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From Cell Biology to Tissue Engineering

Overexpression of EIF5A2 is associated with poor survival and aggressive tumor biology in gallbladder cancer

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Summary. Gallbladder cancer (GBC) is a malignant tumor of the biliary tract. The main problem affecting the treatment of gallbladder cancer is late diagnosis and poor prognosis. EIF5A2 is one of two isoforms of the EIF5A family and is reported to be a new oncogenic protein in many human cancers. In this study, our results showed for the first time that EIF5A2 was overexpressed in GBC samples compared with non-tumor tissue. Overexpression of EIF5A2 was associated with lymph node metastasis, tumor differentiation, UICC (Union for International Cancer Control) staging, histological type, metastasis, and tumor size. Overexpression of EIF5A2 in gallbladder carcinoma tissues is also associated with poor prognosis in patients. The interference of EIF5A2 significantly inhibited the proliferation, cell cycle, migration and colony formation of GBC-SD cells in vitro. Our results suggest that EIF5A2 is a target oncogene and may be an important prognostic biomarker in the pathogenesis of gallbladder cancer.

Key words: EIF5A2, Gallbladder cancer, Proliferation, Prognosis

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Introduction

The incidence of gallbladder cancer varies widely geographically, ethnically, and culturally, suggesting that genetic and environmental factors play a key role in the development of gallbladder cancer (Misra et al., 2003; Andia et al., 2008). Although the incidence of gallbladder cancer is relatively low, most patients are diagnosed at an advanced stage (Li et al., 2014a,b). The lack of gallbladder serosa in the vicinity of the liver leads to liver invasion and metastasis, which is one of the main reasons for its poor prognosis (Hundal and Shaffer, 2014). There are currently no reliable tumor markers for the diagnosis of gallbladder cancer. Currently only two markers, carcinoembryonic antigen (CEA) and CA19-9, are most frequently elevated in advanced stages, but have lower specificity. Therefore, in most cases, they are not used for independent diagnosis of GBC (Srivastava et al., 2013). However, other tumor markers such as CA242, CA125, cancer antigen (CA), CEA (carcinoembryonic antigen), CA199, etc. have also been studied in the diagnosis of gallbladder cancer, but the results are highly inconsistent (Zur et al., 2012; He et al., 2013; Zhang et al., 2013). In addition, previous reports have shown that biomarkers such as CA242, Mac-2BP, CA15-3, RCAS1, and Fragments of cytokeratin¬19 (CYFRA21¬1) are frequently found in the blood of cancer patients, showing a correlation with GBC, but with different sensitivities and specificities (Koopmann et al., 2004; Srivastava et al., 2013; Huang et al., 2015). Therefore,

an understanding of the molecular mechanisms of gallbladder cancer metastasis and recurrence is essential for the development of effective adjuvant therapy.

Eukaryotic translation initiation factor 5A2 (EIF5A2) is a putative oncogene isolated from 3q26.2 using the chromosomal micro-separation-hybridization selection technique (Guan et al., 2001). EIF5A2 is rare in most normal tissues, but is evident in many malignancies, and the association of EIF5A2 with cancer has received considerable attention (Caraglia et al., 2013). The EIF5A2 gene is frequently amplified in different human malignancies, including pancreatic cancer (Griffin et al., 2007), gastric cancer (Guan et al., 2000; Takada et al., 2005), colon cancer (He et al., 2003), liver cancer (Tang et al., 2010), and breast cancer (Forozan et al., 2000). Among the several cancers just mentioned, elevated EIF5A2 is often associated with more severe disease states, increased likelihood of postoperative recurrence and metastasis, and decreased survival.

Here, our results indicate that EIF5A2 is up-regulated in many primary GBC tumors and is strongly associated with poor patient prognosis. The interference of EIF5A2 inhibits proliferation, tumor growth and migration of gallbladder cancer cells. Therefore, we believe that EIF5A2 is a regulator of GBC development, which may be a novel biomarker and potential therapeutic target for GBC patients.

Materials and methods

Patients and clinicopathological data

A total of 40 paraffin-embedded specimens of gallbladder cancer were collected from January 2014 to May 2017 at the Nankai Hospital in Tianjin, China. Another 40 cases of gallbladder cancer tissue chips were purchased from Shanghai OUTDO Biotechnology Company Ltd. (Shanghai, China). All the above gallbladder cancer tissue samples were confirmed by paraformaldehyde, paraffin embedding, sectioning, and HE staining. No patients received radiotherapy or chemotherapy related treatment before surgery. All the 80 patients with gallbladder cancer collected had follow-up information. This study was approved by the Clinical Research Ethics Committee of Tianjin Nankai Hospital.

