting elbow venous blood was collected from patients with cholecystitis and healthy volunteers. All the above clinical blood samples were collected in vacuum coagulation-promoting blood pumping vessels. The blood samples were placed at room temperature for 2h, centrifuged at 3000r/min for 10 min, and the upper serum was collected and frozen in a refrigerator at -80°C for testing.

*Clinical tissue samples*

After operation, the tissue samples were collected without affecting the requirements of pathological diagnosis, tumor tissue samples were collected from 42 patients who underwent radical resection of gallbladder cancer. The postoperative pathological results were confirmed as gallbladder cancer. The tissue samples were staged according to the pathological results, intraoperative findings and the eighth edition of tumor staging guidelines issued by the American Joint Committee on Cancer (AJCC) [8]. Tissue specimens were classified on the basis of clinical and pathological characteristics. In addition, 35 cases of non-tumor gallbladder tissue adjacent to tumor were taken (with a distance of more than 3cm; among them, 7 cases of non-tumor gallbladder tissue adjacent to cancer were missing), and no cancer tissue was found in postoperative pathological examination. All these tissue specimens were frozen in a refrigerator at -80°C. No patient had received chemotherapy, radiotherapy and blood transfusion before surgery.

*Basic data of clinical cases in each group*

There were 42 patients with gallbladder cancer, including 22 males and 20 females, aged between 38 and 72 years (mean 58 years). According to tumor size, 24 cases were <5cm and 18 were ≥ 5cm. According to the level of tissue differentiation, there were 10 cases of low differentiation, 25 cases of medium differentiation and 7 cases of high differentiation. According to the histological classification, the cases could be divided into: 23 cases of adenocarcinoma, 5 cases of papillary carcinoma, 5 cases of adenosquamous carcinoma, 5 cases of squamous cell carcinoma, 3 cases of neuroendocrine carcinoma and 1 case of malignant melanoma. According to AJCC TNM stage, intraoperative findings and postoperative pathological results, there were 22 cases of lymph node metastasis, 14 cases of N1 metastasis, 8 cases of N2 metastasis and 11 cases of distant metastasis. There were no significant differences in gender and age between control group and gallbladder cancer group.

This study was approved by the ethics committee of Henan Provincial People's Hospital, and all subjects in each group have signed the relevant informed consent.

*Main experimental instruments*

| | |
|---|---|
| Ultra-low temperature refrigerator (-80°C) | SANYO (Japan) |
| Ordinary refrigerator | Gree Electric Appliance |
| High-speed refrigerated micro centrifuge | DragonLab |
| Ultramicro spectrophotometer | Thermo |
| Decolorization shaker | Servicebio |
| Microscope | Olympus |
| Pathological slicer | Shanghai Leica instrument |
| Tissue Slice Scanner | 3D HISTECH |

*Main experimental reagents*

| | |
|---|---|
| GDF-15 ELISA kit | CUSABIO |
| Anhydrous ethanol | Sinopharm Chemical Reagent Co |
| Xylene | Sinopharm Chemical Reagent Co |
| Hematoxylin Stain | Servicebio |
| Primary antibody | Beijing Boaosen Biology Technology |
| Secondary antibody | Servicebio |

*Experimental methods*

*ELISA*

1. Sample configuration: All blood test samples in each group were from serum samples obtained by the above method;
2. Preparation of standard working solution: Centrifuge the standard at 10000rpm for 30 sec. Mix 1ml sample dilution solution and mix them evenly to obtain standard working fluid S7. Then take 7 centrifugal tubes (S0-S6) with the size of 1.5ml, arrange them according to the serial number, and add 250μl diluted solution, respectively. Pipette 250μl S7 sample into the first centrifuge tube with a pipette gun (S6). pay attention to gently blowing and mixing with the gun head, and then get the standard S6. Pipette 250μl S6 sample into the second centrifuge tube and mix well to obtain standard S5. By analogy, the standard is diluted proportionally, and the working dilution solution is taken as S0. Finally, the working solution with corresponding concentration is obtained (S7: 500pg/ml, S6: 250pg/ml, S5: 125pg/ml, S4: 62.5pg/ml, S3: 31.2pg/ml, S2: 15.6pg/ml, S1: 7.8pg/ml, S0: 0pg/ml);
3. Specific steps: set standard working fluid and sample holes to be inspected separately, add 100μl of working fluid or sample to be inspected to each hole, cover the plate with stickers, incubate at 37°C for 2 h, pour the liquid in each hole, and spin the plate dry, Take out 100μl of Biotin-antibody, add 100μl into each well, cover it with a new plate, and incubate at 37°C for 1 h; pour out the liquid in each well and soak it in 200μl Wash Buffer for 2 min, repeat three times, and spin the plate dry; 100μl of HRP-avidin was added to each well, cover with a new patch, and incubate at 37°C for 1 h; discard the liquid in the well, wash the plate with 200μl Wash Buffer for 5 times, and spin the plate dry; Add 90μl TMB Substrate to each well in sequence, and develop color at 37°C for 25 min in the dark; add 50μl Stop Solution to each well in order, mix well and stop the reaction; the OD value (optical density) of each reaction hole was measured with a microplate reader at the wavelength of 450nm within 10 min after stopping the reaction.

