Original Paper

MYBL2 is a Potential Prognostic Marker that Promotes Cell Proliferation in Gallbladder Cancer

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Key Words

MYBL2 • Gallbladder cancer • Cell proliferation • Cell cycle • S phase • G2/M phase

Abstract

Background: Gallbladder cancer (GBC) is an aggressive and highly lethal biliary tract malignancy, with extremely poor prognosis. In the present study, we analyzed the potential involvement of MYBL2, a member of the Myb transcription factor family, in the carcinogenesis of human GBC. **Methods:** MYBL2 expression levels were measured in GBC and cholecystitis tissue specimens using quantitative real-time PCR (qRT-PCR) and immunohistochemical (IHC) assays. The effects of MYBL2 on cell proliferation and DNA synthesis were evaluated using Cell Counting Kit-8 assay (CCK-8), colony formation, and 5-ethynyl-2'-deoxyuridine (EdU) retention assay, flow cytometry analysis, western blot, and a xenograft model of GBC cells in nude mice. **Results:** MYBL2 expression was increased in GBC tissues and associated with histological differentiation, tumour invasion, clinical stage and unfavourable overall survival in GBC patients. The downregulation of MYBL2 expression resulted in the inhibition of GBC cell proliferation, and DNA replication *in vitro*, and the growth of xenografted tumours in nude mice. Conversely, MYBL2 overexpression resulted in the opposite effects. **Conclusions:** MYBL2 overexpression promotes GBC cell proliferation through the regulation of the cell cycle at the S and G2/M phase transitions. Thus, MYBL2 could serve as a potential prognostic and therapeutic biomarker in GBC patients.

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Introduction

Gallbladder cancer (GBC), derived from the bile duct epithelium, is the most common malignancy of the biliary tract and the sixth most common gastrointestinal malignancy, accounting for 80–95% of biliary tract cancers [1, 2]. GBC remains a challenging tumour with a poor overall prognosis. Many cases of GBC are not resectable at presentation, the 5-year survival rate for GBC patients is only approximately 5%, and the overall median survival time of these patients is less than 1 year [3, 4]. The major cause of such a poor prognosis is the early infiltration of neoplasm cells through haematogenous, lymphatic, perineural routes and direct invasion into the liver and other adjacent organs [5, 6]. Considering the poor outcomes, GBC patient prognosis must be improved through the development of effective therapeutic targets and biomarkers that predict the therapeutic responses of this fatal disease.

MYBL2 (B-myb, v-Myb avian myeloblastosis viral oncogene homologue-like2), located on chromosome 20q13, is a highly conserved member of the Myb transcription factor family and is ubiquitously expressed in proliferating cells, particularly in embryonic stem cells and adult haematopoietic precursors [7–9]. Previous studies have shown the DREAM complex (DP, RB-like, E2F, and MuvB) represses cell cycle genes expression during quiescence. Upon cell cycle entry, the MuvB core dissociates from P107/P130 and sequentially recruits MYBL2 and FoxM1 to coordinate the expression of the late cell cycle G2/M genes. The expression of MYBL2, which is involved in cell cycle regulation and performs essential functions in proliferating cells, is hardly detectable at G0 phase and is induced at the G1/S transition of the cell cycle [10–16]. MYBL2 is overexpressed in several types of tumours, including hepatocellular carcinoma, lung cancer, breast cancer, cervical cancer and colorectal cancer [17–24]. Additionally, MYBL2 overexpression manifests poor prognosis in patients with breast cancer and prostate cancer [19, 25]. Taken together, these data indicate that MYBL2 is involved in cell proliferation and carcinogenesis. However, MYBL2 expression and its biological functions in GBC have not been previously investigated. These results prompted us to investigate the role of MYBL2 in GBC progression.

In the present study, we evaluated the correlation between ectopic MYBL2 expression and the poor prognoses of GBC patients, and examined its oncogenic function *in vitro* and *in vivo* to explore the potential mechanisms of action of this protein. We determined that MYBL2 acts as an independent predictive factor of poor prognosis in GBC patients, contributing to GBC progression.