Immunohistochemical analysis of gallbladder cancer tissues

Immunohistochemistry (IHC) uses the manufacturer's instructions for the SP-9001 system (ZSGB, China). For antigen retrieval, the antigen was repaired with sodium citrate antigen repair solution at 95°C for 10 minutes, and then slowly cooled. The sections were incubated with rabbit monoclonal anti-EIF5A2 (Abcam, UK) diluted 1:200 and placed in a refrigerator at 4°C overnight. Finally, an appropriate amount of freshly prepared 3,5-diaminobenzidine (DAB) chromogenic solution was added and the staining was observed under the microscope (Leica, Germany). The staining evaluation of EIF5A2 adopted a semi-quantitative scoring standard (Ma et al., 2010), which includes staining intensity and percentage of positive cells. The scoring criteria are as follows: when the positive cells accounted for 0-25%, it was 1 point, 26-50% was 2 points, 51-75% was 3 points, and >76% was 4 points; when the staining intensity is negative, it is 0, weak is 1 point, moderate is 2 points, and strong is 3 points. The multiplication of the two scores is the final score. The staining index ≥4 is divided into positive, and <4 is divided into negative. Pathological section staining was independently assessed by two pathologists.

Cell culture and EIF5A2 interference

GBC-SD cell lines were purchased from the Shanghai Institute of Cell Bank, Chinese Academy of Science (Shanghai, China). GBC-SD cell lines were cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco). The GBC-SD cell line was incubated in a humidified incubator containing 5% carbon dioxide at 37°C.

The double-stranded small interfering RNA (siRNA) were purchased from GenePharma company (Shanghai, China). GBS-SD cells were transfected with siRNA with siRNA Transfection Reagent (Polyplus transfection, France), according to the manufacturer's instructions. The EIF5A2-specific siRNA target sequences were as follows: control-siRNA, 5'-UUCUCCGAACGU GUCACGUTT-3', 5'-ACGUGACACGUUCGGAG AATT-3'; EIF5A2-siRNA-1, 5'-GUGGAGAUGUC AACUUCCATT-3', 5'-UGGAAGUUGACAUCUCC ACTT-3'; EIF5A2-siRNA-2, 5'-GCAUUCAAGAUG GUUACCUTT-3', 5'-AGGUAACCAUCUUGAAUG CTT-3'; EIF5A2-siRNA-3, 5'-GGAUCUUAAACU GCCAGAATT-3', 5'-UUCUGGCAGUUUAAGAUC CTT-3'. After 48h of si-RNA transfection, the gene silencing efficiency was detected by Western blot. Each experiment was repeated three times independently.

Western blot analysis

GBC-SD cells were lysed with cold RIPA buffer (Millipore, USA) supplemented with phosphatase, protease inhibitors, phenylmethanesulfonyl fluoride and aprotinin (Sigma, USA) after transfection of siRNA for 48h. Protein was extracted according to instructions and quantified by BCA protein concentration assay kit (Solarbio, China). 40 μg of total protein was electrophoresed through 10% separation gel, then transferred onto polyvinylidene fluoride membrane (Millipore, USA) by semi-dry electrophoretic transfer cell (Bio-rad, USA). The membranes were blocked and probed with the primary antibodies against β-actin and EIF5A2 (1:1000 dilution) (Cell Signaling Technology, USA) overnight at 4°C. The membranes were incubated with horseradish peroxidase-conjugated secondary

antibodies after three times of washing. The immunoreactivity was detected by chemiluminescent HRP substrate kit (Millipore, USA).

Quantitative real-time PCR

Total RNA was extracted from GBC-SD cells transfected with siRNA by TRIzol reagent (Invitrogen, USA). 2 μg of total RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). All primers were synthesized by Invitrogen (USA), and the primer sequences are as follows: β-actin (forward primer: 5'-ACCGGGC ATAGTGGTTGGA-3'; reverse primer: 5'-ATGGTAC ACGGTTCTCAACATC -3'), EIF5A2 (forward primer: 5'- TGTCCTTCTACTCACAACATGGA -3'; reverse primer: 5'- CTCACGAACTTCACCAGTTTCT -3'). Real-time PCR was performed with GoTaq® qPCR Master Mix (Promega, USA) on ABI 7500 FAST Real-Time PCR System according to the manual. The relative expression of mRNA was calculated by $2^{-\Delta\Delta Ct}$.