The above experimental operations were performed strictly in accordance with the instructions of human growth differentiation factor 15 ELISA Kit. Based on the concentration of the standard working solution, draw a standard curve, obtain the formula: $y=770.04x^2-34.887x+6.3341$ ($R^2=0.9993$), and calculate the expression concentration of GDF-15 in each group.

*Immunohistochemical staining (IHC)*

(1) Section dewaxing: Put the slices into the following solution: xylene for 15 min, repeat for 3 times, anhydrous alcohol for 5 min, repeat for 2 times, 85% alcohol for 5 min, 75% alcohol for 5 min, and wash with distilled water. (2) Antigen repair: Put the sample slices in a container containing citric acid repair solution (pH 6.0), repair the antigen in a microwave oven, heat the samples with medium fire for 8 min until boiling, cease fire for 8 min, keep warm and then turn to medium and low fire for 7 min (pay attention to prevent the repair solution from volatilizing too much, and remember not to dry the slices). Cool down at room temperature, then put the slices in PBS (pH 7.4), and wash with shaking on a decolorizing shaker for 5 mi and repeat for 3 times. (3) Blocking endogenous peroxidase: Place the slices in 3% hydrogen peroxide, incubate for 25 minutes at room temperature in the dark, put the slices in phosphate buffered saline (PBS) (pH 7.4), and wash them for 5 min in a decolorizing shaker. Repeat for 3 times. (4) Serum blocking: 3% bovine serum albumin (BSA) was evenly dropped on the tissue in the histochemical circle, and reacted for 30 min at room temperature (The goat-derived primary antibody was rabbit serum, the others were blocked with BSA). (5) Add primary antibody: Pour out the blocking solution, drop the primary antibody which prepared with PBS on the slice according to the ratio, put the slice in a wet box, and incubate overnight at 4°C (be careful to avoid antibody volatilization). (6) Add secondary antibody: Put the slices in PBS (pH 7.4), shake and wash it on a decolorizing shaker for 5 min, and repeat for 3 times. Dry it slightly, the second antibody (corresponding to the first antibody) labeled with horseradish peroxidase (HRP) was dropped on the tissue in the loop and incubated at room temperature for 50 min. (7) DAB color development: Put the slices in PBS (pH 7.4), shake and wash them on a decolorizing shaker for 5 min, and repeat for three times. Dry it slightly, and drop the diaminobenzidine (DAB) chromogenic liquid into the circle. Grasp the reaction time under microscope observation. It is positive if it turns to brownish yellow, then rinse the slide with running water to stop the reaction. (8) Re-staining the nucleus: Re-staining with hematoxylin for about 3 min, washing it with running water, differentiating with hematoxylin differentiation solution for a few sec, rinsing with running water, returning to blue with hematoxylin blue solution, and washing with tap water. (9) Dehydration and sealing: Put the slices into 75% alcohol for 5 min, 85% alcohol for 5 min and absolute ethyl alcohol for 5 min in sequence, repeat twice, then put them into xylene for 5 min, dehydrate until they are transparent, then take out the slices, turn them upside down to dry, and seal them with neutral gum. (10) Observe with a microscope and collect pictures for analysis.

*Statistics*

First, the Shapiro-Wilk and Levene methods were used to detect the normal distribution and the homoge-

neity of variance of data in each group. The ELISA data satisfy both normal distribution and variance homogeneity test. Therefore, the ELISA results are depicted in the manner of mean ± standard deviation. The mean comparison between the two groups was compared by T test, and the mean comparison between multiple groups was performed by one-way variance analysis; ROC curve was used to analyze the efficacy of serum GDF-15 in the diagnosis of gallbladder cancer and lymph node metastasis; Kaplan-Meier method was used for survival analysis along with log-rank test, and the relationship between the expression of GDF-15 in gallbladder cancer tumor tissue and the prognosis of patients with gallbladder cancer; First univariate Cox regression model was used to analyze the clinicopathological characteristics, TNM staging, grade of tissue differentiation, and GDF-15 expression of gallbladder cancer patients in a single factor, and the next step was multivariate analysis performed to find independent risk factors that affect the prognosis of patients with gallbladder cancer. All data were analyzed by SPSS 20.0 software, and the difference was considered to be statistically significant when $p<0.05$.

