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**Prognostic implications of** **estrogen receptor1 and vascular endothelial growth factor A in primary gallbladder carcinoma**

Zhang LQ *et al*. ER1 and VEGF-A in GBC prognosis

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**Abstract**

**AIM:** To investigate the prognostic significance of estrogen receptor1 (ER1) and vascular endothelial growth factor A (VEGF-A) in primary gallbladder carcinoma (GBC) patients after surgery, so as to identify new prognostic markers for GBC.

**METHODS:** By immunohistochemistry methods, we investigated the ER1 and VEGF-A expressions in 78 GBC and 78 cholelithiasis (CS) tissues. The results were implicated with clinic-pathological features. Univariate and multivariate analysis were performed to evaluate the relationship between ER1 and VEGF-A expressions and patients’ prognosis. Further Kaplan-Meier survival analysis was also performed.

**RESULTS:** ER1 and VEGF-A were higher expressed in the GBC compared with CS (47/78 *vs* 28/78, *P* < 0.05; 51/78 *vs* 33/78, *P* < 0.05). ER1 expression was correlated with gender (*P* < 0.05) and VEGF-A expression was correlated with tumor differentiation in GBC patients (*P* < 0.05). In univariate analysis, age and tumor node metastasis (TNM) stage were factors associated with GBC prognosis (*P* < 0.05). Although there was no statistical difference between the expressions of ER1 and VEGF-A and overall survival, the high expressions of ER1 combined with VEGF-A predicted a poor prognosis for GBC patients (16.30 ± 1.87 *vs* 24.97 ± 2.09, log-rank *P* < 0.05). In multivariate analysis, ER1 combined with VEGF-A, TNM stage were independent prognostic factors for GBC patients (*P* < 0.05).

**CONCLUSION:** ER1 combined with VEGF-A is potential prognostic marker for GBC patients. Clinical detections of ER1 and VEGF-A in surgically resected GBC tissues would provide important reference for decision-making of postoperative treatment programs.

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**Key words:** Gallbladder carcinoma; Estrogen recptor1; Vascular endothelial growth factor A

**Core tip:** Gallbladder carcinoma (GBC) is a serious threat to public health for its poor prognosis. The authors found that estrogen receptor 1 (ER1) and vascular endothelial growth factor A (VEGF-A) were higher expressed in GBC than cholelithiasis tissues, and high expression of ER1 combined with VEGF-A conferred a poor prognosis in GBC patients after surgery. ER1 combined with VEGF-A was an independent factor associated with GBC prognosis. As potential prognostic marker, clinical detections for ER1 and VEGF-A may guide postoperative clinical treatment of GBC patients.

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**INTRODUCTION**

Primary gallbladder carcinoma (GBC), originated from bile duct epithelium, is characterized by poor prognosis[1,2]. Most of GBC patients were asymptomatic until the disease has progressed to an advanced and non-curative stage. According to epidemic investigations, the 5-year survival rate for GBC patients was less than 10%, with the overall mean survival time of 6 months. In clinic, the tumor node metastasis (TNM) staging system sometimes could not predict GBC patients’ prognosis accurately. In spite of this, except for TNM staging system, there were no other molecular markers available to facilitate the evaluation of GBC prognosis. Therefore, it is imperative to explore new predictive factors to guide the postoperative treatments for GBC patients.

Due to the female predominance in GBC incidence, it is speculated that estrogen may play important roles in the genesis and progress of GBC[3-5]. Estrogen execute its biological functions by binding with estrogen receptors (ER), and a number of studies have reported that ER was associated with carcinogenesis[6-10]. ER includes two subtypes, ER1 (or ER-α) and ER2 (or ER-β). In spite of similar molecular structure, ER1 and ER2 exhibited an antagonistic effect in some biological processes. As far as our knowledge, ER1 is able to promote tumor development and indicates poor prognosis, while ER2 usually suppress tumor progression and prefigures good survival[11-13]. Therefore, some researchers assumed that ER1 possibly keep a subtle balance with ER2 in normal conditions[14]. Sumi *et al*[13] have reported the relationship between ER2 and GBC prognosis. However, although ER1 has been detected in GBC samples, the clinical significance is still equivocal.