Two dimensional colony formation assay and cell cycle

Plate clone formation experiment was performed by plating approximately 200-500 cells in 6-well culture dishes for 10-14 days. The cells in the 6-well plate were fixed with 4% paraformaldehyde and then stained with a 0.1% crystal violet solution. The formed cell colonies (≥50 cells) were counted under a microscope.

To examine cell cycle, after transfecting the cells with si-RNA for 48 h, the cells were fixed with a 90% ethanol solution overnight. The cell cycle was detected using a flow cytometer (ACEA Biosciences, Inc.), and the cells were stained using propidium iodine dye (Biolegend, San Diego, USA) according to the manufacturer's protocol.

Cell proliferation assay

Carboxyfluorescein succinimidyl ester (CFSE) is applicable to track cell division. Such dye-labeled cells can distribute the fluorescence equally into the daughter cells with every cell division. Fluorescence intensity of each generation of cells can be detected by flow cytometry. Subsequently, ModFit LT (Verity Software House, USA) software was used to determine the proportion of cells in each generation according to the fluorescence intensity of the initial CFSE-labeled cells, and calculate the proliferation index (PI).

GBC-SD cells were cultured to the logarithmic growth phase and starved overnight with serum-free medium. Next, the cells were collected and incubated with CFSE at the final concentration of 5μM for 10min. After termination of staining, the cells were centrifuged with medium containing 10% FBS. CFSE-labeled cells were then seeded into 6-well plates, while incubated with control-siRNA or EIF5A2-siRNA-3 for 48h. Finally, cell proliferation of two groups was detected by flow cytometry.

The MMT assay measures cell proliferation. Cell viability was detected at 24 h, 48 h, 72 h and 96 h using the MTT solution (Sigma, USA) according to the manufacturer's protocol. The medium was detected using a spectrophotometer with a wavelength of 570 nm.

Wound healing and cell migration assay

Wound healing was assessed by measuring the rate at which cells move in the scratch area. The scratch areas were created by a 10-μl pipette tube. The area of wound was observed after 24 h and 48 h and pictures were taken under the microscope. Cell migration assay was verified using Transwell chamber (Corning Incorporated, USA). 5×10^4 cells suspended in 100 μl of RPMI 1640 medium with 10% FBS were added to the upper chamber. Then 600 ul of RPMI 1640 medium with 20% FBS were added to the lower chamber. After 16h of culture, the cells were fixed with 4% paraformaldehyde and stained with crystal violet. The upper chamber cells were wiped with a cotton swab and three random fields were selected to capture the cells that have migrated through the membrane. The results were analyzed using the ImageJ software (NIH).

Statistical analysis

All experimental data were expressed as mean ± S.E (standard deviation). Statistical analysis was performed using GraphPad Prism Version 5.0 (GraphPad Inc., San Diego CA) and SPSS statistical analysis software (SPSS Inc., Chicago, IL, USA). The chi-square test was used to evaluate the correlation between EIF5A2 protein expression and clinicopathological features in GBC patients. Survival statistics were performed using the Kaplan-Meier method and survival curves were plotted, and the difference in survival rates among the subgroups was compared using the Log-rank test. Multivariate analysis of gallbladder cancer case data was performed using Cox regression analysis model. All data were derived from at least three independent experiments and a P value of less than 0.05 was considered statistically significant.

Results

EIF5A2 is overexpressed in GBC tissues

The expression of EIF5A2 in GBC tissues was investigated by IHC staining. Compared to the adjacent non-tumor tissues, the EIF5A2 was up regulated in tumors (Fig. 1A).

The expression of EIF5A2 is associated with the clinicopathological variables of GBC patients

According to the statistical result from the 80 investigated patients, it was revealed that the expression

of EIF5A2 was high in 75% (60/80) of the tumor tissues, and the level of EIF5A2 was associated with the degree of malignancy (Table 1). As shown in Table 1, high expression of EIF5A2 in GBC was significantly associated with tumor size (P=0.007), histological type (P=0.001), tumor differentiation (P=0.0018), metastasis