## Results

### *ELISA results*

#### *Expression and comparison of GDF-15 in the serum of patients with gallbladder cancer, cholecystitis and healthy volunteers*

The concentration of GDF-15 in the serum of the gallbladder cancer group was significantly higher than that of the cholecystitis group (2985.87±1157.17pg/ml vs 1950.40±892.50pg/ml, p=0.006), and healthy volunteers group (2985.87±1157.17pg/ml vs 1136.01±712.87pg/ml,

**Table 1.** The relationship between the expression of GDF-15 in serum and clinicopathological characteristics in the gallbladder cancer group

| *Clinicopathological features* | *Cases (n)* | *Expression of GDF15 in serum (pg/ml)* | *p* |
|---|---|---|---|
| Age, years | | | 0.670 |
| <58 | 21 | 3063.40±1106.41 | |
| ≥58 | 21 | 2908.34±1228.12 | |
| Gender | | | 0.307 |
| Male | 22 | 3161.71±1316.99 | |
| Female | 20 | 2792.45±947.27 | |
| Grade of tissue differentiation | | | 0.423 |
| High | 7 | 2800.22±1041.47 | |
| Medium | 25 | 3159.61±1033.28 | |
| Low | 10 | 3400.71±1677.90 | |
| Size of tumor, cm | | | 0.478 |
| <5 | 24 | 3097.18±1166.29 | |
| ≥5 | 18 | 2837.45±1161.18 | |
| Histological types | | | 0.422 |
| Adenocarcinoma | 22 | 2847.11±1260.39 | |
| Others | 20 | 3138.51±1042.55 | |
| T staging | | | 0.013 |
| Tis-T2 | 20 | 2465.43±960.62 | |
| T3 | 13 | 3323.38±875.80 | |
| T4 | 9 | 3654.90±1467.87 | |
| N staging | | | <0.001 |
| N0 | 20 | 2204.10±497.35 | |
| N1 | 14 | 3276.37±1026.48 | |
| N2 | 8 | 4431.93±953.97 | |
| M staging | | | 0.014 |
| M0 | 31 | 2729.43±968.53 | |
| M1 | 11 | 3708.58±1377.53 | |
| Clinical staging | | | <0.001 |
| 0-II | 15 | 2122.50±389.78 | |
| III | 9 | 2620.46±644.50 | |
| IV | 18 | 3888.05±1148.34 | |

p<0.001); GDF-15 concentration in serum of the cholecystitis group was slightly higher than that of the healthy volunteer group (1950.40±892.50pg/ml vs 1136.01±712.87pg/ml, p=0.023) (Figure 1).

*The relationship between the expression of GDF-15 in serum and clinicopathological characteristics in gallbladder cancer group*

The expression of GDF-15 in the serum of gallbladder cancer group had no significant correlation with routine clinicopathological factors such as gender and age (all p>0.05 (Table 1), but closely related to T stage (p=0.013), lymph node metastasis (p<0.001), M stage (P=0.014) and clinical stage (p<0.001). The average expression concentration of GDF-15 in the serum of patients without lymph node metastasis was 2204.10±497.35pg/ml, in N1 group it was 3276.37±1026.48pg/ml, and that in N2 group was 4431.93±953.97pg/ml. By comparison, we found that the expression of GDF-15 in serum increased progressively with the increase of N stage, and the average expression concentration of GDF-15 in lymph node metastasis group (N1+N2) was significantly higher than that in the non-lymph node metastasis group (3696.57±1131.07pg/ml vs 2204.10±497.35pg/ml, p<0.001).

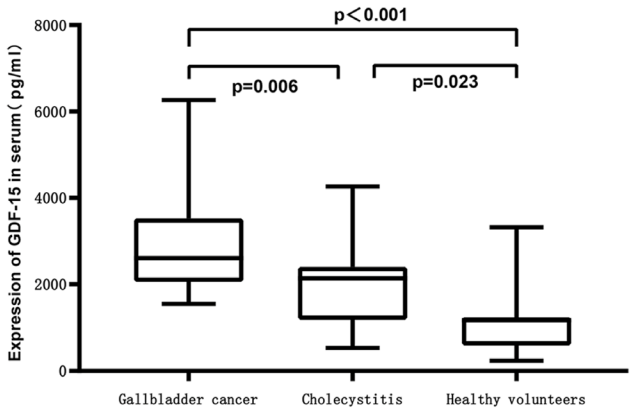


**Figure 1.** Expression of GDF-15 in the serum of patients with gallbladder cancer, cholecystitis and healthy volunteers.

