

## Original Article

# Expression of vimentin and Oct-4 in gallbladder adenocarcinoma and their relationship with vasculogenic mimicry and their clinical significance

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**Abstract:** Vimentin (a marker of epithelial-mesenchymal transition), and Oct-4 (a marker of cancer stem cells) are predictive biomarkers for identifying malignant cell invasion and metastasis. Vasculogenic mimicry (VM), a newly discovered tumor characteristic that is common in highly invasive malignancies, is considered to be an important factor in evaluating the prognosis and metastasis of many malignancies. The following paper analyzes the correlation between vimentin, Oct-4, and VM in gallbladder adenocarcinoma (GBAC) specimens using immunohistochemistry in an attempt to elucidate the survival and clinicopathological parameters of changes in vimentin, Oct-4, and VM. Briefly, significantly higher positive expression rates of vimentin, Oct-4, and VM were observed in GBAC tissues than in the corresponding para-carcinoma tissues. In addition, the levels of vimentin, Oct-4, and VM were positively correlated with tumor grade, lymph node metastasis (LNM), infiltration of the surrounding tissues (STI), and tumor-node-metastasis (TNM) stage, as well as inversely with a patient's overall survival (OS) time. Moreover, the analysis of multiple factors shows that high vimentin, Oct-4, and VM levels, STI, and LNM as well as TNM stage were potential and significant factors for OS time in patients with GBAC. To sum up, the positive expression of vimentin, Oct-4, and VM may be undesirable factors for metastasis, invasion and prognosis, as well as effective therapeutic targets for GBAC.

**Keywords:** Gallbladder adenocarcinoma, VM, vimentin, Oct-4, prognosis

## Introduction

Gallbladder carcinoma (GC) is one of the most aggressive and pernicious tumors in the world [1]. It ranks fifth among all gastrointestinal carcinomas [2]. Gallstones, pebble-like collections of cholesterol and other substances that form in the gallbladder and can cause chronic inflammation, are regarded as important factors in gallbladder carcinoma [1]. Adenocarcinoma accounts for 98% of all gallbladder carcinomas; with two-thirds of these being moderately or poorly differentiated [3]. Females are affected two to six times more often than males [2]. Generally, the average survival time is nearly 6 months, while its 5-year survival rate is only 5% [1].

Poor prognosis of GBAC is associated with tumor cell metastasis and invasion. Epithelial-

mesenchymal transition (EMT) has been shown to have an essential role in cancer invasion and metastasis. EMT is characterized by reduced cell adhesion and increased cell motility [4]. Vimentin, one of the most abundant and highly conserved type III intermediate filament (IF) proteins and a representative marker of EMT, has been shown as essential for vital mechanical and biological cell functions such as cell stiffness, stiffening, contractility, migration and proliferation [4-8]. Its overexpression usually indicates a process of cellular reprogramming in which epithelial cells gain a mesenchymal phenotype that significantly alters their shape and exhibits increased motility. In addition, in recent years, vimentin has been associated with cancer invasion, metastasis, stage of tumor, lymph node metastasis, and patient survival in numerous types of cancer including prostate cancer, clear-cell renal cell carcinoma,

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**Table 1.** Patients characteristics

Patients characteristics	Frequency (n)	Percentage (%)
Age (years)		
≤60	35	33.3
>60	70	66.7
Gender		
Male	38	36.2
Female	67	63.8
Size (cm)		
<2	25	23.8
≥2	80	76.2
Grade		
Well	9	8.6
Moderate	73	69.5
Poor	23	21.9
LNM		
No	69	65.7
Yes	36	34.3
STI		
No	61	58.1
Yes	44	41.9
TNM Stage		
I	22	21.0
II	34	32.4
III	35	33.3
IV	14	13.3
Gallstones		
No	46	43.8
Yes	59	56.2

gastrointestinal tract cancers, breast cancer, cervical cancer, endometrial carcinomas, malignant melanoma, lung cancer, papillary thyroid carcinoma, and certain types of lymphomas [7, 9-12].