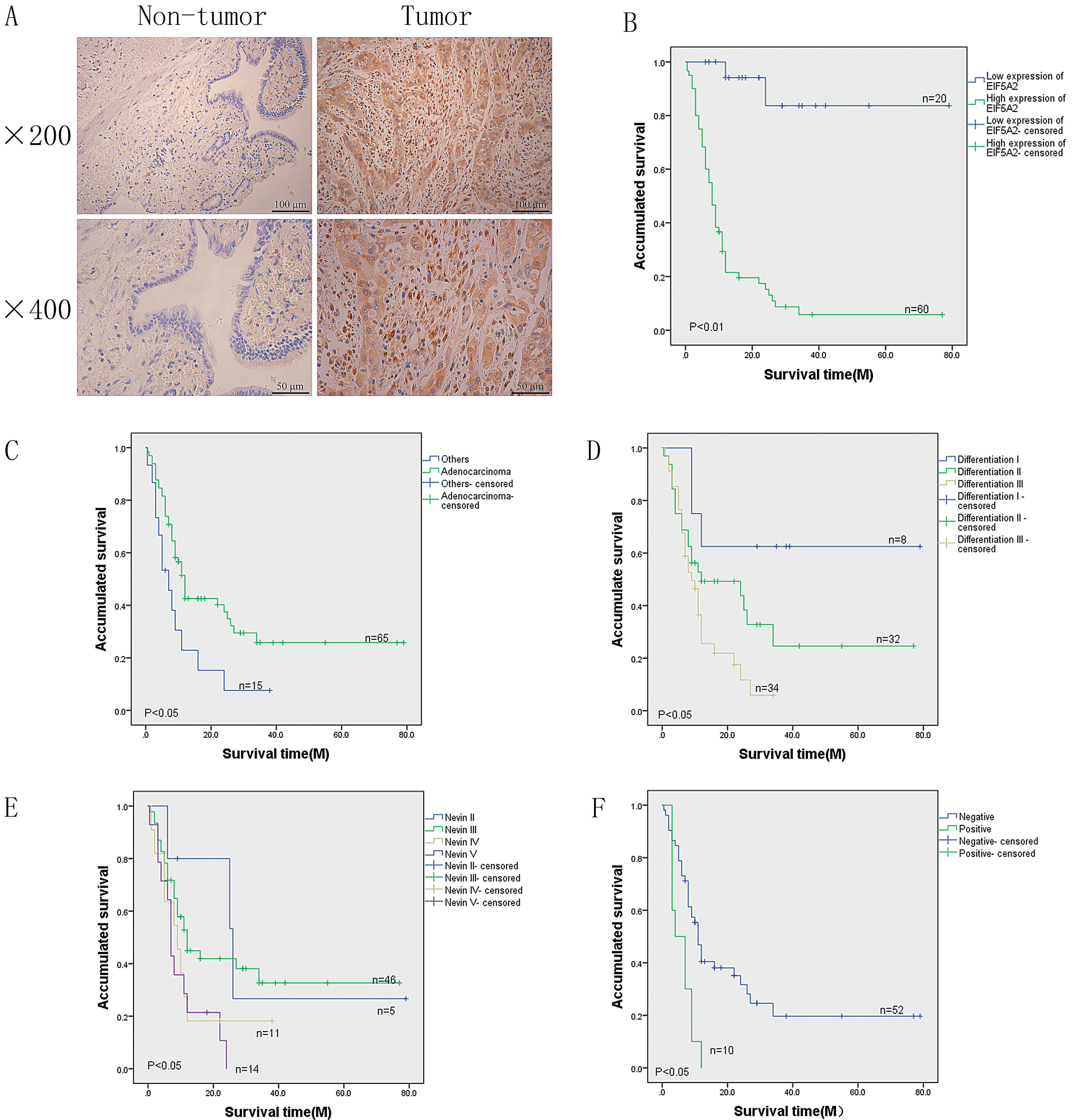

Fig. 1. Immunohistochemical analysis of EIF5A2 in GBC tissues. **A.** EIF5A2 expression level in the gallbladder tumor and adjacent non-tumor tissues. **B.** Kaplan–Meier overall survival curve of GBC patients based on EIF5A2 expression (log-rank test). Overall survival of all patients with GBC: low expression, n=20 cases, mean time=69.3 months; high expression, n=60 cases, mean time=13.6 months. **C-F.** Survival curve for 80 GBC patients according to histological type, tumor differentiation, nevin staging and metastasis.

(P=0.0041), lymph node metastasis (P=0.0093), and UICC staging (P=0.0124).

The expression of EIF5A2 protein is associated with survival and prognosis in GBC patients

The Kaplan-Meier survival curves suggested that the accumulated survival rate of GBC patients with high level of EIF5A2 (n=60, with a mean survival of 13.6 months) was poorer than those with low level (n=20 ,with a mean survival of 69.3 months, P<0.05, Table 2, Fig. 1B).