Angiogenesis is essential for cancer growth, invasion and metastasis. It is well-known that vascular endothelial growth factor (VEGF) is a potent vascular active molecule which directly stimulate the proliferation of vascular endothelial cells[15]. Accumulated evidences suggested that VEGF plays important roles in many kinds of tumors by inducing neoangiogenesis. In human cholangiocarcinoma, VEGF-A was positive expression, and was considered to mediate the proliferative effects of estrogen[16]. Similar to other tumors, adequate blood supply and sufficiant angiogenesis are fundamental requirements for the growth of GBC. In GBC, the VEGF-A single nucleotide polymorphisms were implicated with GBC risk[17]. There was also investigations indicated that VEGF-A was highly expressed in GBC and was correlated with a poor prognosis[18]. Nevertheless, Giatromanolaki *et al*[19] reported that VEGF was not associated with GBC patient survival, yet both VEGF and thymidine phosphorylase (TP) expression were considered as unfavorable prognostic factors. Therefore, it is still controversial in regards of the prognostic significance of VEGF in GBC.

ER1 and VEGF-A play important roles in GBC. Estrogen can modulate VEGF expression[20-23]. However, there was no relevant reports about the prognostic significance of ER1 and VEGF-A in GBC. Hence, we decided to investigate the expression status of ER1 and VEGF-A in resected human GBC tissues, and to evaluate the prognostic values in GBC.

**MATERIALS AND METHODS**

***Tissue specimens***

In the present study, tissue specimens were collected from 156 consecutive patients who had undergone surgical resection of GBC at the First Affiliated Hospital of Medical College, Xi'an Jiaotong University (Xi'an, China) between October 2009 and October 2010: 78 patients with GBC confirmed by postoperative pathological diagnosis, and 78 patients with cholelithiasis (CS) underwent cholecystectomy. None of them received any preoperative radiochemotherapy. The two groups were matched in age and gender. The clinic-pathological information was obtained from the hospital’s medical records. The following data of each patient was included: age, gender, gallstone status, tumor differentiation, TNM (tumor, lymph nodes, and metastases) stage. All GBC patients were closely followed-up after surgery ranged from 4 to 53 months, and we defined that GBC patient’s death was the only positive outcome in our study.

***Immunohistochemistry process***

Immunohistochemistry-streptavidin-peroxidase (SP) method was performed using rabbit polyclonal antibody to ER1 and VEGF-A obtained from Santa Cruz Biotechnology to detect the expression of ER1 and VEGF-A in GBC and CS tissues. The whole process was described briefly as following: formalin-fixed and paraffin-embedded specimens were cut into 4 μm sections, mounted onto slides treated with poly-L-lysine, deparaffinized, and rehydrated; the slides were heated at 96-98 ˚C in a microwave for 15 min in a citrate buffer solution at pH 6.0 and cooled for 30 min in room temperature to retrieve antigen. To quench the endogenous peroxidase activity, sections were dealt with 0.3% H2O2 for 30 min. Subsequently, the sections were treated with 5% normal goat serum in phosphate-buffered saline for 1 h to block nonspecific sites. All sections in a humidified box were incubated overnight at 4 ˚C with specific antibodies detecting ER1 and VEGF-A, and then incubated with biotinylated antirabbit IgG and avidin-biotin-peroxidase complex, respectively. Finally, antibody binding was visualized by exposure to diaminobenzidine (DAB). Then，hematoxylin was used to weakly counterstain sections. And then, the sections were dehydrated in graded alcohol and cleared in transparent solution of xylene in turn. Finally, all sections were mounted with neutral gum.

***Immunohistochemical assess of ER1 and VEGF-A***

According to previous literatures[18,24,25], semiquantitative manner was used to evaluate the staining of ER1 and VEGF-A. All of the sections were assessed by two separate investigators in a blind manner under a transmission light microscope. We assessed both the intensity of staining (IS) and the percentage of positive staining (PS) cells. The IS was scored as 0 (absent), l (weak), 2 (moderate), and 3 (strong). The percentage of tissues PS was scored as 0 (none), 1 (1% to 25%), 2 (26% to 50%), 3 (51% to 75%), and 4 (76% to 100%). 5 fields per case and 100 tumor cells per × 40 field were examined. The mean value obtained was the final score for each case. A final score (FS) was calculated using the formula: FS = IS + PS. Finally, all the sections were defined as “low” expression with FS 0-4 or “high” expression with FS 5-7 for assessment of ER1 and VFGF-A staining. The typical histology of each histological scores used in this study was shown in Figure 1.

