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ORIGINAL ARTICLE

Retrospective Study

Prognostic implications of estrogen receptor 1 and vascular endothelial growth factor A expression in primary gallbladder carcinoma

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Abstract

AIM: To investigate the prognostic significance of estrogen receptor 1 (ER1) and vascular endothelial growth factor A (VEGF-A) expression in primary gallbladder carcinoma (GBC) to identify new prognostic markers for this malignancy.

METHODS: Using immunohistochemistry, we investigated ER1 and VEGF-A expression in 78 GBC and 78 cholelithiasis (CS) tissues. The results were correlated with clinicopathological features. Univariate and multivariate analyses were performed to evaluate the relationship between ER1 and VEGF-A expression and patients' prognosis. Further Kaplan-Meier survival analysis was also performed.

RESULTS: ER1 and VEGF-A expression was significantly higher in 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 expression of ER1 or VEGF-A and overall survival, the high expression 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, combined expression of ER1 and VEGF-A and TNM stage were independent prognostic factors for GBC patients ($P < 0.05$).

CONCLUSION: Combined expression of ER1 and VEGF-A is a potential prognostic marker for GBC patients. Clinical detection of ER1 and VEGF-A in surgically resected GBC tissues would provide an

important reference for decision-making of post-operative treatment programs.

Key words: Gallbladder carcinoma; Estrogen receptor 1; 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) expression was significantly higher in GBC than in cholelithiasis tissues, and high expression of ER1 combined with VEGF-A conferred a poor prognosis in GBC patients after surgery. Combined expression of ER1 and VEGF-A was an independent factor associated with GBC prognosis. Clinical detection of ER1 and VEGF-A may guide postoperative clinical treatment of GBC patients.

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INTRODUCTION

Primary gallbladder carcinoma (GBC), originating from the 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 epidemiological investigations, the 5-year survival rate for GBC patients was less than 10%, with the overall mean survival time of 6 mo. In clinical practice, the tumor node metastasis (TNM) staging system sometimes could not predict GBC patients' prognosis accurately. In spite of this, except for the 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 progression of GBC[3-5]. Estrogen executes its biological functions by binding to estrogen receptor (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 suppresses 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, its 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 stimulates the proliferation of vascular endothelial cells[15]. Accumulating evidence suggested that VEGF plays important roles in many kinds of tumors by inducing neoangiogenesis. In human cholangiocarcinoma, VEGF-A was positively expressed and was considered to mediate the proliferative effects of estrogen[16]. Similar to other tumors, adequate blood supply and sufficient angiogenesis are fundamental requirements for the growth of GBC. In GBC, the VEGF-A single nucleotide polymorphisms were implicated in GBC risk[17]. There was also investigations indicating 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, but combined VEGF and thymidine phosphorylase expression was considered an unfavorable prognostic factor. Therefore, it is still controversial with regards to 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 have been no relevant reports about the prognostic significance of ER1 and VEGF-A in GBC. Hence, we investigated the expression status of ER1 and VEGF-A in resected human GBC tissues, and to evaluate their prognostic value in GBC.

MATERIALS AND METHODS

Tissue specimens

In the present study, tissue specimens were collected from 156 patients who had undergone surgical resection at the First Affiliated Hospital of Medical College, Xi'an Jiaotong University (Xi'an, China) between October 2009 and October 2010, including 78 patients with GBC confirmed by postoperative pathological diagnosis, and 78 patients with cholelithiasis (CS) who underwent cholecystectomy. None of them received any preoperative radiochemotherapy. The two groups were matched in age and gender. The clinicopathological information was obtained from the hospital's medical records. The following data of each patient was included: age, gender, gallstone status, tumor differentiation, and TNM stage. All GBC patients were closely followed after surgery for

4 to 53 mo, and we defined that GBC patient's death was the only positive outcome in our study.