Similarly, cancer stem cells (CSCs) have also been shown to play an important role in GBAC metastasis and invasion. The term CSCs was initially introduced more than 20 years ago [13]. The concept quickly gained attraction as a bona fide biological phenomenon in leukemia, followed shortly by solid evidence of CSCs in multiple solid cancers [13, 14]. CSCs are characterized by self-renewal, multi-directional differentiation, high tumorigenicity and high invasiveness. Oct-4 (octamer-binding transcription factor 4) is a member of the POU family of TF. Its main function is to bind octamer sequence motifs and to activate the expression of target

genes [15]. Oct4 regulates embryonic stem cells' (ESCs) pluripotency and self-renewal ability [16] and is one of the key transcription factors in reprogramming the cell to acquire a pluripotent phenotype [17]. Moreover, the overexpression of Oct-4 has been found in various tumors such as bladder, brain, lung, ovary, pancreas, prostate, kidney, seminoma, and testicular cancer [18]. Furthermore, a high expression of Oct-4 has been associated with poor prognosis in patients with tumors [15, 18, 19]. Also, the overexpression of Oct-4 in tumor tissues is very likely linked to CSCs.

Recent studies have shown that the formation of VM leads to poor prognosis in patients with the tumor, which may be related to the poor prognosis of GBAC. A malignant tumor needs enough blood and nutrients for rapid growth and infiltration. In 1999, Maniotis *et al.* first proposed the concept of VM while they were studying invasive human uveal melanoma and metastatic cutaneous melanoma [20]. VM is a network of blood vessels surrounded by malleable, aggressive tumor cells. VM has been found in several other aggressive tumor types, including breast, prostate, ovarian carcinoma, and liver cancer, gastrointestinal stromal tumors, malignant glioma, colorectal cancer and throat squamous cell carcinoma [21-26]. VM has the following characteristics: negative CD34 staining, positive periodic acid-Schiff (PAS) staining; the pipeline structure without vascular endothelial cells; remodeling of the extracellular matrix; VM and tumor microvascular structure that are interlinked with each other and are both full of blood [20-27]. Some studies have suggested that higher positive rates of VM expression lead to stronger aggressiveness of the tumor and to worsened prognosis [20-28].

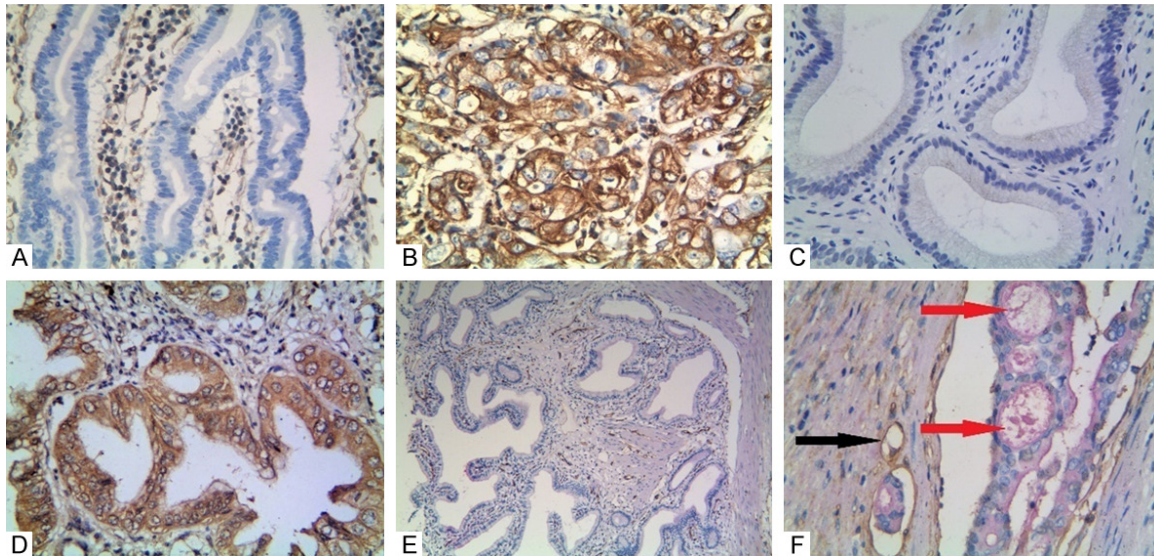
To sum up, vimentin, Oct-4, and VM have been associated with tumor metastasis and prognosis; nevertheless, the relationships between vimentin, Oct-4, and VM in GBAC still remain unexplored. The following study examines the assumption that these factors are mutually correlated and connected with metastasis and prognosis in GBAC.