***Statistical analysis***

Fisher’s exact test or Chi-square test as appropriate was performed to assess the associations between the ER1 and VEGF-A expressions and clinic-pathological variables. Kaplan and Meier method were used to plot survival curves, and the log-rank test was used to determine statistical differences. Multivariate analysis was performed using Cox proportional hazard model.*P* value less than 0.05 was considered statistically significant. All statistical analysis was based on SPSS 13.0 program.

**RESULTS**

***ER1 and VEGF-A*** ***are higher expressed in GBC tissues compared with CS tissues***

The expression status of ER1 and VEGF-A were shown in Figure 2. VEGF-A was expressed in cytoplasmic compartment, and ER1 was expressed in cell nucleus. The expression of ER1 and VEGF-A were both significantly higher in GBC compared with CS (Table 1). Higher ER1 expression was observed in more GBC (47/78, 60.3%) than in CS (28/78, 35.9%) (*P* = 0.002). Similarly, higher expression of VEGF-A was observed in more GBC (51/78, 65.4%) than CS (33/78, 42.3%) (*P* = 0.004). In GBC patients, there was no statistical significance between the histological scores of ER1 and VEGF-A (*r* = 0.176, *P* = 0.124).

***Relationship between*** ***the expressions of ER1 and VEGF-A and clinic-pathological GBC features***

ER1 expression was associated with gender. ER1 expression was more frequent in female than male (*P* = 0.022). In addition, VEGF-A expression was correlated with tumor differentiation (*P* = 0.01). No other significant difference was found between the expression of ER1 and VEGF-A with clinic-pathological factors (Table 2).

***Expressions of ER1 and VEGF-A and GBC prognosis***

By univariate analysis (Table 3), age and TNM stage were associated with GBC prognosis (*P* < 0.05). Patients with GBC in stage 2 have a better survival than stage 3 and stage 4 (Figure 3A). Although there was no statistical difference between ER1 and VEGF-A expression status and GBC prognosis, respectively (Figure 3B and 3C, *P* > 0.05), the expression of ER1 combined with VEGF-A was correlated with postoperative survival of GBC patients (Figures 3D and 4, *P* < 0.05). GBC patients with simultaneously high expressions of ER1 and VEGF-A have a poorer prognosis. By multivariate analysis, TNM stage, ER1 combined with VEGF-A expression were identified as independent prognostic factors (*P* < 0.05) (Table 4). There was no statistical significance between ER1 and VEGF-A expression and GBC recurrence (*P* > 0.05).

**DISCUSSION**

The present study examined the expression status of ER1 and VEGF-A in resected human GBC and CS tissues. The main findings are following aspects: (1) ER1 and VEGF-A expressions were both higher expressed in GBC than CS tissues; ER1 level was implicated with gender, and VEGF-A expression was associated with tumor differentiation; and (2) high expression of ER1 combined with VEGF-A in GBC predicted a poor prognosis. This is the first time to report prognostic significance of ER1 combined with VEGF-A in GBC.

GBC’s poor prognosis caused wide public attentions. Despite of rapid improvement in medical technology in past decades, the survival time of GBC patients are far from satisfactory. Based on lots of clinical and molecular investigations about GBC, we speculated that the dismal prognosis of GBC patients may be attributed to the following aspects: (1) early diagnosis is difficult and most GBC patients are confirmed at an advanced stage, so as to lose the best surgical chances; (2) In respect of treatment options, GBC is relatively resistant to chemotherapy and radiation; apart from surgical resection, lack other effective measures; and (3) Postoperative therapy for GBC patients should be selected according to patients’ prognosis. Despite a number of studies have been conducted about GBC molecular mechanisms, there were no effective prognostic biomarkers for GBC to guide postoperative treatment. The present study exhibited that ER1 combined VEGF-A were associated with GBC prognosis, and would favor postoperative treatment.

