

Overexpression of *MYBL2* predicts poor prognosis and promotes oncogenesis in endometrial carcinoma

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ABSTRACT

Endometrial cancer (EC) is the most common gynecologic malignancy and still remains clinically challenging. We aimed to explore the potential biomarkers of EC and provide a theoretical basis for early screening and targeted therapy. The available transcriptome data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) were analyzed to identify differentially expressed genes. Immunohistochemistry was performed to detect gene expression. We analyzed the associations of *MYBL2* with clinicopathological features and survival time and the biological effect of *MYBL2* on the proliferation of EC cells. The effect of *MYBL2* silencing on the transcriptome of EC cell model was analyzed by RNA-Seq. *MYBL2* was significantly upregulated with obvious copy number alteration in EC. Copy number amplification significantly increased *MYBL2* mRNA expression, which led to a poor prognosis and severe pathological types of EC. Additionally, *MYBL2* silencing significantly inhibited proliferation and induced apoptosis and G₁-phase cell cycle arrest in EC cell lines. Our results indicate that *MYBL2* is closely related to the cell cycle and apoptosis pathways in EC. The findings in this study provide evidence that *MYBL2* can serve as a new candidate prognostic marker and a target for future therapeutic intervention in EC.

Key words: Endometrial carcinoma; *MYBL2*; copy number; proliferation; prognosis.

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Introduction

Endometrial carcinoma (EC) is one of the most lethal gynecological cancers and is the most common malignant tumor of the female genital tract in the world. There are 319,500 new cases each year, with a high mortality rate that exceeds 23%.¹ Surgery is the main treatment for EC patients, while radiotherapy, hormone therapy, chemotherapy and other auxiliary methods are also used.^{2,3} However, the poor prognosis, high local recurrence rate, high metastasis rate, and chemoresistance of EC remain to be addressed.³ Hence, it is urgent to explore the potential molecular mechanisms of EC and to identify novel biomarkers and therapeutic targets. Genetic factors play an important role in the etiology of EC. Cancer evolution is a complex developmental process that is mainly related to the activation of proto-oncogenes and the loss of suppressor genes.^{4,5} The identification of proto-oncogenes or tumor suppressor genes can partly elucidate the pathogenesis of tumors, including chromosomal alterations, copy number alterations (CNAs), and epigenetic changes.^{6,7}

Copy number amplification is a form of structural variation defined as the CNA of DNA fragments from 1 Kb to several Mb in the human genome.⁶ DNA deletion, insertion, replication and complex multisite mutations are involved in copy number alteration (CNA).⁷ CNA is a hallmark of cancer, leading to oncogene activation or inactivation of tumor suppressor genes in various malignant tumors.⁸ Previous research has found that high-risk germline mutations are likely to occur during the mismatch repair of the *MSH2*, *MSH6* and *PMS2* genes, and some rare germline copy number deletions will also occur in the early phase of EC.⁹ The study found that *GSTT1* copy number amplification increased EC risk with substrate specificity. The intermediates produced by *GSTT1* may be genotoxic to cells.¹⁰

In recent years, through analysis of data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) for comprehensive genomic and transcriptome analysis of EC, new molecular types of EC have been discovered. The classification system greatly contributes to the development of targeted therapy for specific genetic abnormalities.¹¹ In this study, based on the EC mRNA expression profiles in the TCGA and GEO databases, we conducted a comprehensive analysis to identify potential biomarkers of EC and provided a potential target for EC therapy.

Materials and Methods

Clinical samples

According to the minimum sample rule of mathematical statistics, thirty-pairs of EC tissues and the adjacent tissues were acquired from the Second Affiliated Hospital of Nanchang University. The tissues were frozen in liquid nitrogen and then immediately stored at -80°C. The Ethics Committee of the Second Affiliated Hospital of Nanchang University approved the protocol with approval number: Review [2018] No. (014), and all patients provided consent for the utilization of their tissue samples in this study. The patients were registered in the Ethics Committee of our hospital; thus, we got the approval of the ethic approval document. The study was conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000).

Data collection and comprehensive analysis

We downloaded EC RNA-seq data and phenotype data from the TCGA database (<https://xenabrowser.net/datapages/>), which includes 174 EC specimens and 13 normal endometrial specimens.