Materials and Methods

Patients, clinicopathological data, and cell lines

This study was approved by the Ethics Committee of Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University, and all patients enrolled in the present study provided informed consent. GBC tissue specimens were obtained from 65 patients who underwent radical cholecystectomy without prior radiotherapy or chemotherapy from 2008 to 2014 in the Department of General Surgery, Xinhua Hospital. In addition, 65 cholecystitis patients undergoing simple cholecystectomy were included as controls. The GBC and cholecystitis tissue specimens were fixed in 4% formalin immediately after removal and embedded in paraffin for IHC staining. Fresh GBC tissue samples and corresponding non-cancerous tissue samples obtained from 36 GBC patients. These samples were prepared for qRT-PCR assay. Fresh tissue samples were processed within 15 min after removal. Each sample was stored in liquid nitrogen.

Among the 65 GBC cases used for IHC analysis, there were 22 males and 43 females with an average age of 68.5 years (range 46 to 85 years). All specimens including GBC and cholecystitis tissues were confirmed through pathological diagnosis and staged according to the 7th AJCC-TNM classification of malignant tumours. The median follow-up period was 14 months (range 1 to 36 months). The GBC cell lines NOZ, SGC-996, GBC-SD, EH-GB-1 and OCU-1 were obtained from the Health Science Research Resources Bank (Osaka, Japan). GBC-SD, EH-GB-1 and OCU-1 were cultured in high-glucose DMEM (Gibco, Grand Island, NY, USA), NOZ was cultured in William's medium E (Gibco, Grand Island, NY, USA), and SGC-996 was cultured

in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FBS; Gibco, Grand Island, NY, USA) and 100 U/ml of penicillin/streptomycin (Gibco, Grand Island, NY, USA). All cells were cultivated in a humidified incubator at 37 °C and 5% CO₂.

Immunohistochemistry analysis and evaluation of MYBL2 expression

IHC staining was performed using a standard immunoperoxidase staining procedure, and MYBL2 expression in cholecystitis and GBC specimens was estimated according to the methods of Pinheiro et al [26]. Briefly, the tissue sections were dehydrated with ethanol, washed three times with phosphate-buffered saline (PBS) (pH 7.4) and boiled for 8 min in a pressure cooker for antigen retrieval. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 min at 26 °C. The sections were further blocked with 3% normal goat serum for 10 min. After the serum had been discarded, the sections were incubated overnight with Primary rabbit anti-MYBL2 polyclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) in a humid chamber at 4 °C. The following day, the sections were incubated with secondary antibody-coated polymer peroxidase complexes (Abcam, Cambridge, UK) for 30 min at room temperature. After three 3 min washes with PBS, the sections were developed using diaminobenzidine (Abcam), and slides were counterstained with hematoxylin for long-term storage. The intensity of the staining was scored by optical density using the semi-quantitative software Image-Pro Plus software 6.0 (Media Cybernetics Co, USA). The sections were semi-quantitatively scored for the extent of immunoreaction using the following scale: 0, 0% immunoreactive cells; 1, <5% immunoreactive cells; 2, 5-50% immunoreactive cells; and 3, >50% immunoreactive cells. In addition, the staining intensity was semi-quantitatively scored as 0 (negative), 1 (weak), 2 (intermediate), or 3 (strong). The final immunoreaction score was defined as the sum of both extension and intensity parameters, and the samples were classified based on negative(0), weak (1–2), moderate (3), and strong (4–6) staining. For statistical purposes, only the final scores of moderate and strong were considered positive, and the other final scores were considered negative.

Quantitative real-time PCR

Total RNA was extracted from tissue samples using TRIzol reagent (Takara, Shiga, Japan). cDNA was synthesized from 2 µg of total RNA using random primers and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). RNA expression was measured through qRT-PCR using the SYBR-Green method (Takara) according to the manufacturer's instructions. The relative expression level of the target gene was calculated using the 2^{-ΔCT} (ΔCT = CT^{target} - CT^{GADPH}) method and normalized to the relative expression detected in the corresponding control cells, defined as 1.0. For the correlation study, the expression level (defined as a fold-change) of MYBL2 was calculated using the 2^{-ΔΔCT} (ΔΔCT = ΔCT^{tumour} - ΔCT^{nontumour}) method. The primer sequences are listed in Table 1.