ER1 has been investigated in several tumors as a promising factor. In ER-negative breast cancer, ER1 expression was necessary and sufficient in the bone marrow-derived cells themselves to promote tumor formation in response to estrogen[12]. In biliary tract cancers (including tumors of the gallbladder, bile duct and ampulla of Vater), the single nucleotide polymorphisms of gene coding ER1 were correlated with risks of these tumors[26]. In our study, the results showed that ER1 was higher expressed in GBC compared with CS. This indicated that ER1 probably play an important role in GBC, despite that the exact mechanisms are unclear at present. In addition, ER1 expression in GBC tissue exhibited a female predominance. It is well known that the overall level of estrogen in female is obviously higher than male. So, it is likely that estrogen induced the ER overexpression in female. In the meanwhile, our findings may partially explain why GBC is more frequent in female. Nevertheless, there was no statistical difference between ER1 expression and postoperative survival.

VEGF-A, a classic biological molecule in the angiogenesis, has been investigated in various kinds of cancers. In human intra-hepatic cholangiocarcinoma, VEGF-A mediated the proliferative effect of estrogen to promote cholangiocarcinoma growth[16]. As to VEGF-A and GBC，there have been many literature reports[17,18,24,25]. Recently, there was a report revealed that VEGF-A was highly expressed in GBC and correlated with poor prognosis[18]. Additionally, there was another report showed that VEGF-A expression in GBC tissues is correlated with histologic differentiation and is independent prognostic factor[24]. Our results were inconsistent with these previous investigations. Nevertheless, of note in our results, the high expression of ER1 combined with VEGF-A in GBC tissues predicted a poor prognosis. Based on this finding, we speculated that there were potentially synergistic effects between VEGF-A and ER1 in GBC progress. From perspective of biological significance, the assumption is possible. Estrogen binding with ER can promote production of VEGF as mentioned before. Increasing VEGF can induce the angiogenesis to provide plenty of oxygen and nutrients, and then promote GBC growth, invasion and metastasis, and finally lead to a poor survival. Of course, this assumption needs to be confirmed through further investigations.

Some limitations should be taken into account. Firstly, the sample size of this study is small. Secondly, our study is not deep, and very little information is about molecular mechanisms.

In conclusion, our study suggested that ER1 combined with VEGF-A confers a particularly poor post-operative survival outcome, and represents potential prognostic biomarker for GBC. Clinical detections for ER1 and VEGF-A in surgically resected GBC tissues may provide reference for decision-making of postoperative treatment programs. GBC patients，once have high expressions of ER1 and VEGF-A, deserve a close surveillance to reduce postoperative mortality.

***Prospect***

Although ER1 and VEGF-A have been considered to be involved in many kinds of tumor progress, the roles of ER1 and VEGF-A in GBC development has not been reported. Further investigations to explore the potential roles of ER1 and VEGF-A in GBC progress is meaningful for clarifying the molecular mechanism of GBC. In addition, ER1 combined VEGF-A may represent potential therapeutic targets and adjuvant endocrine therapy may be new approaches for GBC.

**COMMENTS**

***Background***

Primary gallbladder carcinoma (GBC) is characterized by poor prognosis. In clinic, there was no effective biomarker to predict the prognosis of GBC patients. Estrogen receptor1 (ER1) and vascular endothelial growth factor A (VEGF-A) are involved in several kinds of malignancies. However, the prognostic significance of ER1 and VEGF-A in GBC are controversial, and need further to be confirmed.

***Research frontiers***

According to epidemiology, the 5-year postoperative survival of GBC patients is less than 10%. Therefore, it is a current hotspot that exploring effective prognostic markers to guide postoperative treatment for GBC patients so as to improve survival after surgery.

***Applications***

Clinical detections of ER1 and VEGF-A expressions can predict prognosis of GBC patients, and provide references for making-decision of postoperative treatment programs. In addition, the identification of ER1 and VEGF-A expressions in human GBC tissues would help to investigate deeply into molecular mechanisms of GBC.