*ROC curve analysis of GDF-15 in serum for diagnosis of gallbladder cancer*

To further analyze the efficacy of serum GDF-15 in the diagnosis of gallbladder cancer, we drew the receiver operating characteristic curve (ROC curve) of GDF-15. The curve showed that the area under the curve of GDF-15 was AUC=0.838(95 The percent confidence interval was 0.756-0.921 (p<0.001). Youden index (sensitivity+specificity), was obtained by calculation, and the maximum value is selected as its cut-off value. After calculation, it was found that when 1897.76pg/ml was selected as the cutoff value, the value of GDF-15 for the diagnosis of gallbladder cancer was as follows: sensitivity 83.3%, specificity 72.7% (Figure 2).

*ROC curve analysis of GDF-15 in serum for diagnosis of lymph node metastasis of gallbladder cancer*

Lymph node metastasis is the most common transfer method of gallbladder cancer. By describing the ROC curve of GDF-15 in serum and lymph node metastasis, it can be obtained that the area under the curve was AUC=0.909 (95% confidence interval: 0.819-0.999, P<0.001), when 2474.615pg/

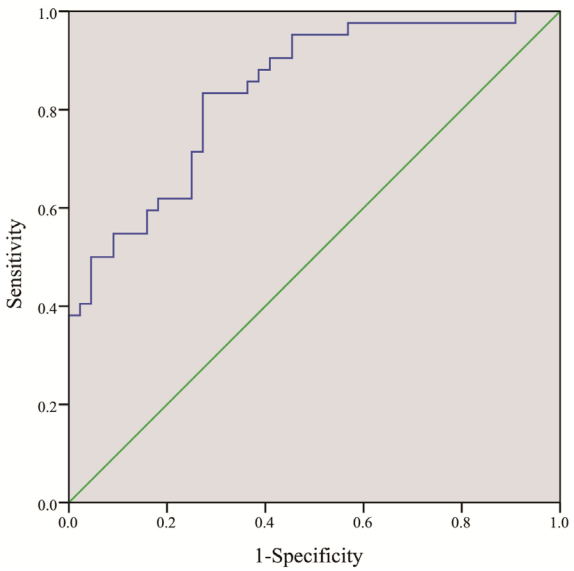


**Figure 2.** ROC curve analysis of GDF-15 in serum for diagnosis of gallbladder cancer.

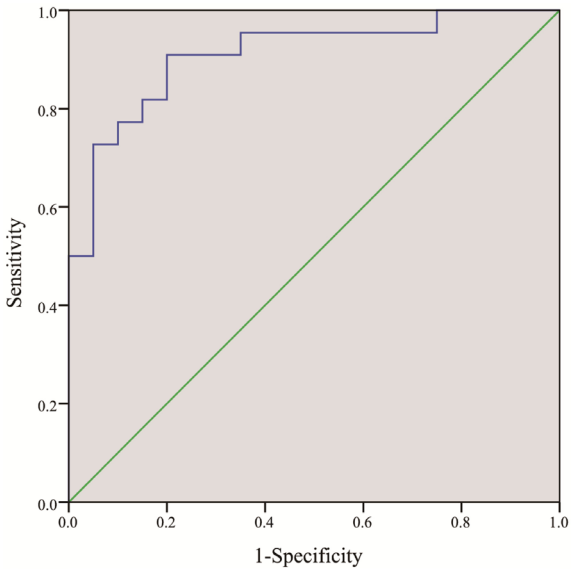


**Figure 3.** ROC curve analysis of GDF-15 in serum for diagnosis of lymph node metastasis of gallbladder cancer.

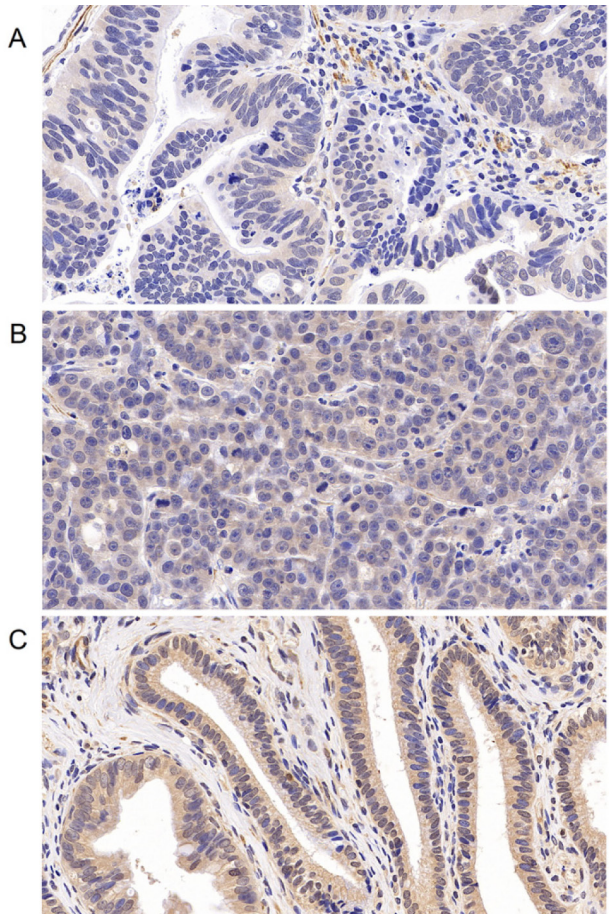


**Figure 4.** The Figure shows **(A)** the weak positive, **(B)** medium positive and strong positive **(C)** expression of GDF-15 in tissues.

ml is taken as the cut-off value, the value of GDF-15 in serum in diagnosing lymph node metastasis of gallbladder cancer is as follows: sensitivity 90.9%, specificity 80% (Figure 3).

*Immunohistochemistry results*

*1) Expression and comparison of GDF-15 in gallbladder cancer tissue and adjacent non-tumor gallbladder tissue*