Univariate and multivariate analyses were performed to investigate the relationship between survival rates and clinicopathological prognostic features of GBC patients. Survival and prognosis was significantly associated with histological type (P<0.05, Table 2, Fig. 1C), tumor differentiation (P<0.05, Table 2, Fig. 1D), nevin staging (P<0.05, Table 2, Fig. 1E), and metastasis (P<0.05, Table 2, Fig. 1F). Subsequently, multivariate statistical analysis was performed using the Cox regression model to examine independent risk factors for each clinicopathological feature. The result demonstrated that a high level of EIF5A2 was an independent prognostic factor with low overall survival (P=0.036, Table 3) in GBC patients, like tumor differentiation and metastasis.

Table1. Correlation between EIF5A2 and clinicopathological characteristics in GBC patients (Chi-square test).

Variables	All cases	EIF5A2 expression		P value
	(n=80)	Low(20)(%)	High(60)(%)	
Gender				
female	47	10(50.0)	37(61.7)	0.359
male	33	10(50.0)	23(38.3)	
Age, (y)				
<65	37	12(60.0)	25(41.7)	0.154
≥65	43	8(40.0)	35(58.3)	
Tumor size (cm) †‡				
≤4. 5	23	3(15.0)	20(33.3)	0.007*
>4.5	34	9(45.0)	25(41.7)	
Histological type				
Adenocarcinoma	65	19(95.0)	46(76.7)	0.001*
other	15	1(5.0)	14(23.3)	
Tumor differentiation‡				
I,	8	4(20.0)	4(6.7)	0.0018*
II, III	66	14(70.0)	52(86.7)	
Nevin staging‡				
I, II	5	2(10.0)	3(5.0)	0.528
III, IV, V	71	16(80.0)	55(91.7)	
metastasis‡				
Negative	52	12(60.0)	40(66.7)	0.0041*
Positive	10	0(0.0)	10(16.7)	
Lymph node metastasis‡				
Negative	36	9(45.0)	27(45.0)	0.0093*
Positive	19	2(10.0)	17(28.3)	
UICC staging				
I, II,III	69	19(95.0)	50(83.3)	0.0124*
IV	11	1(5.0)	10(16.7)	

†: Tumor size was measured by the length of the largest tumor nodule. ‡: Partial data not available; statistics based on available data. *: P<0.05 was considered signifcant.

Table 2. Univariate analysis of different prognostic parameters in 80 patients with GBC (log-rank test).

Variable	All cases	Mean survival(months)	P value
Gender			0.754
female	47	23.6	
male	33	29.4	
Age, (y)			0.540
< 65	37	24.3	
≥65	43	27.2	
Tumor size (cm) ‡			0.954
≤4.5	23	24.8	
>4.5	34	23.4	
Histological type			0.017*
Adenocarcinoma	65	29	
Others	15	10.1	
Tumor differentiation‡			0.010*
I	8	53.1	
II	32	28.8	
III	34	11.9	
Nevin staging‡			0.046*
I	0		
II	5	35.9	
III	46	32.3	
IV	11	12.6	
V	14	9.8	
metastasis‡			0.002*
Negative	52	25.1	
Positive	10	6.0	
Lymph node metastasis‡			0.233
Negative	36	25.0	
Positive	19	12.5	
UICC staging			0.368
I	4	30.0	
II	17	27.5	
III	48	18.5	
IV	11	8.1	
EIF5A2			0.000*
Low expression	20	69.3	
High expression	60	13.6	

*: P<0.05 was considered signifcant. ‡: Partial data not available; statistics based on available data.

Table 3. Multivariate analysis on overall survival (Cox regression model)

Variable	Hazard ratio	95%confidence interval	P value
Tumor differentiation	2.214	1.091-4.495	0.028*
Metastasis	4.667	1.408-15.468	0.012*
EIF5A2	8.756	1.147-66.854	0.036*

*: P<0.05 was considered signifcant.

Downregulation of EIF5A2 inhibits the growth of GBC cell lines

EIF5A2 was highly expressed and downregulated by siRNA in GBC cell line. Western blot and qPCR were used to detect the interference efficiencies of three siRNAs targeting EIF5A2. As shown in Fig. 2A,B, EIF5A2-siRNA-3 downregulated the expression of EIF5A2 effectively and was chosen for the following experiments. Subsequently, clone formation experiment was performed to evaluate the growth of GBC cell line, after the cells were transfected by EIF5A2-siRNA-3 or