Immunohistochemical staining

The 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. 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 ℃ in a microwave for 15 min in a citrate buffer solution at pH 6.0 and cooled for 30 min at room temperature to retrieve antigen. To quench the endogenous peroxidase activity, sections were treated with 0.3% H_2O_2 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 ℃ with specific antibodies detecting ER1 and VEGF-A, and then incubated with biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complex, respectively. Antibody binding was visualized by exposure to diaminobenzidine. Hematoxylin was used to weakly counterstain sections. The sections were dehydrated in graded alcohol and cleared in xylene. Finally, all sections were mounted with neutral gum.

Immunohistochemical assessment of ER1 and VEGF-A

According to the previous literature[18,24,25], a semi-quantitative manner was used to evaluate the staining of ER1 and VEGF-A. All of the sections were assessed independently by two investigators in a blind manner under a transmission light microscope. The intensity of staining (IS) and the percentage of positively stained (PS) cells were evaluated. The IS was scored as 0 (absent), l (weak), 2 (moderate), and 3 (strong). The percentage of PS cells was scored as 0 (none), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). Five 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 if FS was 0-4 or "high" expression if FS was 5-7 for assessment of ER1 and VFGF-A staining. The typical histology corresponding to each histological score used in this study is shown in Figure 1.

Statistical analysis

Fisher's exact test or χ^2 test as appropriate was performed to assess the associations between the ER1 and VEGF-A expression and clinicopathological variables. Kaplan-Meier method was 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. A *P*-value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 13.0 program.

RESULTS

ER1 and VEGF-A expression is significantly higher in GBC tissues compared with CS tissues

The expression status of ER1 and VEGF-A is shown in Figure 2. VEGF-A was expressed in the cytoplasmic compartment, and ER1 was expressed in the nucleus. The expression of both ER1 and VEGF-A was significantly higher in GBC compared with CS (Table 1). Higher ER1 expression was observed in more GBC (47/78, 60.3%) than in CS tissues (28/78, 35.9%) (P = 0.002). Similarly, higher expression of VEGF-A was observed in more GBC (51/78, 65.4%) than in CS tissues (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 expression of ER1 and VEGF-A and clinicopathological features of GBC

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

Expression of ER1 and VEGF-A and GBC prognosis

Univariate analysis (Table 3) revealed that age and TNM stage were significantly associated with GBC prognosis (P < 0.05). Patients with stage 2 GBC had a better survival than those with stages 3 and 4 disease (Figure 3A). Although there was no statistical difference between ER1 or VEGF-A expression status and GBC prognosis (Figure 3B and 3C, P > 0.05), combined expression of ER1 and VEGF-A was correlated with postoperative survival of GBC patients (Figures 3D and 4, P < 0.05). GBC patients with simultaneous high expression of ER1 and VEGF-A had a poorer prognosis. By multivariate analysis, TNM stage and combined ER1 and 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 of ER1 and VEGF-A in resected human GBC and CS tissues. The main findings are: (1) ER1 and VEGF-A