The level of immunohistochemical staining showed that GDF-15 was significantly higher in cancer tissues than in adjacent tissues, and the difference was statistically significant (p=0.003). Among them, the expression rates of strong positive, medium positive, and weak positive in gallbladder cancer tissue were: 40.5% (17/42), 35.7% (15/42), 23.8% (10/42), and the expression rates of strong positive, medium positive, and weak positive in non-tumor gallbladder tissue adjacent to cancer were: 16.7% (7/42), 42.8% (18/42), 40.5% (17/42). The strong positive rate of GDF-15 in gallbladder cancer tissues was significantly higher than that in adjacent tissues (40.5% vs 16.7%) (Figure 4).

*2) The relationship between the expression of GDF-15 and clinicopathological features in gallbladder carcinoma*

The expression of GDF-15 in tumor tissues of gallbladder cancer had no significant correlation

**Table 2.** Relationship between expression of GDF-15 and clinicopathological features in cancer tissues

| *Clinicopathological features* | *Cases (n)* | *Expression of GDF-15 in cancer tissues* | | | *p* |
|---|---|---|---|---|---|
| | | + | ++ | +++ | |
| Gender | | | | | 0.781 |
| Male | 22 | 6 | 7 | 9 | |
| Female | 20 | 4 | 8 | 8 | |
| Age, years | | | | | 0.380 |
| <58 | 21 | 7 | 6 | 8 | |
| ≥58 | 21 | 3 | 9 | 9 | |
| Size of tumor, cm | | | | | 0.955 |
| <5 | 24 | 6 | 9 | 9 | |
| ≥5 | 18 | 4 | 6 | 8 | |
| Histological types | | | | | 0.864 |
| Adenocarcinoma | 22 | 5 | 9 | 8 | |
| Others | 20 | 5 | 6 | 9 | |
| TNM staging | | | | | 0.002 |
| 0-II | 15 | 5 | 5 | 5 | |
| III-IV | 27 | 5 | 10 | 12 | |
| Grade of differentiation | | | | | 0.005 |
| Low | 10 | 1 | 1 | 8 | |
| Medium | 25 | 6 | 12 | 7 | |
| High | 7 | 3 | 2 | 2 | |

**Table 3.** Univariate and multivariate Cox regression analysis of prognostic factors of gallbladder carcinoma

| *Variable* | *Univariate analysis* | | | *Multivariate analysis* | | |
|---|---|---|---|---|---|---|
| | *HR value* | *95% confidence interval* | *p value* | *HR value* | *95% confidence interval* | *p value* |
| Gender | 0.942 | 0.422-2.105 | 0.885 | | | |
| Age | 1.321 | 0.589-2.964 | 0.498 | | | |
| Size of tumor | 0.515 | 0.213-1.244 | 0.133 | | | |
| Histological types | 0.652 | 0.291-1.458 | 0.294 | | | |
| Grade of tissue differentiation | 4.526 | 1.181-17.348 | 0.028 | 2.552 | 0.609-10.695 | 0.200 |
| TNM staging | 4.277 | 1.459-12.537 | 0.008 | 3.240 | 1.087-9.655 | 0.035 |
| The expression of GDF-15 in tissues | 10.460 | 2.246-48.723 | 0.003 | 7.846 | 1.669-36.886 | 0.009 |