***Terminology***

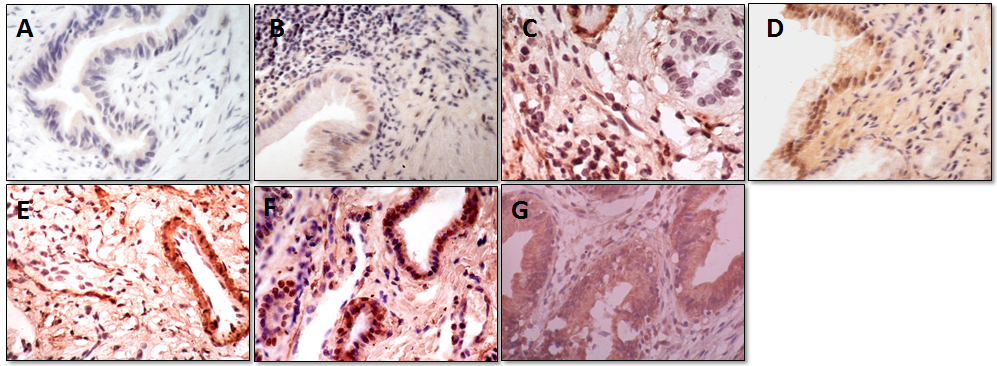
ER1 also named estrogen receptor alpha, a kind of ligand-regulated transcription factor, mediated biological actions of estrogen. ER1 is implicated in several kinds of tumors. VEGF-A (vascular endothelial growth factor A), can promote physiological and pathological angiogenesis, and is believed to play an important role in various tumors.

***Peer review***

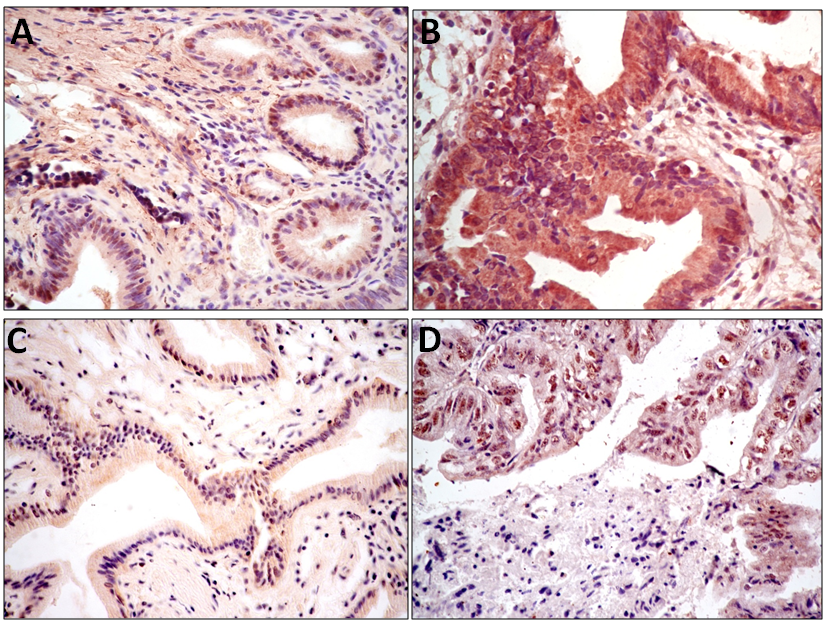
The authors reported that ER1 combined with VEGF-A assessed by immunohistochemistry was potential prognostic marker for GBC patients after surgery. Their findings were useful for the postoperative clinical treatment of GBC patients.