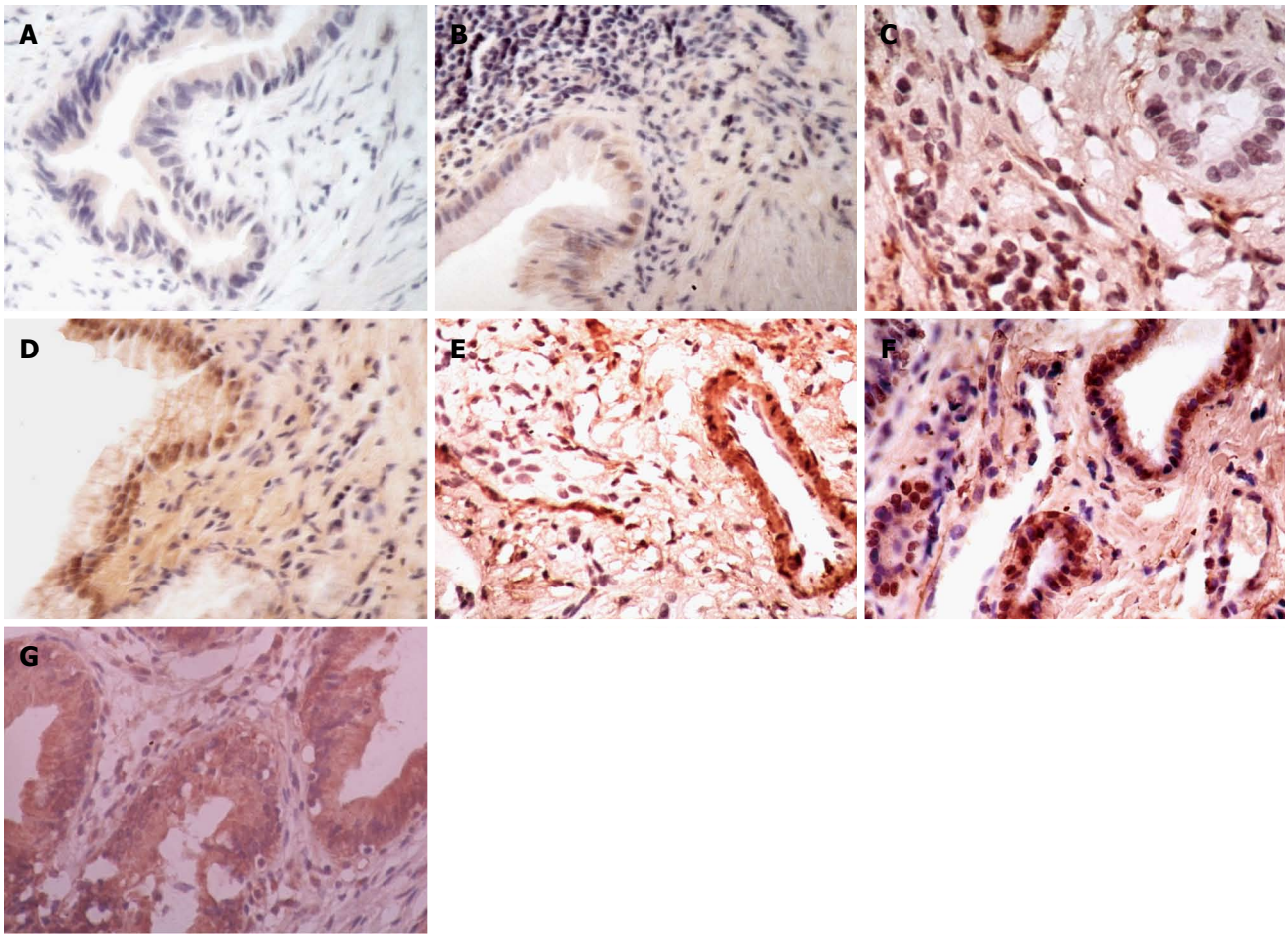

Figure 1 Typical histology corresponding to each histological score. A, B, C, D, E, F, and G were scored as 0, 2, 3, 4, 5, 6, and 7, respectively.