Lentivirus-mediated RNA interference

The short hairpin RNA-2 (shRNA-2) listed in Table 1 were used to target MYBL2. A non-silencing shRNA, with the scrambled sequence 5'-TTCTCCGAACGTGTCACGT-3', was used as a negative control. The shMYBL2 and scrambled shRNA were synthesized and inserted into the pFH1UGW lentivirus core vector containing a cytomegalovirus-driven enhanced green fluorescent protein (EGFP) reporter gene. The shRNA Expression was driven through the H1 promoter. Recombinant lentiviruses expressing MYBL2-shRNA or scrambled shRNA (Lv-shMYBL2 and Lv-shNC, respectively) were produced at Genomeditech (Shanghai, China). SGC-996 and NOZ cells were infected with concentrated virus in serum-free medium. The supernatant was replaced with complete culture medium after 24 h. The infection efficiency was confirmed using qRT-PCR analysis after 120 h.

Construction of plasmids and transfection

The full-length MYBL2 cDNA (GenBank accession number NM_002466) was cloned into the GV230 expression vector (Genechem, Shanghai, China) and transfected into GBC-SD cells. Stable MYBL2-expressing clones were selected for 2 weeks using neomycin (Genechem), and MYBL2 expression was determined using qRT-PCR and western blot. Empty vector-transfected cells (MOCK) were used as a control. The primer sequences used for vectors construction are listed in Table 1.

Table 1. The nucleotides applied in the study

Description	Name	Sequence
Primers for qRT-PCR	MYBL2-F	5'-AAACAGTGAGGAGGAAC-3'
	MYBL2-R	5'-CAGGGAGGTCAAATTTAC-3'
	GADPH-F	5'-AGAAGGCTGGGGCTCATTTG-3'
	GADPH-R	5'-AGGGCCATCCACAGTCTTC-3'
shRNA for MYBL2	shRNA-1	5'-CACCCUGUCAGGUCAAAA-3'
	shRNA-2	5'-GAGGUGAAGAAGUCUUGCU-3'
	shRNA-3	5'-GUUAAGAAGUAUGGCACAA-3'
	shRNA-4	5'-GAGGUGAAGAAGUCUUGCU-3'
Primers for vectors construction	GV230-MYBL2-F	5'-GAACCGTCAGATCCGCTAGCATGTCTCGGCGGACGCGCTG-3'
	GV230-MYBL2-R	5'-TCACCATGGTGGCGACCGGTGGGGACAAGATGAGGGTCCGAG-3'

Cell proliferation assay

Approximately 1×10^3 NOZ or SGC-996 cells, and 0.8×10^3 GBC-SD cells were seeded onto 96-well plates. Cell proliferation was assessed using the CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The cell proliferation curves were plotted using the absorbance at each time point.

Colony formation assay

A total of 1,000 SGC-996 cells, and 500 NOZ or GBC-SD cells were seeded onto 6cm culture dishes. The cells were cultured for approximately 14 days, fixed with 4% paraformaldehyde, and subsequently stained with 0.1% crystal violet (Sigma, St. Louis, MO, USA). The total number of colonies (>50 cells/colony) was counted.

EdU retention assays

5-ethynyl-2'-deoxyuridine (EdU) retention assays were performed to examine the effect of MYBL2 on DNA synthesis. The dissociated cells were exposed to 25 μ M of EdU (RiboBio, Guangzhou, China) for 2h at 37°C, and the cells were then fixed in 4% paraformaldehyde. After permeabilization with 0.5% Triton-X, the cells were reacted with 1× Apollo reaction cocktail (RiboBio) for 30 min. Subsequently, the cellular DNA content was stained with Hoechst 33342 for 30 min and visualized under a fluorescence microscope [5].

Flow cytometry cell cycle analysis

GBC-SD, NOZ, and SGC-996 cells were harvested through trypsinization, washed twice with cold PBS, and subsequently fixed in 70% ethanol at 4 °C overnight. After fixation, the cells were washed and resuspended in cold PBS and incubated in a solution containing 10 mg/mL of RNase and 1 mg/mL of propidium iodide (Sigma-Aldrich, USA) at 37 °C for 30 min in the dark. The DNA content was determined using flow cytometry (BD Biosciences, USA). The percentage of the cells in the G0/G1, S and G2/M phases was determined using Cell Quest acquisition software (BD Biosciences, USA).