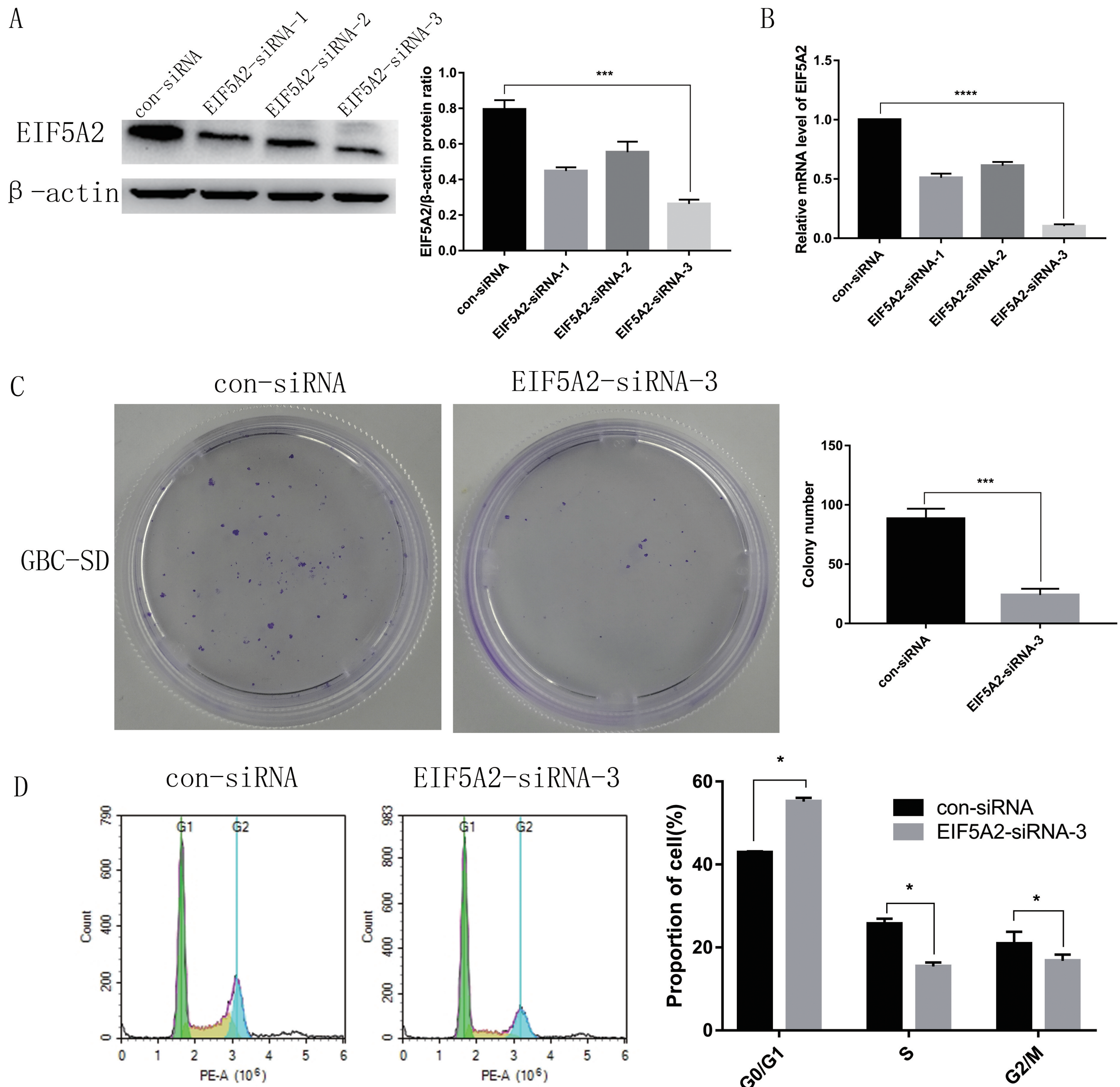

Fig. 2. Inhibition of EIF5A2 expression by RNA interference and EIF5A2 promotes GBC cell growth. **A, B.** The efficiency of EIF5A2 interference was evaluated by western blot and qPCR after GBC-SD cells were transfected with EIF5A2-siRNA; EIF5A2-siRNA-3 is considered to have the best down-regulation effect (***: P<0.001, ****: P<0.0001). All subsequent experiments were performed using EIF5A2-siRNA-3 in combination with control-siRNA. **C.** Colony formation was inhibited in EIF5A2-siRNA-3 cell lines (***: P<0.001). **D.** Flow cytometry was used to analyze the cell cycle of EIF5A2-siRNA-3 and control-siRNA cell lines (*: P<0.05). These data were reported as mean ± SD and three separate experiments were performed.