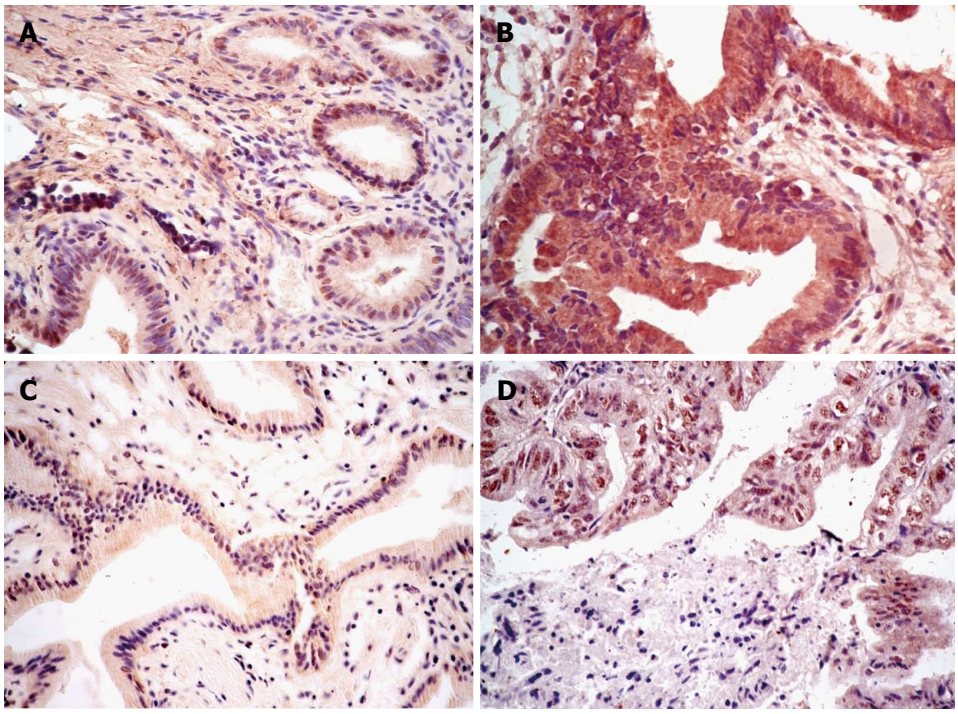

Figure 2 Immunohistochemical staining of estrogen receptor 1 and vascular endothelial growth factor A in gallbladder carcinoma and cholelithiasis specimens. A: Low vascular endothelial growth factor A (VEGF-A) expression in cholelithiasis (CS) tissue; B: High VEGF-A expression in gallbladder carcinoma (GBC) tissue; C: Low estrogen receptor 1 (ER1) expression in CS tissue; D: High ER1 expression in GBC tissue.

Table 1 Comparison of expression of estrogen receptor 1 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 expression and clinicopathological characteristics of gallbladder carcinoma

Characteristic	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 (yr)			0.151			0.095
≤ 55	18	17		23	12	
> 55	29	14		28	15	
Gallstones			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	
Moderate/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 (yr)		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	
Moderate/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 with VEGF-A		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.

expression were significantly higher in GBC than in CS tissues, ER1 expression was significantly associated 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 study to report prognostic significance of expression of ER1 combined with VEGF-A in GBC.

The poor prognosis of GBC has caused wide public attentions. Despite rapid improvement in medical technology over past decades, the survival time of GBC patients are far from satisfactory. Based on many 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 cases are diagnosed at an advanced stage and have lost the best surgical chance; (2) GBC is relatively resistant to chemotherapy and radiation; apart from surgical resection, other effective measures are lacking; and (3) postoperative therapy for GBC patients should be selected according to patients' prognosis. Despite a number of studies have been conducted about the molecular mechanisms of GBC, there have been no effective prognostic biomarkers for GBC to guide postoperative treatment. The present study exhibited that combined ER1 VEGF-A expression was associated with GBC prognosis, which would favor postoperative treatment.

ER1 as a promising prognostic factor has been investigated in several tumors. 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 the gene coding ER1 were correlated with risks of these tumors[26]. In our study, the results showed that ER1 expression was significantly higher in GBC compared with CS. This indicated that ER1 probably plays 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 females is obviously higher than in males. It is likely that estrogen induces ER overexpression in females. Thus, our findings may partially explain why GBC is more frequent in females. Nevertheless, there was no significant correlation between ER1 expression