According to Diskin's research, the clinical information, CNA spectrum, and gene sequencing details of EC patients were obtained.¹² Moreover, two gene expression profile matrix files were downloaded from the GEO database. GSE63678 includes 7 EC tissues and 5 normal endometrial tissues.¹³ GSE17025 includes 91 EC tissues and 12 normal endometrial tissues.¹⁴ The platforms for these two data sets are GPL571 and GPL570. According to the annotation information on the platform, the probe is converted into the corresponding gene symbol, and log₂ conversion is performed on all gene expression data.

We used the method of linear model empirical Bayes statistics in the limma package in R software to perform nonspecific filtering on the EC data set to screen out the differentially expressed genes (DEGs) between tumor and adjacent tissues.¹⁵ We used GEO2R to screen DEGs in GSE63678 and GSE17025, where genes with p values <0.05 and log (FC) >1 were considered DEGs.

Furthermore, the mRNA expression and clinical data were extracted from the TCGA with cBioPortal (www.cbioportal.org). These data were preprocessed and analyzed. To prove the relationship between *MYBL2* copy number status or mRNA expression level and survival rate, the mean expression value of each gene was compared between CNA groups, and the significance analysis was assessed with *t*-test. A Kaplan-Meier diagram was constructed to prove the relationship between genetic disorders and patient survival. Additionally, various clinical and pathological features were also detected, including the age of initial diagnosis, menstrual conditions, pathological type, FIGO stage and pathological grade. The data were subjected to the chi-square test after quantification and analysis.

Immunohistochemistry

We stained the tumor tissue samples using rabbit monoclonal antibodies against MYBL2 (1:200, Abcam, Cambridge, UK), CDK1, and CCNB1 (1:200, Proteintech, Rosemont, IL, USA) based on the standard Immunohistochemistry (IHC) protocol. Briefly, 5- μ m-thick paraffin-embedded tissue sections were deparaffinized with xylene and graded alcohol, and endogenous peroxidase activity was quenched with 3% H₂O₂ in methanol. We washed the tissues with Tris-borate saline Tween-20 (TBST). After washing, slides were incubated with DAB (3,3'-diaminobenzidine tetrahydrochloride) (Sigma-Aldrich, St. Louis, MO, USA) and immediately washed under tap water after color development. Slides were then counterstained with hematoxylin. Slides were mounted with dibutyl phthalate xylene (DPX) and were then observed under a light microscope (Carl Zeiss, Oberkochen, Germany). Two independent pathologists evaluated the slices. Positive staining of MYBL2 was observed in the nucleus and cytoplasm. Five fields of view were randomly selected for each assessment and viewed at 400 times magnification (each field had at least 200 cells). MYBL2, CDK1 and CCNB1 staining were scored based on the percentage of positive cells and fluorescence intensity (0, negative; 1, weak; 2, medium; 3, strong). The product of the intensity score and the distribution score was used as the staining score. A specimen with a final staining score of 0-1 was considered negative, and a specimen with a score of 2 was considered positive.

Cell culture

We used HEC-1-B and AN3CA cell lines for *in vitro* experiments, and they were purchased from Shanghai Enzyme Biotechnology (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fresh fetal bovine serum, 100 μ g/ml penicillin and 100 μ g/ml streptomycin. Cells in media were placed at 37°C with 5.0% CO₂ in a humidified incubator and sub-cultured when the cells reached 70% confluence.

RNA extraction, transfection and qRT-PCR

HEC-1-B and AN3CA cells were plated from cell culture flasks into 6-well plates. The shRNA plasmid was transfected into HEC-1-B and AN3CA cells with TurboFect transfection reagent (Life Technologies Co., Carlsbad, CA, USA) when the cells grew to 50%-60%. The cells were then stained with EdU reagent and Hoechst reagent after 48 h. The shRNA sequences targeting *MYBL2* were sh*MYBL2*-1, GUUAUGGACCAAAGAGGAATT and sh*MYBL2*-2, CCGUCCCUCCUACCAUAAATT. The positive control shRNA (shPC) sequence was UUCUCCGAACGUGU-CACGUTT.