### Materials and methods

#### Specimens

105 GBAC and the corresponding para-carcinoma tissues were collected at the Department of

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**Figure 1.** Immunostaining of vimentin, Oct-4, and VM in GBAC as well as the control tissue. A. Negative staining of vimentin in the control tissues (400 magnification); B. Positive staining of vimentin in the membrane and cytoplasm of cancer cells (400 magnification); C. Negative staining of Oct-4 in the control tissue (400 magnification); D. Positive staining of Oct-4 in the cytoplasm of cancer cells (400 magnification); E. Negative staining of VM in the control tissue (100 magnification); F. Positive staining of VM in the GBAC tissue (400 magnification, the red arrow is the VM structure and contains red cells; the black arrow is the microvessel).

Pathology, First Affiliated Hospital of Bengbu Medical University, from January 2009 to December 2012. The selected patients had never received chemo- or radio-therapy before the operation. All tissue samples were accessed after receiving each patient's informed consent. The study was approved by the Ethical Committee of Bengbu Medical University and was conducted in line with the ethical guidelines of the Declaration of Helsinki. The para-carcinoma tissues were taken from each patient, avoiding necrotic tissue, and from circumambient GBAC tissue at least 5 cm away from the resection margin. The research subjects included 105 people, 38 males and 67 females. Their ages ranged from 35 to 89 (the average age was  $63.7 \pm 10.9$  years old). All patients who completed the clinical, pathological and follow-up (6-month intervals by phone, message, or email) data showed a scattered distribution. Overall survival (OS) time was estimated as the time from the operation to death or till December 2017 (mean OS time 28.04 months; range 5-66 months). The TNM tumor stage was based on the *Cancer Staging Manual*, 8th edition, of the American Joint Committee on Cancer (AJCC). The tumor grade was evaluated according to WHO tumor differentiation standards. All patients' characteristics are shown in **Table 1**.

### Immunohistochemistry

Immunohistochemistry was performed according to the Elivision™ Plus detection kit instructions (Lab Vision, USA). All GBAC and the corresponding para-carcinoma tissues were fixed in 10% buffered formalin, embedded in paraffin and then cut into 4  $\mu\text{m}$  thick sections. All pathological sections were deparaffinized in xylene, dehydrated with graded alcohol, and consequently washed in a phosphate buffer saline (PBS, pH 7.2) for 10 min [30]. Endogenous peroxidase activity was quenched by incubating sections in methanol containing 3%  $\text{H}_2\text{O}_2$  at room temperature (RT) for 10 min. Samples were then placed in a citrate buffer (pH 6.0) and heated at  $95^\circ\text{C}$  for 30 min [29]. After washing with PBS, all sections were blocked with goat serum at RT for 30 min, following incubation with a mouse monoclonal antibody against human CD34 (DAKO, USA), Oct4 (DAKO, USA), and vimentin (DAKO, USA) at  $37^\circ\text{C}$  for 1 h [29]. All samples were subjected to periodic acid-Schiff (PAS)-CD34 dual staining to determine endothelial cells in the glycosylated basement membranes of vessels, as well as vessel like (VM) structures.

Yue's method was used to evaluate VM in the GBAC and the corresponding para-carcinoma

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**Table 2.** The associations between VM, vimentin and Oct-4 expression, and clinicopathological characteristics of gallbladder adenocarcinoma (GBAC)

Variables	VM		P	Vimentin		P	Oct-4		P
	-	+		-	+		-	+	
Age (years)			0.880			0.454			0.326
≤60	25	10		28	7		12	23	
>60	49	21		60	10		31	39	
Gender			0.922			0.223			0.553
Male	27	11		34	4		17	21	
Female	47	20		54	13		26	41	
Size (cm)			0.003			0.175			0.412
<2	23	2		23	2		12	13	
≥2	51	29		65	15		31	49	
Grade			<0.001			<0.001			<0.001
Well	8	1		9	0		7	2	
Moderate	59	14		70	3		35	38	
Poor	7	16		9	14		1	22	
LNM			<0.001			<0.001			<0.001
No	65	4		69	0		43	26	
Yes	9	27		19	17		0	36	
STI			<0.001			<0.001			<0.001
No	58	3		60	1		43	18	
Yes	16	28		28	16		0	44	
TNM Stage			<0.001			<0.001			<0.001
I	22	0		22	0		21	1	
II	34	0		34	0		22	12	
III	17	18		28	7		0	35	
IV	1	13		4	10		0	14	
Gallstones			0.266			0.058			0.948
No	35	11		42	4		19	27	
Yes	39	20		46	13		24	35	

**Table 3.** Correlation among VM, expression of vimentin and Oct-4 in GBAC

Variable	VM		r	P	Oct-4		r	P
	-	+			-	+		
VM							0.539	<0.001
-					43	31		
+					0	31		
Vimentin			0.679	<0.001			0.366	<0.001
-	74	14			43	45		
+	0	17			0	17		

tissues. Vimentin stains were mainly seen in tumor cell membranes and cytoplasm. Oct-4 stains were mainly observed in tumor cell cytoplasm.