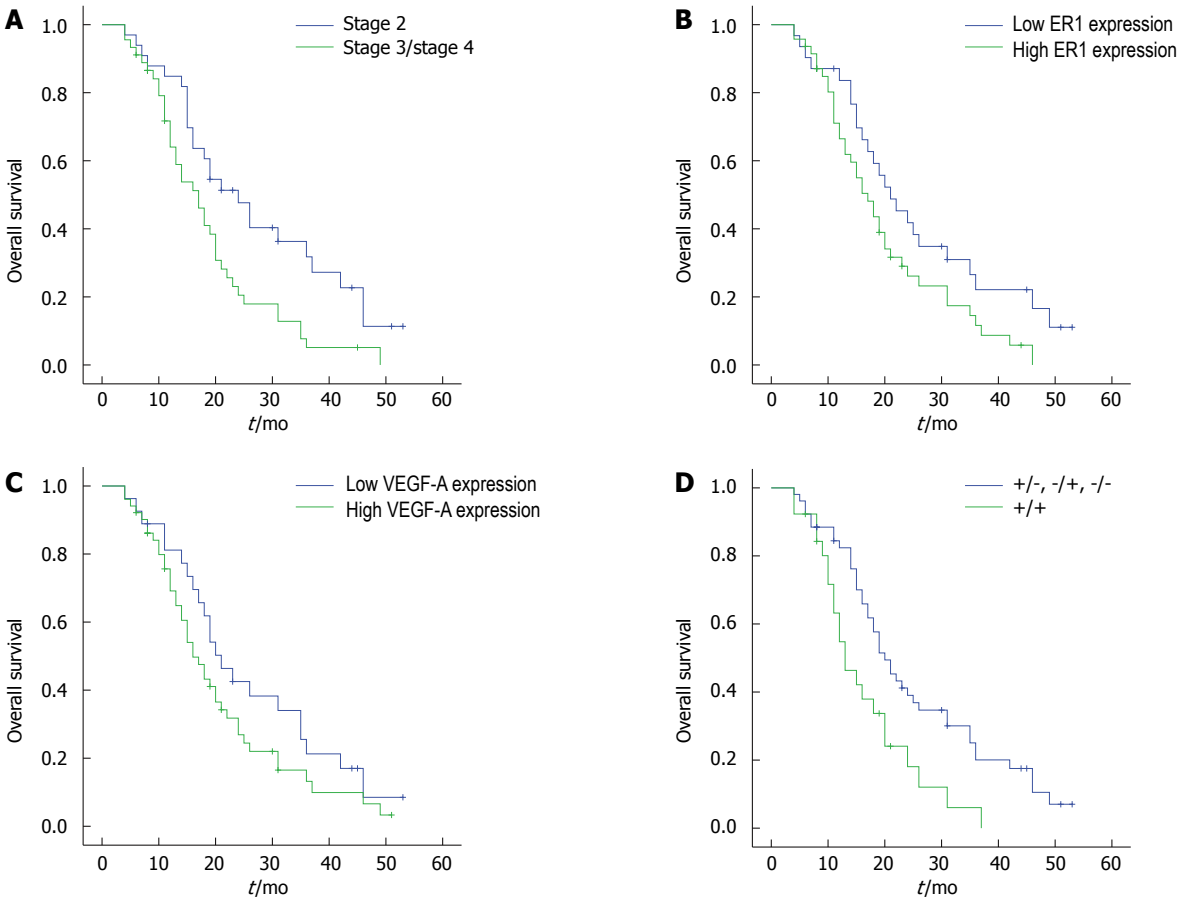

Figure 3 Kaplan-Meier survival curves. A: Stratified for tumor node metastasis stage. Patients with stage 2 disease had a better prognosis than patients with stages 3 and 4 disease (P = 0.07); B: Stratified for estrogen receptor 1 expression status. Low estrogen receptor 1 (ER1) expression was associated with a better survival time than high ER1 expression, but there was no statistical significance between the two groups (P = 0.053); C: Stratified for vascular endothelial growth factor A expression status. Low vascular endothelial growth factor A (VEGF-A) expression was associated with a better survival time than high VEGF-A expression, but there was no statistical significance between two groups (P = 0.155); D: Stratified for estrogen receptor and vascular endothelial growth factor A expression. 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 had a worse prognosis than low 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.