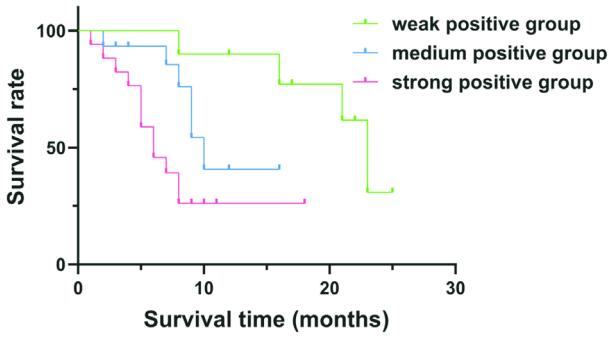


**Figure 5.** Relationship between GDF-15 expression in tissues and prognosis of patients with gallbladder cancer. The average survival time of the strong positive group was 6.65 months, which was lower than that of the medium positive group (8.2 months, p=0.045) and significantly lower than that of the weak positive group (17.7 months, p=0.003).

with basic pathological factors such as gender, age, tumor size and histological type (all p>0.05;Table 2), but was closely related to tumor differentiation level (p=0.005) and TNM stage (p=0.002). The expression of GDF-15 in poorly differentiated cancer tissues was markedly higher than that in moderately differentiated (p=0.003) and well-differentiated cancer tissues (p=0.005).

*Prognosis analysis*

42 cases of gallbladder cancer patients were followed up after the operation, with December 2019 as the cut-off time for observation. The results showed that the average survival time after surgery was 9.8 months (median 8 months). Based on the expression intensity of GDF-15 in gallbladder cancer tissues, the patients were divided into three groups, namely weak positive group (n=10, 95% confidence interval: 13.68-21.72), medium positive group (n=15, 95% confidence interval: 6.2-10.2), and strong positive group (n=17, 95% confidence interval: 4.6-8.7). The survival analysis by Kaplan-Meier method plus log rank test showed that the average survival time of the strongly positive group was 6.65 months, which was lower than that of the moderately positive group (8.2 months, p=0.045) and significantly lower than that of the weakly positive group (17.7 months, p=0.003) (Figure 5). Univariate Cox regression analysis showed that TNM stage, tissue differentiation grade and GDF-15 expression were related to the prognosis of patients with gallbladder cancer. Multivariate analysis showed that the expression level of GDF-15 in tumor tissues and TNM stage were independent risk factors for the prognosis of gallbladder cancer (p=0.009, p=0.035) (Table 3).

## Discussion

At present, with the rapid development of medicine, research and understanding of gallbladder cancer are deepening, but the pathogenesis of gallbladder cancer is still unclear [3]. Primary gallbladder cancer is the most common malignant tumor of the biliary system, which is characterized by lack of specificity of early symptoms. Because of its hidden symptoms and early metastasis through vascular invasion and lymph nodes, most of clinically diagnosed patients are in advanced stage [9]. At present, surgical radical resection is still the first choice for the treatment of disease, while patients with advanced gallbladder cancer usually have no opportunity for operation, so the prognosis is considerably poor. Early diagnosis and early treatment can significantly improve the survival rate of the gallbladder cancer patients. Therefore, it is imperative to improve the early diagnosis rate this disease.

Many patients have already multiple metastases when they go to the hospital. Even if surgery is performed to reduce tumors, it cannot improve the patient survival [10]. Therefore, many patients with advanced gallbladder cancers can only be treated with palliative treatment. Most of these patients have biliary obstruction or the digestive tract obstruction. The purpose of palliative treatment is

only to relieve the obstruction, so as to improve the patient's quality of life. For example, patients with digestive tract obstruction could be offered gastrojejunostomy, and patients with obstructive jaundice should be offered percutaneous transhepatic cholangial drainage (PTCD) operation [11]. For now, there is no uniform radiotherapy and chemotherapy regimen for gallbladder cancer. Relevant studies have shown that palliative chemotherapy of gemcitabine combined with cisplatin can prolong the limited survival time for patients with biliary tumors that cannot be treated by surgery [12]. For radiotherapy, there is not enough evidence to prove that it can improve the survival rate, but can only relieve the pain symptoms of some patients with gallbladder cancer [13]. Therefore, new treatment ideas are urgently needed for patients with unresectable advanced gallbladder cancer.