### Evaluation of immunostaining data

Immunostaining results were assessed semi-quantitatively by two experienced pathologists who were blinded to the patients' follow-up and clinicopathological data. Ten representative fields at high-power-fields (HPF) from different areas of each GBAC slide were analyzed to avoid any intratumoral heterogeneity of antibody expression. The results were scored based on intensity (no staining count 0; weak staining count 1; moderate staining count 2; strong staining count 3) and extent (<10% positive cells count 1; 11-50% positive cells count 2; 51-75% positive cells count 3; >75% positive cells count 4). The results for the intensity and extent were multiplied to get final scores that ranged from 0-12. Those scores ≥3 were regarded as positive. For areas that were positive for both vimentin and Oct-4, an average of the final score of each area was taken.

### Statistical analysis

Relationships between clinicopathological variables and the expression of vimentin, Oct4 or VM were compared using Fisher's exact test or Chi-square test. Associations among the expressions of vimentin, Oct4 and VM were compared using Spearman's coefficient test. The effects of vimentin+, Oct4+ or VM+ on survival were determined by univariate and multivariate analyses. Independent prognostic factors were determined using the Cox regression model for multivariate analysis. The Kaplan-Meier method with log rank test for univariate analysis was used to assess associations between OS time and vimentin+, Oct4+ or VM+ results or clinicopathological characteristics, using SPSS 24.0 software (New York, IBM). P<0.05 was regarded as statistically significant.

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**Table 4.** Results of univariate analyses of overall survival (OS) time

Variable	N	Mean OS (months)	Log-rank	P value
VM			115.337	<0.001
Negative	74	35.80±15.51		
Positive	31	9.52±2.58		
Oct-4			92.875	<0.001
Negative	43	46.60±8.92		
Positive	62	15.16±8.51		
Vimentin			123.450	<0.001
Negative	88	31.92±16.83		
Positive	17	7.94±1.39		
Age (years)			0.729	0.393
≤60	35	25.69±16.81		
>60	70	29.21±18.24		
Gender			0.066	0.798
Male	38	29.58±17.75		
Female	67	27.16±17.87		
Size (cm)			3.497	0.061
<2	25	33.84±18.31		
≥2	80	26.23±17.33		
Grade			48.583	<0.001
Well	9	45.00±19.69		
Moderate	73	30.58±16.35		
Poor	23	13.35±10.64		
LNM			104.329	<0.001
No	69	37.29±14.98		
Yes	36	10.31±3.24		
STI			104.544	<0.001
No	61	39.90±13.75		
Yes	44	11.59±4.88		
TNM Stage			136.130	<0.001
I	22	49.95±10.76		
II	34	37.06±9.94		
III	35	13.09±5.04		
IV	14	9.07±2.90		
Gallstones			1.316	0.251
No	46	29.80±18.40		
Yes	59	26.66±17.31		

### Results

#### *Connections between vimentin, Oct-4, and VM, and clinicopathological characteristics*

For purposes of evaluating the effects of vimentin, Oct-4, and VM to GBAC, the results were immunohistochemically evaluated for both GBAC and the corresponding para-carcinoma

tissues specimens. These statistical results were compared with the clinicopathological characteristics of the patients. Briefly, significantly higher vimentin expression was observed in the GBAC tissues (16.2%, 17/105) compared to the corresponding para-carcinoma tissues (0%, 0/105;  $P<0.001$ ; **Figure 1A** and **1B**). The positive rate of vimentin expression in GBAC was associated with tumor grade, LNM, STI, TNM stage, but not with the patient's age, gender, size, and potential presence of gallstones (**Table 2**).