The total RNA of each group of cells was extracted according to the TRIzol reagent instructions (Invitrogen), and a reverse transcription kit (TaKaRa, Dalian, Liaoning, China) was used to reverse transcribe the RNA. qRT-PCR was performed with SYBR green (Applied Biosystems, Foster City, CA, USA) by means of a ViiA7 real-time PCR system (Applied Biosystems). The reaction system of qRT-PCR was as follows: 10 μ L SYBR, 0.4 μ L ROX enzyme, 1.6 μ L upstream and downstream primers, 6 μ L DEPC water, and 2 μ L cDNA template. We calculated the relative mRNA expression by the comparative Ct method using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control. The following primers were used for *GAPDH*: forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CACCTGTTGCTGTAGCCAAA-3'. The following primers were used for *MYBL2*: forward, 5'-CCGGAGCAGAGGGATAGCA-3' and reverse, 5'-CAGTGGGTTAGGGAAGTGG-3'.

Western blotting

We extracted proteins from each group of transfected cells by

using radioimmunoprecipitation lysis buffer (Roche, Nutley, NJ, USA). Protein lysates were quantified with a BioRad kit (Bio-Rad, Hercules, CA, USA). Microgram protein samples were separated *via* 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Burlington, MA, USA). Then, the NC membrane was blocked with 5% skim milk at 37°C for 1 h. The membranes were immunoblotted with primary antibodies overnight at 4°C followed by incubation with the respective secondary antibodies for 4 h. The ECL kit was used to detect the bands. The antibodies used were as follows: *MYBL2* (1:1,000; Absin Bioscience, Shanghai, PRC); *GAPDH* (1:5,000; Epitomics, Burlingame, CA, USA); and anti-rabbit IgG antibody (1:4,000; Epitomics).

Cell proliferation and clone formation

EC cells were cultured in DMEM with 10% (v/v) FBS (Gibco). Cells in culture flasks were placed in an incubator (37°C, 5% CO₂) and subcultured every 3 days. To perform the cell proliferation assay, we divided EC cells into 96-well plates at a density of 3×10³ cells/well and cultured them with 0.5 mL DMEM (with 10% FBS) for 48 h. Then, the cells were transfected with constructed shRNA expression vectors. After transfection, the EdU assay was performed according to the manufacturer's protocol for the EdU colorimetric cell proliferation assay kit (RiboBio, Guangzhou, China). Cell activity was detected by CCK8 colorimetry. Three thousands (3000) cells per well were inoculated in a 96-well plate, cultured for 48 h after transfection, 10 μ L CCK8 reagent was added to each well, and incubated for two hours. The absorbance was then measured with a microplate reader at 562 nm. The growth