Xenograft studies

Nude nu/nu mice, 4-6 weeks old, were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). All mice were housed under specific pathogen-free conditions following the guidelines of the Ethics Committee of Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University. To characterize the effects of MYBL2 on tumour growth *in vivo*, 1×10^6 SGC-996 cells (Lv-shNC and Lv-shMYBL2) and 1×10^6 GBC-SD cells (MOCK and MYBL2) were subcutaneously implanted into the left axilla of nude mice (5 mice/group). Tumour growth was monitored weekly and measured in two dimensions. The tumour volume was calculated using the following formula: tumour volume = $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$, where the width and length were the shortest and longest diameters, respectively. After 4 weeks, the mice were sacrificed, and the tumours were dissected and weighed. Ki-67 and MYBL2 expression were evaluated in xenograft tumors through IHC detection.

Antibodies and western blot

A rabbit anti-MYBL2 polyclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-Cyclin A2, anti-Cyclin B1, rabbit anti-Cdc2, anti-Plk1, and anti-GADPH antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Cellular protein was extracted in lysis buffer (Beyotime, Shanghai, China), and the cytosolic and nuclear fractions were extracted using the Nuclear and Cytoplasmic

Protein Extraction Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, equal quantities of cellular protein were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked and subsequently probed with primary antibodies overnight at 4 °C. After incubation with a secondary antibody, the blots were visualized using enhanced chemiluminescence (Millipore, Billerica, MA, USA). GAPDH was used as a loading control [27].

Statistical analyses

SPSS software (version 18.0) was used for statistical analyses. The MYBL2 mRNA levels in GBC and paired non-tumour tissues were compared using the paired Student's t-test. Student's t-test for independent samples was performed to compare the means of two groups. Pearson's χ^2 test was used to analyze the association of MYBL2 expression with clinicopathological parameters. Kaplan-Meier plots and log-rank tests were used for survival analyses. Univariate and multivariate analysis (Cox proportional hazard regression model) were conducted to analyze independent prognostic factors. Each experimental value was expressed as the mean $\pm$ standard deviation (SD), and $P < 0.05$ was considered significant. All data points represent the mean of triplicate data points.

Results

High MYBL2 expression correlates with poor outcomes in GBC patients

To evaluate MYBL2 expression in GBC, we compared MYBL2 mRNA in 36 pairs of GBC samples (tumour and corresponding non-tumour tissues) using qRT-PCR. The relative expression of MYBL2 mRNA in tumour tissues was markedly higher than that in corresponding non-tumour tissues ($P < 0.01$; Fig. 1A and B). MYBL2 protein levels were further evaluated in 65 samples of archived paraffin-embedded GBC tissues and 65 cholecystitis tissues using

Fig. 1. MYBL2 overexpression correlated with poor clinical outcomes in GBC patients. (A) Scatterplots of the relative expression of MYBL2 in GBC tissues and corresponding non-tumour tissues. MYBL2 expression was calculated and depicted as the MYBL2/GAPDH expression ratio ($2^{-\Delta\Delta CT}$). (B) Comparison of the MYBL2 expression levels between GBC tissues and their non-tumour counterparts. (C) Representative image of IHC staining for MYBL2 protein in cholecystitis and GBC tissues. (D) Kaplan-Meier overall survival curve of GBC patients based on MYBL2 expression.

Table 2. Expressions of MYBL2 in GBC and cholecystitis tissues (Chi-square test) [n (%)]

Proteins	GBC tissues	cholecystitis tissues	P value
MYBL2			
+	47 (72.3)	15 (23.1)	<0.001
-	18 (27.7)	50 (76.9)	

Table 3. Association of MYBL2 expression with the clinicopathological features of GBC patients. **P < 0.05