GDF-15 is a growth differentiation factor and a member of BMP family of the TGF-β superfamily14. It is widely distributed in the blood and tissues of mammals, and its biological functions is not yet fully defined. However, some studies have found that GDF exerts multiple functions in the progression of various diseases, such as inflammatory diseases, cancer, and cardiovascular diseases [15].

As a multifunctional growth differentiation factor, GDF-15 promotes the occurrence and development of various cancers. Liu et al [16] analyzed the expression level of GDF-15 in the serum of 1014 subjects, and found that, compared with the healthy control group, the expression of GDF-15 in the serum of patients with liver cancer and liver cirrhosis was significantly higher, and concluded that GDF-15 is a new serum marker of liver cancer [17]. Michal Vocka et al found that the level of GDF-15 in the serum of colorectal cancer patients was significantly higher than that in the healthy control group ($p<0.001$). In addition, the level of GDF-15 is related to the degree of liver involvement, and the prognosis of patients with high level of GDF-15 is obviously poor ($p<0.0001$) [18]. Wang et al [19] selected 3966 patients with digestive system tumors from multiple online clinical databases, evaluated the relationship between the expression of GDF-15 and clinical prognosis through meta-analysis, and found that GDF-15 was independent of other traditional biomarkers and could be used as a new tumor marker for the diagnosis of the digestive system tumors. Through research, Wallentin et al [20] found that when GDF-15 was used in the diagnosis of cardiovascular diseases and cancers in elderly men, its diagnostic efficiency significantly exceeded that of conventional biomarkers. Furthermore, combining the expression level of GDF-15 in serum with the current routine serological tumor markers can improve the specificity of cancer diagnosis. Brown et al [21] found that the specificity of diagnosis of prostate cancer was significantly improved by combining the detection of GDF-15 and free prostate specific antigen, thus potentially reduced unnecessary biopsy by 27%.

Through immunohistochemical staining and RT-PCR, Zhang et al found that the expression of GDF-15 in colorectal cancer tissues was significantly higher than that in adjacent normal tissues [22]. Wang et al reported that the over-expression of GDF-15 promotes cell viability, cell invasion, migration and angiogenesis in hepatocellular carcinoma, while downregulation of the expression of GDF-15 can significantly inhibit the proliferation and distant migration of HCC cells [23]. This implies us that GDF-15 may be a potential target for the treatment of liver cancer. In addition, a number of authors have found abnormal high expression of GDF-15 in gastric cancer, pancreatic cancer and other digestive system malignant tumors, which is closely related to the prognosis of patients with cancers [24-26]. On the contrary, Wang et al found that increasing the expression of GDF-15 can inhibit the metastasis of breast cancer. It is suggested that GDF may be a tumor suppressor of breast cancer [27]. These results suggest that different tumors may lead to different expression of GDF-15.

In this study, the concentration of GDF-15 in the serum of patients with gallbladder cancer, cholecystitis and healthy volunteers was determined. Through comparison, we found that the concentration of GDF-15 in the serum of patients with gallbladder cancer was significantly higher than that of patients with cholecystitis and healthy volunteers, and the concentration of GDF-15 in the serum of patients with cholecystitis was slightly higher than that of healthy volunteers. After further analysis of the relationship between the expression of GDF-15 in the serum and various clinicopathological factors in patients with gallbladder carcinoma, we found that the high expression of GDF-15 was closely related to T stage ($p=0.013$), N stage ($p<0.001$), M stage (0.014) and clinical stage ($p<0.001$) of tumor. The level of GDF-15 in the serum of gallbladder cancer patients with lymph node metastasis was significantly higher than that of patients without lymph node metastasis ($p<0.001$), and with the increase of lymph node metastasis, the concentration of GDF-15 showed a progressive increasing trend. The above results are consistent with the conclusions of other authors in other malignant tumors. Zhang et al found that GDF-15 was highly expressed in patients with epithelial ovarian cancer, but not in the control group.