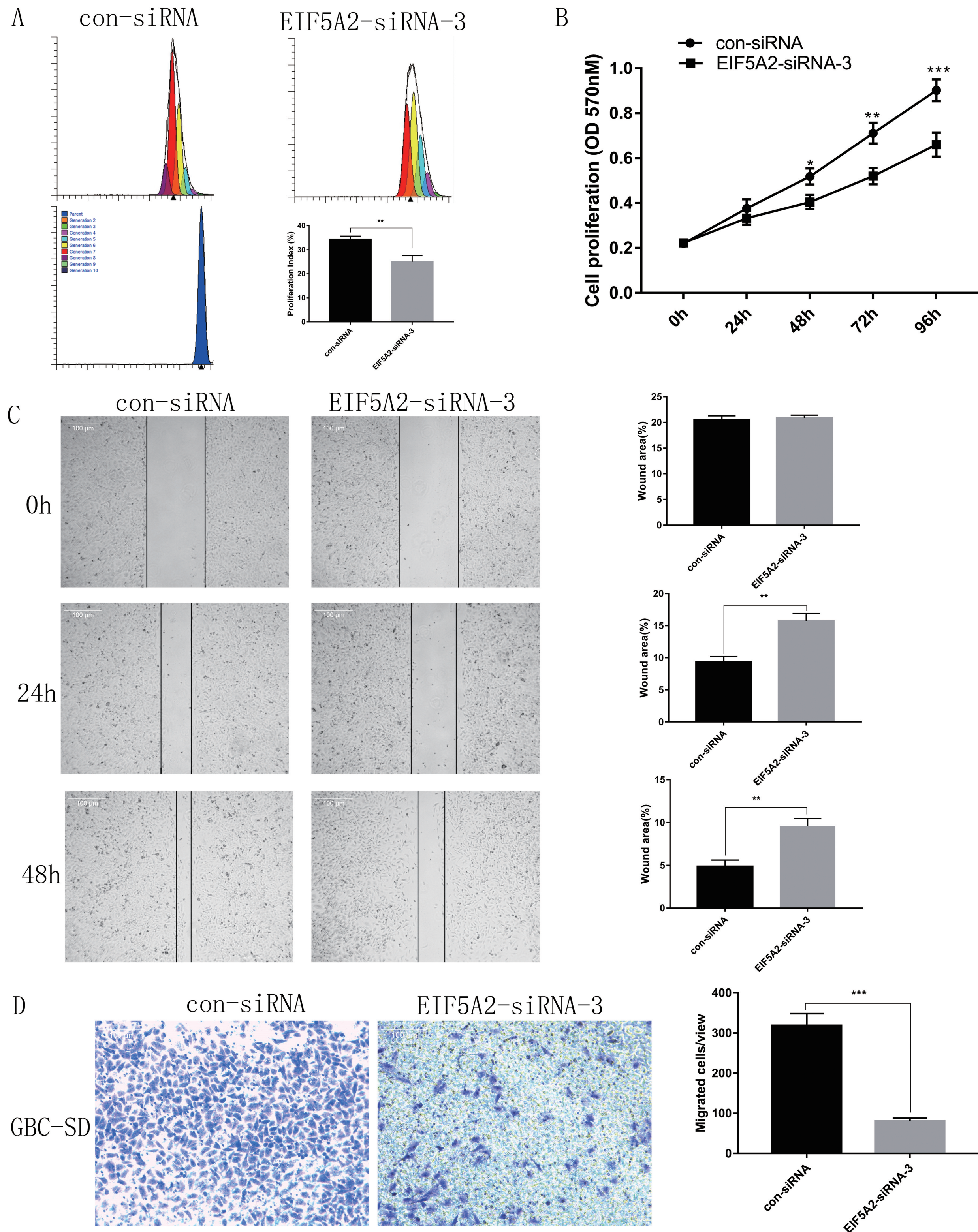

Fig. 3. Silencing of EIF5A2 decreases cell proliferation and cell migration. **A.** Effects of EIF5A2-siRNA-3 and control-siRNA cell lines on proliferation index after 48h transfection si-RNA, different colored peaks represent different generations (**: P<0.01). **B.** The proliferative capacity of EIF5A2-siRNA-3 and control-siRNA cell lines were compared using MTT assays (*: P<0.05, **: P<0.01, ***: P<0.001). **C.** The most representative photographs of wound healing were observed under the microscope at 0h, 24h and 48h (**: P<0.01). Relative wound area was shown in the bar graph. **D.** Cell migration rates of EIF5A2-siRNA-3 and control-siRNA cell lines were compared using transwell migration assays (***: P<0.001). The graph shows the migration rates of EIF5A2-siRNA-3 and control-siRNA cell lines after 16h of culture on transwell. These data are reported as mean ± SD and three separate experiments were performed.