Features	No. of cases	MYBL2		
		No. of positive cases (%)	χ^2	P
Age				
<60	17	13 (76.5)	0.017	0.896
≥60	48	34 (70.8)		
Gender				
Male	22	17 (77.3)	0.120	0.729
Female	43	30 (69.8)		
Associated gallstones				
Present			0.690	0.406
Absent	38	26 (68.4)		
	27	21 (77.8)		
Histological differentiation				
Well			21.426	<0.001**
Moderately	20	7 (35.0)		
Poorly	31	29 (93.5)		
	14	11 (78.6)		
Tumor invasion				
Tis-T1	12	4 (33.3)	8.905	0.003**
T2-T4	53	43 (81.1)		
Lymph node metastasis				
Present			0.461	0.497
Absent	26	20 (76.9)		
	39	27 (69.2)		
TNM stage				
0-I	10	3 (30.0)	8.215	0.004**
II-IV	55	44 (80.9)		

Table 4. Univariate and multivariate analyses of prognostic factors in GBC patients. **P < 0.05, CI, confidence interval; HR, hazard ratio

parameters	Univariate analysis		Multivariate analysis	
	HR (95%CI)	p	HR (95%CI)	p
Age				
(< 60 vs. ≥ 60)	0.840 (0.460-1.536)	0.572	-	-
Gender				
(Female vs. Male)	1.414 (0.819-2.440)	0.214	-	-
Associated gallstones				
(Present vs. Absent)	1.099 (0.644-1.877)	0.729	-	-
Histological differentiation				
(Well vs. Moderate vs. Poor)	0.992 (0.718-1.371)	0.963	-	-
Tumor invasion(AJCC)				
(Tis-T1 vs. T2-T4)	2.298 (1.121-4.713)	0.023**	0.891 (0.204-3.895)	0.878
Lymph node metastasis				
(Present vs. Absent)	2.260 (1.270-4.020)	0.006**	1.963 (1.059-3.641)	0.032**
TNM stage(AJCC)				
(0-I vs. II-IV)	2.783 (1.262-6.136)	0.011**	1.770 (0.322-9.713)	0.511
MYBL2 overexpression in tumor				
(Negative vs. Positive)	2.557 (1.345-4.860)	0.004**	2.193 (1.102-4.367)	0.025**

**P < 0.05, CI, confidence interval; HR, hazard ratio.

IHC (Fig. 1C). Based on the IHC staining score, 72.3% (47/65) of the GBC samples exhibited positive MYBL2 staining in tumour cells. In contrast, only 23.1% (15/65) of the cholecystitis tissue samples showed positive staining for MYBL2 (P<0.001; Table 2). Next, we assessed the relationship between MYBL2 expression and clinicopathological parameters from GBC

Fig. 2. Expression of MYBL2 mRNA in GBC cell lines. (A) The expression of MYBL2 mRNA in five GBC cell lines was detected using qRT-PCR. Data are presented as mean $\pm$ SD ($n = 3$). (B) qRT-PCR analyses of knockdown and overexpression efficiency in the indicated cells (mean $\pm$ SD, $n = 3$, * $P < 0.05$, ** $P < 0.01$). (C) western blot analyses of knockdown and overexpression efficiency in the indicated cells.

patients. As shown in Table 3, MYBL2 expression was significantly associated with histological differentiation ($P < 0.001$), tumour invasion ($P = 0.003$) and clinical stage ($P = 0.004$), but not age, gender, associated gallstones or lymph node metastasis. Furthermore we evaluated the correlation between MYBL2 expression and overall survival in GBC patients using the Kaplan-Meier analysis. The results showed that high MYBL2 expression negatively correlated with post-operative overall survival in GBC patients (Fig. 1D). Multivariate cox regression analysis further confirmed that MYBL2 expression negatively correlated with post-operative survival, indicating that MYBL2 expression was an independent prognostic marker in GBC patients (hazard ratio, 2.193; 95% confidence interval, 1.102-4.367; $P = 0.025$; Table 4).