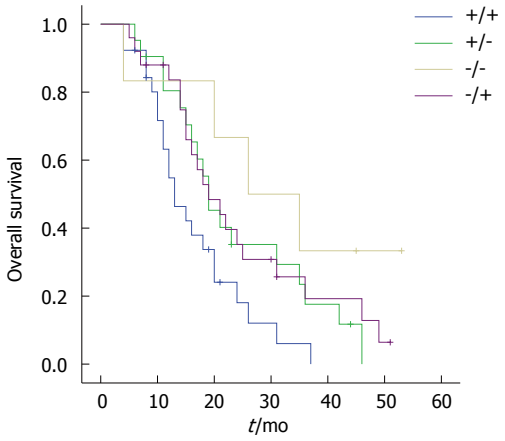

Figure 4 Kaplan-Meier survival curves stratified for estrogen receptor 1 and vascular endothelial growth factor A expression. Patients with high expression of estrogen receptor1 (ER1) combined with vascular endothelial growth factor A (VEGF-A) (+/+) had worst prognosis than other groups (+/-,-/+,-/-) (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 4 Multivariate analysis of factors associated with survival in patients with gallbladder carcinoma

Item	Hazard ratio	95% CI	*P*-value
Age, yr			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 with VEGF-A			0.042
+/+ *vs* +/-, -/+, -/-	1.773	1.021-3.080	

ER1: Estrogen receptor 1; VEGF-A: Vascular endothelial growth factor; 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.

and postoperative survival.

VEGF-A, a classic biological molecule in angiogenesis, has been investigated in various kinds of cancer. 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 reports[17,18,24,25]. Recently, a study revealed that VEGF-A was highly expressed in GBC and correlated with poor prognosis[18]. Additionally, another study showed that VEGF-A expression in GBC tissues is correlated with histologic differentiation and is an 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 speculate that there were potentially synergistic effects between VEGF-A and ER1 in GBC progression. From the perspective of biological significance, this assumption is possible. Estrogen binding to ER can promote production of VEGF as mentioned before. Increasing VEGF can induce angiogenesis to provide plenty of oxygen and nutrients, and thus promote GBC growth, invasion and metastasis, finally leading to a poor survival. Of course, this assumption needs to be confirmed by further investigations.

Some limitations of this study should be taken into account. The sample size of this study was small. In addition, our study was not mechanistic, and there was very little information about molecular mechanisms.

In conclusion, our study suggested that expression of ER1 combined with VEGF-A confers a particularly poor postoperative survival outcome, and represents a potential prognostic biomarker for GBC. Clinical detection of ER1 and VEGF-A in surgically resected GBC tissues may provide a reference for decision-making of postoperative treatment programs. GBC patients having high expression 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 progression of many kinds of tumors, their roles in GBC development have not been reported. Further investigations are required to explore the potential roles of ER1 and VEGF-A in GBC progression to clarify the molecular mechanism of GBC. In addition, ER1 and 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 clinical practice, there has been no effective biomarker to predict the prognosis of GBC patients. Estrogen receptor 1 (ER1) and vascular endothelial growth factor A (VEGF-A) are involved in the progression of several kinds of malignancies. However, the prognostic significance of ER1 and VEGF-A in GBC is controversial, and needs further investigation.

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 detection of ER1 and VEGF-A expression can predict prognosis of GBC patients, and provides a reference for making-decision of postoperative treatment programs. In addition, the identification of ER1 and VEGF-A expression in human GBC tissues would help to investigate the molecular mechanisms of GBC.

Terminology

ER1 also named estrogen receptor alpha, is a ligand-regulated transcription factor and mediates 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 expression of ER1 combined with VEGF-A assessed by immunohistochemistry was a potential prognostic marker for GBC patients after surgery. Their findings were useful for the postoperative clinical treatment of GBC patients.

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