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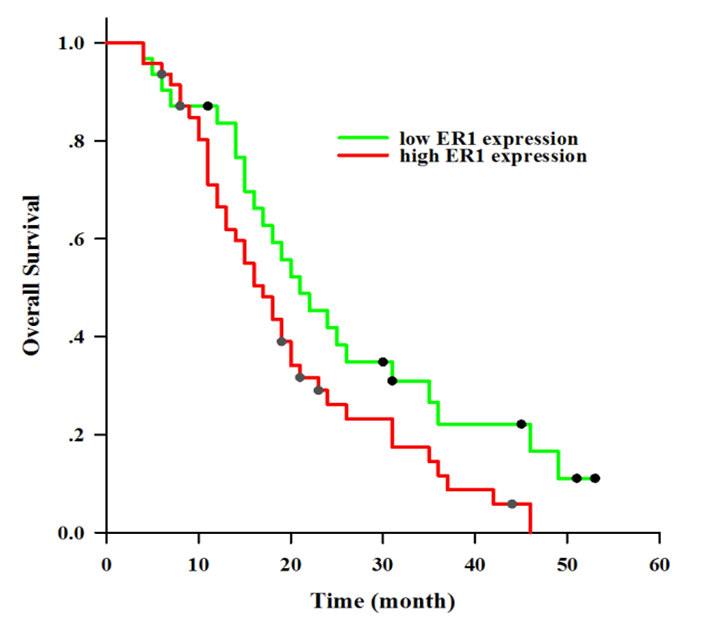
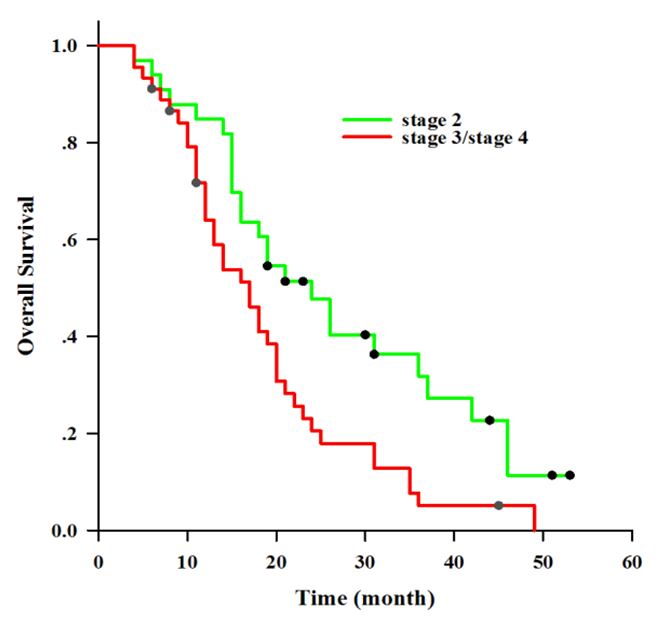
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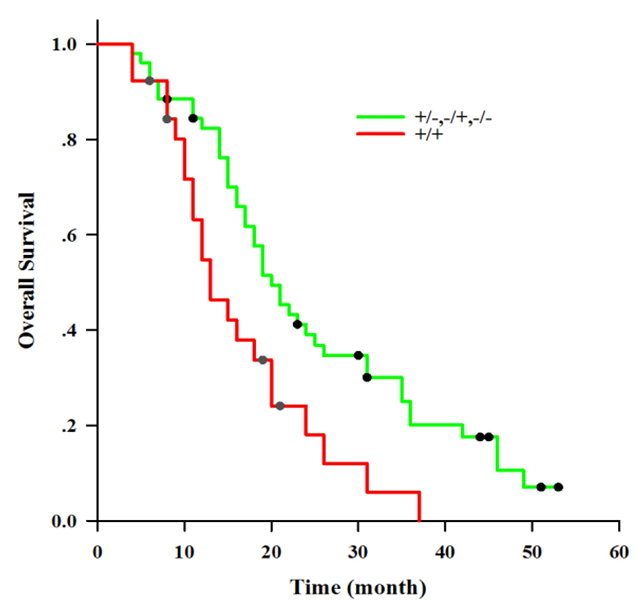
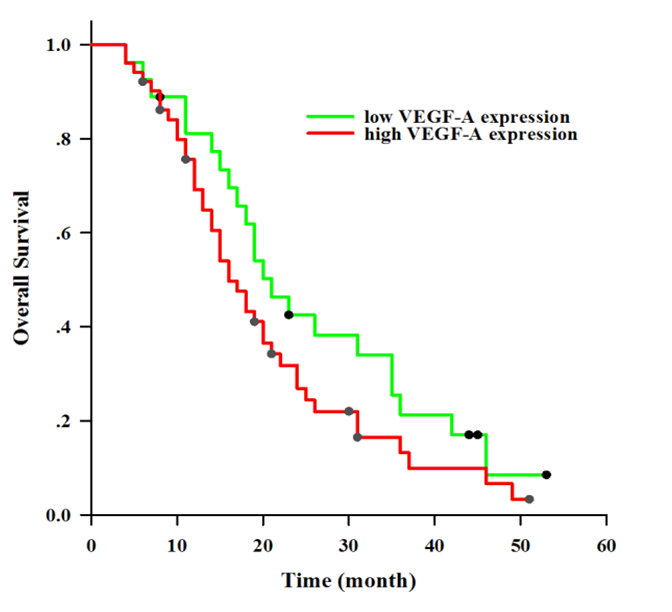
**Figure 1 Typical histology of each histological scores.** A, B, C, D, E, F, G, H was scored as 0, 2, 3, 4, 5, 6, 7, respectively.