MYBL2 promotes GBC cell proliferation in vitro

MYBL2 mRNA expression in five GBC cell lines was detected using qRT-PCR (Fig. 2A). To further explore the oncogenic potential of MYBL2, we selected the NOZ and SGC-996 cell lines that were transfected with Lv-shNC and Lv-shMYBL2 and exhibited higher MYBL2 expression levels, and GBC-SD cell lines that were transfected with MYBL2-expression vector and exhibited lower MYBL2 expression levels. The transfection efficiency was confirmed using qRT-PCR and western blot (Fig. 2B and C). To explore the effects of MYBL2 on GBC cell proliferation, we performed CCK-8 and colony-formation assays. As shown in Fig. 3A, the cell growth of NOZ and SGC-996 cells was inhibited after MYBL2 knockdown compared with the negative control ($P < 0.05$). Additionally, colony formation assays showed that the number of colonies formed by NOZ and SGC-996 cells was significantly decreased after transfection with Lv-shMYBL2 ($P < 0.05$; Fig. 3C). Conversely, MYBL2-transfected GBC-SD cells exhibited increased growth viability and higher colony-forming ability compared with empty vector-transfected cells (Fig. 3B and D). To investigate the potential mechanisms underlying the effects of MYBL2 on GBC cell proliferation, EdU retention assays were performed to examine the regulatory influence of MYBL2 on DNA synthesis. Following transfection with Lv-shMYBL2, the percentage of EdU-positive cells decreased in NOZ and SGC-996 cells compared with the negative control. Compared with empty vector-transfected cells, the percentage of EdU-positive cells increased in GBC-SD cell transfected with MYBL2. Collectively, these results showed that the deregulation of MYBL2 levels resulted in corresponding changes in the rate of DNA synthesis (Fig. 4A and B).

Fig. 3. Effects of MYBL2 silencing and overexpression on the growth of GBC cells *in vitro*. (A and B) The cells growth rates were detected using CCK-8 cell viability assays at various time points (* $P < 0.05$). Data are presented as mean $\pm$ SD ($n = 5$). (C and D) Representative images of colony formation induced through Lv-shNC and Lv-shMYBL2 in NOZ and SGC-996 cells, and in MOCK-GBC-SD, and MYBL2-GBC-SD cells. The clone numbers were counted and are depicted in the bar chart(** $P < 0.01$). Data are presented as mean $\pm$ SD ($n = 3$).

Effects of MYBL2 knockdown and overexpression on GBC cell cycle distribution

To investigate the effects of MYBL2 depletion and overexpression on cell cycle progression, we performed flow cytometry analysis. The proportion of G2/M phase cells increased after MYBL2 knockdown in NOZ and SGC-996 cells. MYBL2 depletion might induce G2/M phase cell cycle arrest. Additionally, The proportion of S phase cells significantly increased after MYBL2 overexpression in GBC-SD cells (Fig. 5A). Furthermore, we detected the expression of cell cycle-related G2/M genes such as cyclinB1, cyclinA2, CDC2 and Plk1 using western blot (Fig. 5B). As shown in Fig. 5B, cyclinB1, cyclinA2, CDC2 and Plk1 protein levels were downregulated following MYBL2 protein suppression. In contrast, the protein levels of these four genes were upregulated following MYBL2 protein overexpression, and the levels of relative mRNA and protein expression were quantified (Fig. 6A-H). These results suggested that MYBL2 plays a vital role in GBC cell cycle progression.

MYBL2 promotes GBC cell growth in a xenograft mouse model

To explore the effects of MYBL2 on GBC growth *in vivo*, we established xenograft mouse models. As shown in Fig. 7A and B, the tumour volume and weight of MYBL2-depleted xenografts were significantly inhibited compared with negative control group. IHC analysis indicated that MYBL2 and Ki67 expression was markedly decreased in MYBL2-depleted implanted tumour tissues (Fig. 7E and F). Conversely, the MYBL2 overexpression group showed a higher tumour volume and weight compared with the empty vector group (Fig. 7C and D), consistent with the results of the IHC analysis (Fig. 7E and F).

Fig. 4. The regulatory influence of MYBL2 on DNA synthesis. (A) The downregulation of MYBL2 inhibited DNA synthesis in NOZ and SGC-996 cells compared with NC as determined using the EdU retention assay. The forced up-regulation of MYBL2 increased DNA synthesis in GBC-SD cells. Representative fluorescent images are shown. (B) The numbers of positive cells were counted and are depicted in the bar chart (* $P < 0.05$). Data are presented as mean $\pm$ SD ($n = 3$).