Like vimentin, the positive rate of Oct-4 expression was significantly higher in GBAC tissues (59.0%, 62/105) than that in the corresponding para-carcinoma tissues (1.9%, 2/105;  $P<0.001$ ; **Figure 1C** and **1D**). The positive rate of Oct-4 expression was positively correlated with tumor grade, LNM, STI, and TNM stage. In addition, there was no correlation between the Oct-4 level and the patient's gender, age, size, and potential presence of gallstones (**Table 2**).

The positive rate of VM (small lumen, the lumen was PAS-positive but CD34-negative) in the GBAC specimens (29.5%, 31/105) was obviously higher compared to the corresponding para-carcinoma tissues specimens (0%, 0/105;  $P<0.001$ ; **Figure 1E** and **1F**). In 31 cases of VM positive biliary GBAC tissue, random 10 HPF of vision, we found 967 lumens (36 lumens were filled in some red cells). The positive rate of VM in GBAC was positively associated with tumor size, grade, LNM, STI and TNM stage, but not with the patient's age, gender, or potential presence of gallstones (**Table 2**).

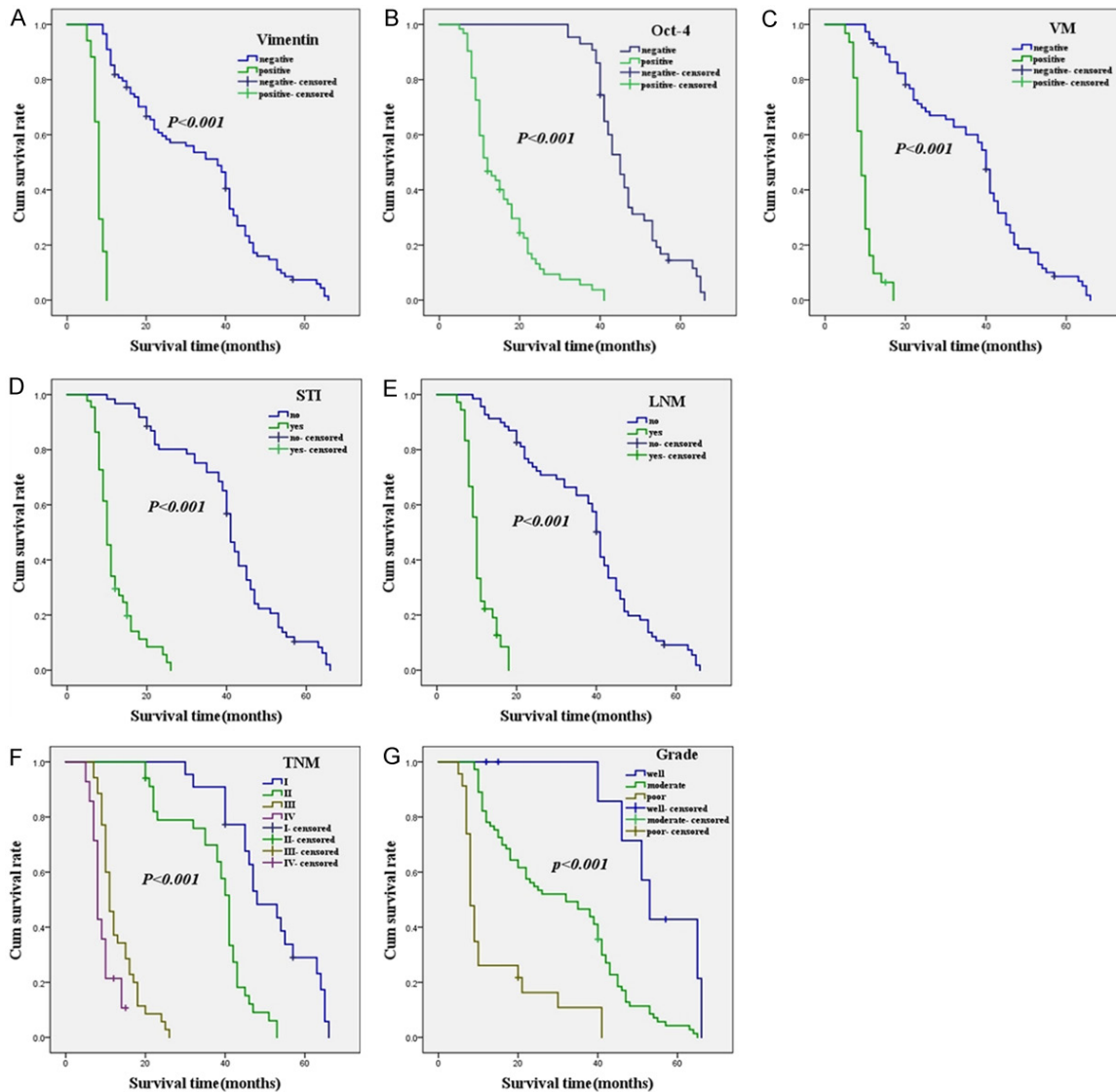
#### *Correlations among expression of vimentin, Oct-4, and VM in GBAC*