**Figure 2 Immunohistochemical staining of estrogen receptor1 and vascular endothelial growth factor A expressions in gallbladder carcinoma and cholelithiasis specimens.** A and B show expression status of vascular endothelial growth factor A (VEGF-A) (A: low VEGF-A expression in CS tissues; B: high VEGF-A expression in GBC tissues); C and D show estrogen receptor 1(ER1) expression status (C: low ER1 expression in CS tissues; D: high ER1 expression in GBC tissues).

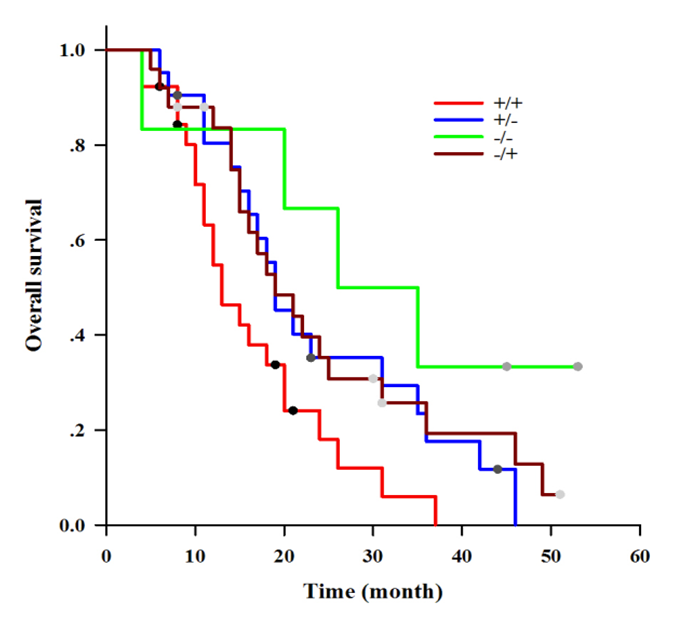


A B



C D

**Figure 3 Kaplan–Meier survival curves.** A: stratified for estrogen receptor 1 expression status. Low estrogen receptor1 (ER1) expression has a better survival time than high ER1 expression, but there was no statistical significance between the two groups (*P* = 0.053); B: stratified for vascular endothelial growth factor A expression status. Low vascular endothelial growth factor A (VEGF-A) expression has a better survival time than high VEGF-A expression, but there was no statistical significance between two groups (*P* = 0.155); C: stratified for tumor node metastasis stage. Patients in stage 2 have a better prognosis than patients in stage 3 and stage 4 (*P* = 0.07); D: stratified for estrogen receptor and vascular endothelial growth factor A expressions. All patients were clarified into two groups: high expression of estrogen receptor1 (ER1) and vascular endothelial growth factor A (VEGF-A) group (+/+), and low expression of ER1 and VEGF-A group (+/-, -/+, -/-). Patients in high expression of ER1 and VEGF-A group have a worse prognosis than high expression of ER1 and VEGF-A group (*P* = 0.009). +/+: high ER1 expression and high VEGF-A expression; +/-: high ER1 expression and low VEGF-A expression; -/+: low ER1 expression and high VEGF-A expression; -/-: low ER1 expression and low VEGF-A expression.



**Figure 4 Kaplan–Meier survival curves stratified for estrogen receptor1 and** **vascular endothelial growth factor A expressions.** Patients with high expression of estrogen receptor1 (ER1) combined with vascular endothelial growth factor A (VEGF-A) (+/+) have worst prognosis than other groups (+/-,-/+,-/-) (*P* = 0.007). +/+: high ER1 expression and high VEGF-A expression; +/-: high ER1 expression and low VEGF-A expression; -/+: low ER1 expression and high VEGF-A expression; -/-: low ER1 expression and low VEGF-A expression.

**Table 1 Comparison of expressions of estrogen receptor1 and vascular endothelial growth factor A between gallbladder carcinoma and cholelithiasis**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Group** | **ER1 expression** | | ***P* value** | **VEGF-A** **expression** | | ***P* value** |
| High | Low | High | Low |
| GBC | 47 | 31 | 0.002 | 51 | 27 | 0.004 |
| CS | 28 | 50 |  | 33 | 45 |  |

GBC: Gallbladder carcinoma; CS: Cholelithiasis; ER1: Estrogen receptor 1; VEGF-A: Vascular endothelial growth factor A.