Fig. 5. The regulatory effect of MYBL2 on the cell cycle. (A) The depletion of MYBL2 might induce G2/M phase cell cycle arrest in NOZ and SGC-996 cells. MYBL2 overexpression induced S phase cell cycle increase in GBC-SD cells (* $P < 0.05$, ** $P < 0.01$). Data are presented as mean $\pm$ SD ($n = 3$). (B) The protein levels of MYBL2, Plk1, Cyclin A2, Cyclin B1 and Cdc2 were detected in NOZ and SGC-996 cells transfected with Lv-shNC, and Lv-shMYBL2, and in MOCK-GBC-SD and MYBL2-GBC-SD cells using western blot analysis. GAPDH was used as a loading control.

Fig. 6. Relative mRNA and protein expression quantities in GBC cell lines. (A) The mRNA levels of MYBL2, Plk1, Cyclin A2, Cyclin B1 and Cdc2 were detected using qRT-PCR in NOZ cells transfected with Lv-shNC, and Lv-shMYBL2. (B) The mRNA levels of MYBL2, Plk1, Cyclin A2, Cyclin B1 and Cdc2 were detected using qRT-PCR in SGC-996 cells transfected with Lv-shNC, and Lv-shMYBL2. (C) The mRNA levels of MYBL2, Plk1, Cyclin A2, Cyclin B1 and Cdc2 were detected using qRT-PCR in MOCK-GBC-SD and MYBL2-GBC-SD cells. (D) The relative protein expression quantities of MYBL2 in GBC cell lines were shown. (E) The relative protein expression quantities of Plk1 in GBC cell lines (mean $\pm$ SD, $n = 3$) were shown. (F) The relative protein expression quantities of Cyclin A2 in GBC cell lines were shown. (G) The relative protein expression quantities of Cyclin B1 in GBC cell lines were shown. (H) The relative protein expression quantities of Cdc2 in GBC cell lines were shown. Data represent the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, $n = 3$.

Discussion

GBC carcinogenesis involves several genetic alternations, including the amplification or mutation of oncogenes, loss of function or mutation in tumour suppressor genes, microsatellite instability, and other collateral mechanisms of oncogenic cellular activation [28-30]. Sustaining chronic proliferation is the most fundamental hallmark of cancer cells [31].

The MYBL2 gene is a member of the MYB family of transcription factors, involved in cell cycle regulation and essential functions in cell proliferation and the maintenance of genomic integrity [14, 15, 32, 33]. Studies have reported that MYBL2 overexpression in different tumours, including HCC [18, 22, 23], and MYBL2 has been implicated in the progression of breast cancer and colorectal cancer [19, 20, 24]. However, the expression and biological function of MYBL2 in GBC have not been previously investigated. Intriguingly, the results

Fig. 7. Effects of MYBL2 silencing and overexpression on GBC cells growth *in vivo*. (A and C) Representative examples of tumours formed in nude mice implanted with the indicated cells. (B and D) The tumour growth curves are summarized in the line chart. A statistical plot of the average tumour weights in the subcutaneous xenograft model (** $P < 0.01$). Data are presented as mean $\pm$ SD ($n = 3$). (E) MYBL2 and Ki67 expression levels in harvested tumour tissues were determined using an IHC staining assay. (F) The relative expression quantities of MYBL2 and Ki67 in harvested tumour tissues (mean $\pm$ SD, $n = 3$) were shown (* $P < 0.05$, ** $P < 0.01$).

of a previous microarray analysis of differential gene expression indicated that MYBL2 expression was slightly elevated in GBC compared with the corresponding non-tumour gallbladder tissues (unpublished results).

In the present study, we investigated the expression which was primarily localized in the nucleus, and biological function of MYBL2 protein in GBC. The results showed that MYBL2 protein was overexpressed in GBC tissues compared with non-cancerous tissues, suggesting that MYBL2 might play a significant role in GBC progression. Based on clinicopathological data, MYBL2 overexpression was associated with an unfavourable overall survival time after surgical dissection. Moreover, Multivariate Cox regression analysis identified MYBL2 and lymph node metastasis as independent poor prognostic factors in GBC patients. Thus, these

data provided the first evidence that MYBL2 acts as an independent predictive factor of poor prognosis in GBCs.