The expression of GDF-15 gradually increased with the increase of clinical stage of ovarian cancer, and was significantly correlated with lymph node metastasis (p=0.003). Their further study found that patients with ovarian cancer resistant to chemotherapy showed higher expression of GDF15 than those with chemotherapy sensitivity (p=0.030) [28]. Engerud et al [29] used ELISA method to detect the expression of GDF-15 in 235 patients with endometrial cancer before operation, and 78 patients with endometrial hyperplasia were used as the control. When cancer recurred, the serum was collected to detect the expression of GDF-15. Through comparison, it was found that the GDF-15 concentration in the serum was significantly higher in patients with recurrent tumors than that of patients without recurrence, and the level of GDF-15 in the serum of recurrent patients was higher than that of preoperation. The authors concluded that high expression of GDF-15 in the serum can independently predict the recurrence and lymph node metastasis of endometrial cancer.

In this study, the expression of GDF-15 in gallbladder cancer tissues and its adjacent non-tumor tissues was also detected by immunohistochemical staining. The results showed that the expression of GDF-15 in gallbladder cancer tissues was significantly higher than that in adjacent non-tumor tissues (p=0.003). Next, we analyzed the relationship between the expression of GDF-15 in tumor tissues and various clinicopathological factors and found that tumor TNM stage (p=0.002) and tissue differentiation level (p=0.005) were closely related to the abnormal expression of GDF-15, and with the increase of TNM stage, the expression of GDF-15 in gallbladder carcinoma was gradually increased. The expression of GDF-15 in the poorly differentiated carcinoma was significantly higher than that in the moderately differentiated (p=0.003) and the well differentiated (p=0.005) cancer tissues. The same experimental results also appeared in the study of other malignant tumors. Zhao et al [30] found that the expression of GDF-15 in lung cancer tissues was significantly higher than that in non-tumor tissues adjacent to lung cancer, and was closely related to tumor size, lymph node metastasis and TNM staging. They also found that GDF-15 plays an indispensable role in the proliferation of non-small cell lung cancer (NSCLC) and it may become a target for this disease. Liu et al [31] recruited 152 patients with stage I-II NSCLC, 48 patients with benign lung disease and 105 healthy volunteers. The GDF-15 level in the serum was measured by enzyme-linked immunosorbent assay (ELISA), and its relationship with clinical and prognostic characteristics was analyzed. The results showed that the level of GDF-15 in the serum of patients with NSCLC was significantly higher than that in the healthy control group and patients with benign lung diseases (p<0.001). They further used Cox regression model to conduct multivariate analysis, and found that high level of GDF-15 in the serum was an independent risk factor for reducing the overall survival rate of patients with lung cancer (HR=3.37, 95% CI:1.09-10.42, p=0.035).

In this study, the prognostic analysis showed that the high expression of GDF-15 in tumor tissues was associated with poor prognosis of patients with gallbladder cancer. Univariate analysis by Cox regression model showed that the expression of GDF-15 (p=0.003), TNM stage (p=0.008) and tumor differentiation level (p=0.028) were significantly associated with the prognosis of patients with gallbladder cancer. Furthermore, multivariate analysis showed that the overexpression of GDF-15 (p=0.009) was an independent risk factor for the prognosis of this disease. Urakawa et al found the same result in esophageal squamous cell carcinoma. They analyzed the prognosis of 69 patients with esophageal cancer and the results showed that the overall survival time of patients with high expression of GDF-15 was significantly shorter than that of patients with low expression of GDF-15 [32].

Although this study is the first to analyze the correlation between GDF-15 and gallbladder cancer, there are still some limitation on this subject, which need further improvement. First of all, gallbladder cancer is a relatively rare disease, the clinical samples are relatively limited, and the lack of sample data will lead to the increase of the confidence interval, so the reliability of the experimental results may be insufficient, which still needs to be confirmed by large-scale multi-center clinical studies. Second, this study confirmed the abnormal expression of GDF-15 from the serological and the histological levels, but did not detect the cell level. Furthermore, this study did not count the patients' serum during the postoperative reexamination. Comparing the expression of GDF-15 before and after operation may help detect tumor recurrence and help improve the postoperative comprehensive treatment of patients.

In conclusion, GDF-15 plays an important role in many kinds of cancers. GDF-15 was highly expressed in the serum and tissues of patients with gallbladder carcinoma, and was closely related to tumor TNM stage, differentiation level and distant metastasis. These results suggest that GDF-15 can be used as a serum tumor marker for the prediction of this disease in early-stage, and may providing

evidence for the prediction of recurrence of this disease. We infer that if the expression of GDF-15 is artificially down-regulated, it may inhibit the occurrence and development of gallbladder cancer, thus providing a new target for targeted therapy of this disease. However, the specific biological mechanism and whether GDF-15 can be down-regulated in clinical treatment still need further study.

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## Conflict of interests

The authors declare no conflict of interests.

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