Spearman correlation coefficient analysis indicated a positive correlations between VM+ expression and vimentin ( $r=0.679$ ,  $P<0.001$ ), and Oct-4 ( $r=0.539$ ,  $P<0.001$ ). In addition, the expression of vimentin was positively correlated to the positive rate of Oct-4 ( $r=0.366$ ,  $P<0.001$ ; **Table 3**).

#### *Univariate and multivariate analyses*

Analysis results showed that OST was significantly lower in GBAC patients with vimentin+ specimens ( $7.94±1.391$  months) compared to those with vimentin- ( $31.92±16.828$  months;

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**Figure 2.** Kaplan-Meier analysis of the survival rate of patients with GBAC. The y-axis represents the percentage of patients; the x-axis, their survival in months. (A) Overall survival of all patients in relation to vimentin expression (log-rank =123.450,  $P<0.001$ ); (B) Overall survival of all patients in relation to Oct-4 expression (log-rank =92.875,  $P<0.001$ ); (C) Overall survival of all patients in relation to VM (log-rank =115.337,  $P<0.001$ ); In (A-C), the green line represents patients with positive vimentin, Oct-4, and VM, respectively; the blue line represents the negative vimentin, Oct-4, and VM group, respectively. Kaplan-Meier analysis of the survival rate of patients with GBAC. The y-axis represents the percentage of patients; the x-axis, their survival in months. In (D), the blue line represents the tumor without the STI group, while the green line represents the tumor with the STI group. In (E), the blue line represents patients without the LNM group; the green line represents patients with the LNM group. In (F), the blue line represents patients with a stage I tumor; the green line represents patients with a stage II tumor; the brown line represents patients with a stage III tumor; the red line represents patients with a stage IV tumor. In (G), the blue line represents patients in the well grade group, the green line patients in the moderate grade group, the brown line represents patients in the poor grade group.

log-rank =123.450,  $P<0.001$ ; **Table 4** and **Figure 2A**). Analogously, OST was significantly lower in Oct-4+ patients ( $15.16\pm 8.511$  months) compared to Oct-4- patients ( $46.6\pm 8.915$  months; log-rank =92.875,  $P<0.001$ ; **Table 4**

and **Figure 2B**). Moreover, the OST of VM+ patients ( $9.52\pm 2.580$  months) was significantly lower compared to VM- patients ( $35.80\pm 15.514$  months; log-rank =115.337,  $P<0.001$ ; **Table 4** and **Figure 2C**). In univariate analysis,

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**Table 5.** Results of multivariate analyses of overall survival (OS) time

Covariate	B	SE	P	Exp (B)	95% CI
Vimentin	1.077	0.519	0.038	2.934	1.061-8.117
Oct-4	1.344	0.430	0.020	3.834	1.649-8.912
VM	1.125	0.521	0.031	3.080	1.109-8.553
Grade	1.190	0.281	<0.001	3.286	1.894-5.701
STI	1.511	0.483	0.002	4.533	1.758-11.687
LNM	1.139	0.536	0.033	3.124	1.093-8.929
TNM	0.610	0.272	0.025	1.841	1.080-3.137

OST was obviously associated with clinicopathological characteristics, including tumor STI (log-rank =104.544,  $P<0.001$ , **Table 4** and **Figure 2D**), LNM (log-rank =104.329,  $P<0.001$ , **Table 4** and **Figure 2E**), TNM stage (log-rank =136.130,  $P<0.001$ , **Table 4** and **Figure 2F**) and grade (log-rank =48.583,  $P<0.001$ , **Table 4** and **Figure 2G**).

Additionally, the multivariate analysis showed that vimentin+, Oct-4+, and VM+ samples, tumor grade, STI, LNM and TNM stage, were pivotal prognostic factors for GBAC (**Table 5**).