Considering that MYBL2 acts as a transcription factor, we hypothesized that MYBL2 might be closely associated with GBC proliferation. The results of *in vitro* and *in vivo* experiments provided evidence for the oncogenic traits of MYBL2 in the GBC carcinogenesis, however the potential mechanisms underlying the effects of MYBL2 on GBC remain unclear. Previous studies have demonstrated the involvement of the cell cycle in carcinogenesis [31]. There are several cyclin proteins involved in the carcinogenesis of GBC, and these proteins are regulated through various transcription factors in the cell cycle. Aberrations of these genes render autonomous cell cycle transition, eventually resulting in carcinogenesis [28, 29]. The results of flow cytometry and western blot analyses demonstrated that suppressed MYBL2 expression might induce G2/M phase arrest and inhibit the expression of related cyclin proteins and cyclin-dependent kinases (CDKs), which are critical for the entry into and progression through mitosis [14, 34]. In contrast, MYBL2 overexpression induced S phase cell cycle increase, which simultaneously might induce the G2-to-M cell cycle transition. The results are consistent with previous studies showing that MYBL2 together with MuvB and FoxM1 regulated the expression of several key genes required for the G2/M transition [12, 16, 35].

Cells respond to DNA damage from exogenous and endogenous lesion factors through the activation of cell cycle checkpoints to delay proliferation and facilitate DNA repair. The critical function of the checkpoint in the G2 phase is the inhibition of CDK activity, which drives the G2-to-M cell cycle transition to block entry into mitosis in the presence of damaged DNA. Based on the work reported here and in previous studies [12, 36, 37], The MuvB core sequentially recruits MYBL2 and then FoxM1 to promote the expression of G2/M cell cycle genes. The balance between the DREAM complex and the MYBL2-MuvB complex are frequently perturbed in several cancers. Ectopic MYBL2 expression may accumulate crucial mitosis-promoting proteins to bypass the G2 checkpoint, leading to the abrogation of checkpoints in G2 and mitosis and the failure to arrest GBC cells proliferation, eventually resulting in unscheduled mitotic entry and the inappropriate proliferation of cells with a damaged genome in GBC.

In addition to the classical role of MYBL2 as a transcription factor at the G2/M-transition, the results indicated that MYBL2 might also be involved in processes related to DNA synthesis or repair in GBC, consistent with previous reports [38-40]. In the present study, we examined the effect of silencing of MYBL2 expression on DNA synthesis in GBC cell lines, and the results suggested that DNA synthesis and/or entry into the S-phase was compromised in cells after MYBL2 knockdown. In contrast, the forced expression of MYBL2 promoted DNA synthesis in GBC cell lines. Furthermore, IHC analysis of xenograft tumour demonstrated that the variation of Ki67 expression was consistent with MYBL2 expression. Collectively, these results suggest that another mechanism through which MYBL2 contributes to the aggressive behaviour of GBC cells may influence DNA synthesis in S-phase. Unfortunately, the direct link between MYBL2 and this potential downstream pathway remains elusive.

Considering the complex etiology of GBC, multigenic and multifactorial variations contribute to GBC development. Based on the confirmed aberration of genes, such as P53, P16 and PTEN [28, 36], ectopic MYBL2 overexpression results in a disordered cell cycle progression at the S and G2/M phase transition. The accumulation of these genetic changes disrupts cell cycle regulation and contributes to continuous GBC cell proliferation and carcinogenesis.

In summary, we demonstrated that MYBL2 expression is associated with poor prognosis in GBC patients and promotes GBC cell proliferation *in vitro* and *in vivo*. Furthermore, we propose that MYBL2 may play an important role during GBC progression through the regulation of the cell cycle at the S and G2/M phase transition. Therefore, MYBL2 may serve as a potential prognostic and therapeutic biomarker in GBC patients.

Ethics approval

This study was approved by the ethics committee of Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University. Written informed consent was obtained from all of the patients enrolled in this study. The use of animals and the experimental protocol were approved by the Institutional Animal Care and Use Committee of Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University. All experiments were performed in accordance with relevant guidelines and regulations for the welfare and use of animals in cancer research.

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Disclosure Statement

The authors declare to have no conflicts of interest.

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