**Table 2 Association between estrogen receptor 1 and vascular endothelial growth factor A expressions and clinic-pathological characteristics of patients with gallbladder carcinoma**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Characteristics** | **ER1 expression** | | ***P* value** | **VEGF-A expression** | | ***P* value** |
| **High** | **Low** | **High** | **Low** |
| Gender |  |  | 0.022 |  |  | 0.099 |
| Male | 15 | 18 |  | 25 | 8 |  |
| Female | 32 | 13 |  | 26 | 19 |  |
| Age |  |  | 0.151 |  |  | 0.095 |
| ≤ 55 | 18 | 17 |  | 23 | 12 |  |
| > 55 | 29 | 14 |  | 28 | 15 |  |
| Gallstone |  |  | 0.370 |  |  | 0.056 |
| Present | 32 | 24 |  | 33 | 23 |  |
| Absent | 15 | 7 |  | 18 | 4 |  |
| TNM stage |  |  | 0.177 |  |  | 0.781 |
| Ⅱ | 17 | 16 |  | 21 | 12 |  |
| Ⅲ/Ⅳ | 30 | 15 |  | 30 | 15 |  |
| Differentiation |  |  | 0.205 |  |  | 0.010 |
| Well | 16 | 15 |  | 15 | 16 |  |
| Moderately/Poor | 31 | 16 |  | 36 | 11 |  |

ER1: estrogen receptor 1; VEGF-A: vascular endothelial growth factor A; TNM: tumor node metastasis.

**Table 3 Univariate analysis of prognostic factors associated with overall survival in patients with gallbladder carcinoma**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Risk factor** | **Survival time(month) (mean ± SE)** | | | ***P* value (Log-rank test)** |
| Gender | |  | 0.682 | |
| Male | | 23.24 ± 4.09 |  | |
| Female | | 21.97 ± 1.72 |  | |
| Age(year) | |  | 0.015 | |
| ≤ 55 | | 26.34 ± 2.71 |  | |
| > 55 | | 18.77 ± 1.71 |  | |
| Gallstones | |  | 0.068 | |
| Present | | 17.69 ± 2.06 |  | |
| Absent | | 23.96 ± 2.02 |  | |
| TNM stage | |  | 0.007 | |
| Ⅱ | | 27.16 ± 2.77 |  | |
| Ⅲ/Ⅳ | | 18.59 ± 1.74 |  | |
| Differentiation | |  | 0.685 | |
| Well | | 23.19 ± 2.94 |  | |
| Moderately/Poor | | 21.65 ± 1.86 |  | |
| ER1 level | |  | 0.053 | |
| High | | 19.81 ± 1.79 |  | |
| Low | | 25.85 ± 2.86 |  | |
| VEGF-A level | |  | 0.155 | |
| High | | 20.35 ± 1.87 |  | |
| Low | | 25.65 ± 2.86 |  | |
| ER1 combined VEGF-A level | |  | 0.007 | |
| +/+ | | 16.30 ± 1.87 |  | |
| +/-, -/+, -/- | | 24.97 ± 2.09 |  | |

ER1: Estrogen receptor 1; VEGF-A: Vascular endothelial growth factor A; TNM: Tumor node metastasis; +/+: High ER1 expression and high VEGF-A expression; +/-: High ER1 expression and low VEGF-A expression; -/+: Low ER1 expression and high VEGF-A expression; -/-: Low ER1 expression and low VEGF-A expression.

**Table 4 Multivariate analysis of factors associated with survival in patients with gallbladder carcinoma**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Items** | **Hazard ratio** | **95% CI** | ***P* value** |  |
| Age |  |  | 0.076 |  |
| ≤ 55 *vs* > 55 | 0.615 | 0.359-1.053 |  |  |
| TNM stage |  |  | 0.031 |  |
| Ⅲ/Ⅳ *vs* Ⅱ | 1.781 | 1.054-3.011 |  |  |
| ER1 combined VEGF-A |  |  | 0.042 | |
| +/+ *vs* +/-, -/+, -/- | 1.773 | 1.021-3.080 |  |  |

ER1: Estrogen receptor 1; VEGF-A: Vascular endothelial growth facto; TNM: Tumor node metastasis; +/+: High ER1 expression and high VEGF-A expression; +/-: High ER1 expression and low VEGF-A expression; -/+: Low ER1 expression and high VEGF-A expression; -/-: Low ER1 expression and low VEGF-A expression.