control-siRNA. The result demonstrated that the colony formation capability of EIF5A2 downregulated cells was attenuated compared to the control cells (Fig. 2C). To explore the mechanism of the above phenomenon, flow cytometry was used to analyze the change in the cell cycle after EIF5A2 was downregulated. According to the result, the percentage of S and G2/M phases was significantly reduced in EIF5A2 downregulated cells, but the percentage of the G1 phase was increased in them (Fig. 2D). These results indicated that the inhibition of cell growth resulted from G1 phase arrest induced by downregulating EIF5A2.

Downregulating EIF5A2 surpresses cell proliferation and cell migration

We further examined the effect of EIF5A2-siRNA-3 on GBC-SD cell lines by flow cytometry. After CFSE-labeled cells were treated with control-siRNA or EIF5A2-siRNA-3, the EIF5A2-siRNA-3 group cell proliferation and the proliferation index was significantly decreased compared with the control-siRNA group (Fig. 3A). As shown in Fig. 3B, the MTT assay revealed that the viability of GBC-SD cells was suppressed in the EIF5A2-siRNA-3 group compared with the control-siRNA group. In the wound healing assay, EIF5A2-siRNA-3 group significantly suppressed the wound healing at 24h and 48h compared with control-siRNA group (Fig. 3C). In addition, the transwell migration assays indicated that interference EIF5A2 markedly suppressed cell migration ability in the GBC-SD cell lines (Fig. 3D). These data were consistent with our previous conclusion that GBC patients with high EIF5A2 expression is significantly associated with metastasis.

Discussion

Despite improvements in the diagnostic and therapeutic strategies of GBC, the prognosis of GBC patients is still unsatisfactory. EIF5A2 is considered a candidate oncogene, but there is currently no information on the role of EIF5A2 in gallbladder cancer. In the present study, we for the first time found that EIF5A2 was upregulated in GBC tissues compared with the non-tumor counterparts. Overexpression of EIF5A2 in GBC was signifcantly associated with tumor size, histological type, tumor differentiation, metastasis, lymph node metastasis, and UICC staging. Furthermore, the Kaplan-Meier survival curves suggested that the accumulated survival rate of GBC patients with high EIF5A2 expression was poorer than those with low EIF5A2 expression. In vitro experiments showed that the knockdown of EIF5A2 remarkably inhibited the proliferation, tumor growth and migration capacity of GBC-SD cells.

In cancers that have been studied, higher expression of EIF5A2 is associated with poorer clinical features and outcomes. Many malignant tumors, including esophageal squamous cell carcinoma, hepatocellular carcinoma, ovarian cancer, colorectal carcinoma, gastric cancer, lung cancer, and bladder cancer, have been reported to be associated with decreased survival (Chen et al., 2009; Yang et al., 2009; He et al., 2011; Zhu et al., 2012; Li et al., 2014b; Wang et al., 2014; Meng et al., 2015). These data indicate that knockdown of EIF5A2 can inhibit tumor growth and metastasis in a variety of cancers. Consistent with these findings, our results demonstrate EIF5A2 knockdown significantly inhibits GBC-SD cell proliferation and migration in vitro. A previous study reached a conclusion that EIF5A2 is one of the three genes that predict lymph node metastasis in gastric cancer (Marchet et al., 2007). Our clinicopathological data also showed that high EIF5A2 expression is significantly associated with metastasis and lymph node metastasis. The relationship between EIF5A2 and metastasis is also seen in esophageal cancer (Li et al., 2014b) and colorectal cancer (Xie et al., 2008). These data support the use of EIF5A2 as a prognostic indicator for various malignant diseases.

Compared with EIF5A1, another member of the EIF5A gene family which is widely expressed, EIF5A2 is expressed only in a few specific parts such as testis and adult brain. And the eIF5A2 isoform is not necessary for embryonic development and organism viability in adults (Meng et al., 2019). However, it is up-regulated in multiple malignant tumor tissues including GBC and is associated with poor prognosis. Therefore, EIF5A2 may be a novel biomarker and potential therapeutic target for GBC patients. But it is necessary to elucidate the mechanism regulating EIF5A2 expression and its downstream pathways.

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Conflict of Interest. The authors declare no conflict of interest.

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