### Discussion

GBAC has a very poor prognosis and a low 5-year survival rate, which is why finding new biological therapy is very important. Vimentin is a mesenchymal marker that is up-regulated in migratory cells and expressed at elongation sites, which suggest its putative role in facilitating migration [11]. As shown in **Figure 1B**, vimentin+ GBAC cells are fusiform, which is conducive to the migration of cancer cells. Furthermore, Kaplan-Meier survival analysis proved that vimentin+ GBAC patients had a clearly lower OST compared to vimentin-patients. These results indicated that a positive expression of vimentin should enhance GBAC invasion and metastasis, thus leading to a poor prognosis. Our research results were in line with previous studies [5, 7, 9-12, 30, 31].

The human Oct-4 gene is located on chromosome 6 [32]. It is a key transcription factor which participates in maintaining pluripotency and self-renewal of undifferentiated embryonic stem cells [33]. Our study showed that the rate of Oct-4 positive expression was obviously higher in GBAC compared to corresponding para-carcinoma tissues. In addition, Oct-4 overexpression showed a significantly correlation

with GBAC differentiation, LNM, STI and TNM stage. Also, Oct-4 overexpression was shown to be associated with the invasion, metastasis, and poor prognosis of GBAC. Moreover, an obviously shorter survival time was found in patients with overexpressed Oct-4 compared to the control group. In addition, a multivariate Cox regression analysis indicated that a high expression of Oct-4 was an independent factor for poor prognosis in GBAC. Our research results are consistent with previous research findings [15, 18, 19, 34-36].

VM is mainly found in highly aggressive malignant tumors, and it has been shown to be negatively correlated with a patient's prognosis [20, 27, 37]. In this study, we found that VM was positively correlated with tumor size, grade, LNM, STI and TNM stage. Also, a Kaplan-Meier survival analysis showed that VM+ GBAC patients had a significantly shorter OST than did VM- patients. These results suggested that VM has a nonnegligible role in GBAC progression, metastasis, and thus should be regarded as a reliable biomarker in regulating GBAC [20, 38, 39]. It is deficient for patients to be treated by anti-vascular endothelial cell growth. In order to solve this problem, VM should be regarded as a possible therapeutic target for GBAC. Some other studies had similar findings [20, 27, 28, 38, 40, 41].

The TNM staging system provides a guide for the treatment of GBAC patients. But this guide is not unique. Hence, it is necessary to identify novel effective biomarkers for the detection of the behavior and prognosis in patients with GBAC. Our data suggested that a positive expression of vimentin, Oct-4, VM as well as grade, STI, LNM and TNM stage are all independent, significant factors for patients with GBAC. In addition, our results indicated that vimentin, Oct-4, and VM are useful biomarkers for GBAC, particularly for metastasis and prognosis detection.

In order to improve the survival rate of GBAC, the discovery of an effective therapeutic target is urgently required. The invasive and metastatic potential of GBAC is one of the main reasons for its poor prognosis. At present, several treatment options for GBAC have been developed; nevertheless, their effectiveness remains debatable. This phenomenon may be related to the development of the tumors. Our study

showed that a poorer differentiation and a higher TNM stage led to a higher expression of CSCs markers in GBAC. Considering that CSCs can exhibit their own plasticity, this characteristic contributes to the development of EMT. In some conditions, during the course of EMT, cells in an epithelial layer can alter its shape i.e. changing from adenoid shape to fusiform shape, in order to facilitate the metastatic behavior [42, 43]. Hence, eliminating EMT by inhibiting CSCs will be a promising avenue for the improvement of cancer therapy. On the other hand, due to the ability of own plasticity CSCs can make a contribution to the formation of VM [20, 44]. VM not only provides tumor cells with sufficient energy, but also promotes tumor cell metastasis throughout the bloodstream. Therefore, targeting to reduce the plasticity of CSCs may effectively inhibit the formation of VM.

From our experimental results and related studies, we found that the mutual promotion of EMT, CSCs and VM reduces the survival rate of patients with GBAC. Therefore, to inhibit GBAC invasion and metastasis, it is useful to take various measures to restrain the development of cancer.

### Conclusions

Our findings imply that vimentin, Oct-4, and VM affect GBAC evolution. In addition, vimentin, Oct-4, and VM may be undesirable factors for metastasis, invasion, and prognosis, as well as effective therapeutic targets for GBAC.

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### Disclosure of conflict of interest

None.

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