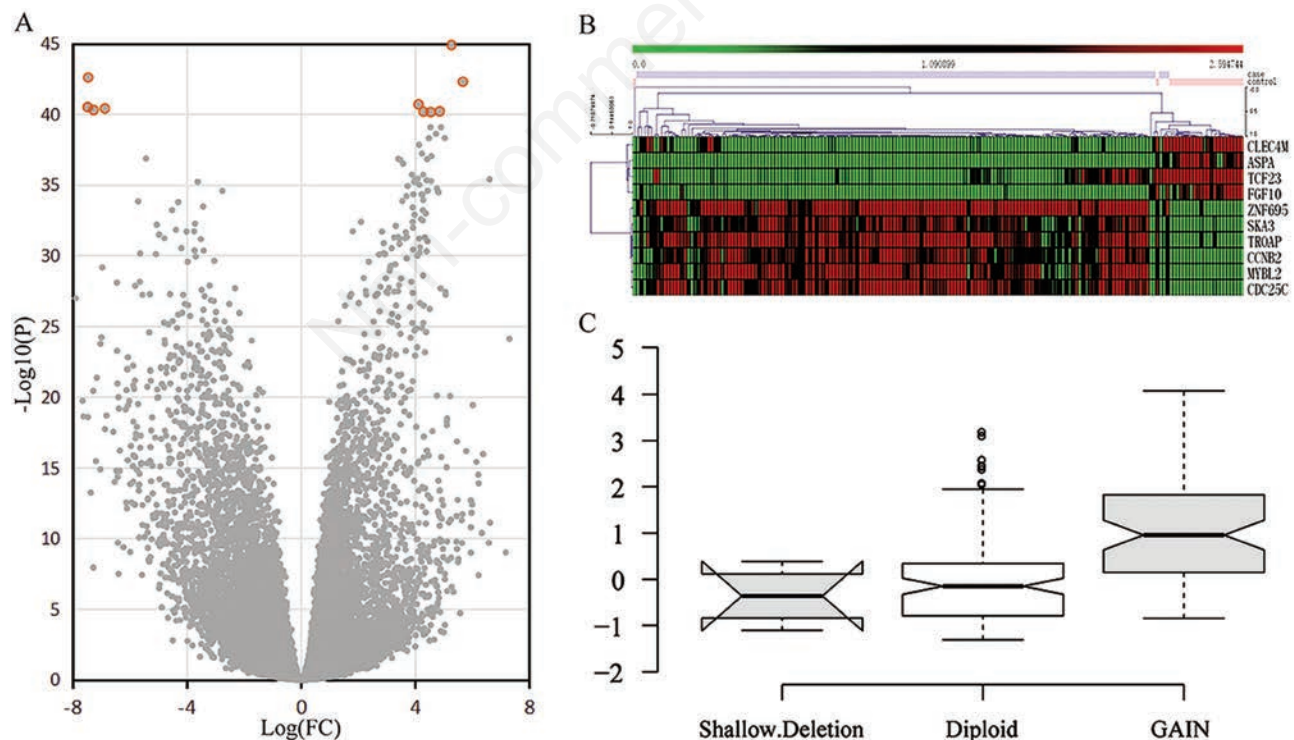


Figure 1. Summary of EC genome-wide integration analysis based on TCGA and GEO data sets. A) In the screening of DEGs in EC, red circles represent ten significant genes. B) In the TCGA data set, the top ten genes expressed most prominently in EC patients were identified; each column represents a sample, while each row represents the expression level of the gene; the color scale represents the original Z score, ranging from green (low expression) to red (high expression). C) Among the top 10 DEGs, *MYBL2* had the most significant CNA in EC, and copy number amplification led to upregulated expression levels of *MYBL2*.

curve generated according to the OD values. For the clonogenic assay, EC cells (3×10^3 per well) were plated in 6-well plates and cultured for 2 weeks. All cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet (w/v) at room temperature, and the colony numbers were determined. Cell cycle distribution was analyzed by propidium iodide (PI) staining and flow cytometry.

Cell cycle

We collected EC cells (2×10^6) with or without sh-*MYBL2* treatment. Half of these cells were fixed with 500 μ l 70% cold ethanol for 2 h overnight according to the manual for the cell cycle detection kit (Keygen Biotech Co., Ltd., Nanjing, Jiangsu, China) and stored at 4°C. The cells were centrifuged by vortexing at 1500 rpm for 5 min, the supernatant was removed, and the hydrated cells were resuspended for 10 min in 0.5 mL PBS (precooled at 4°C). RNase was also added at 37°C for 10 min. We added 400 μ L of PI (final concentration: 50 μ g/mL) and incubated it for 15 min at room temperature in the dark.

The cell cycle was detected on a flow cytometer (BD Accuri C6, Ann Arbor, MI, USA) after cell digestion by assessing red fluorescence at a wavelength of 480 nm and analyzed by FlowJo software. We performed at least 3 independent experiments to determine the percentage of cells in G₀, G₁, and G₂/M.

RNA-seq analysis

We extracted and purified total RNA from cells under various culture conditions, removing rRNA to build libraries. We collected

10 Gb of sequencing data for each condition after ABI SOLiD sequencing for data processing. We identified all the transcription units (TUs) in the genome and calculated the expression abundance. The expression levels of the genes were calculated from the reads per kilobase of transcript per million mapped reads (RPKM) value, and the TU shear mode was determined by comparing the sequences. DEGseq software was used to compare the expression levels of each group of genes to determine the DEGs and to analyze the biological pathways.

Statistics

Data analyses were performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). We used Student's *t*-test or analysis of variance for the statistical analyses; *p*-values <0.05 were considered significant.

Results

MYBL2 was overexpressed in EC according to whole-genome screening and clinical experiments

We downloaded the EC data set from the TCGA, which contains information on 20,530 genes. After screening, we selected 12,047 significant genes (*p*<0.05). We compared the gene expression levels between EC tissue and nontumor tissue, and the DEGs were screened out. Among the top 10 most significant DEGs, the downregulated genes in EC were *CLEC4M*, *ASPS*, *TCF23*, and

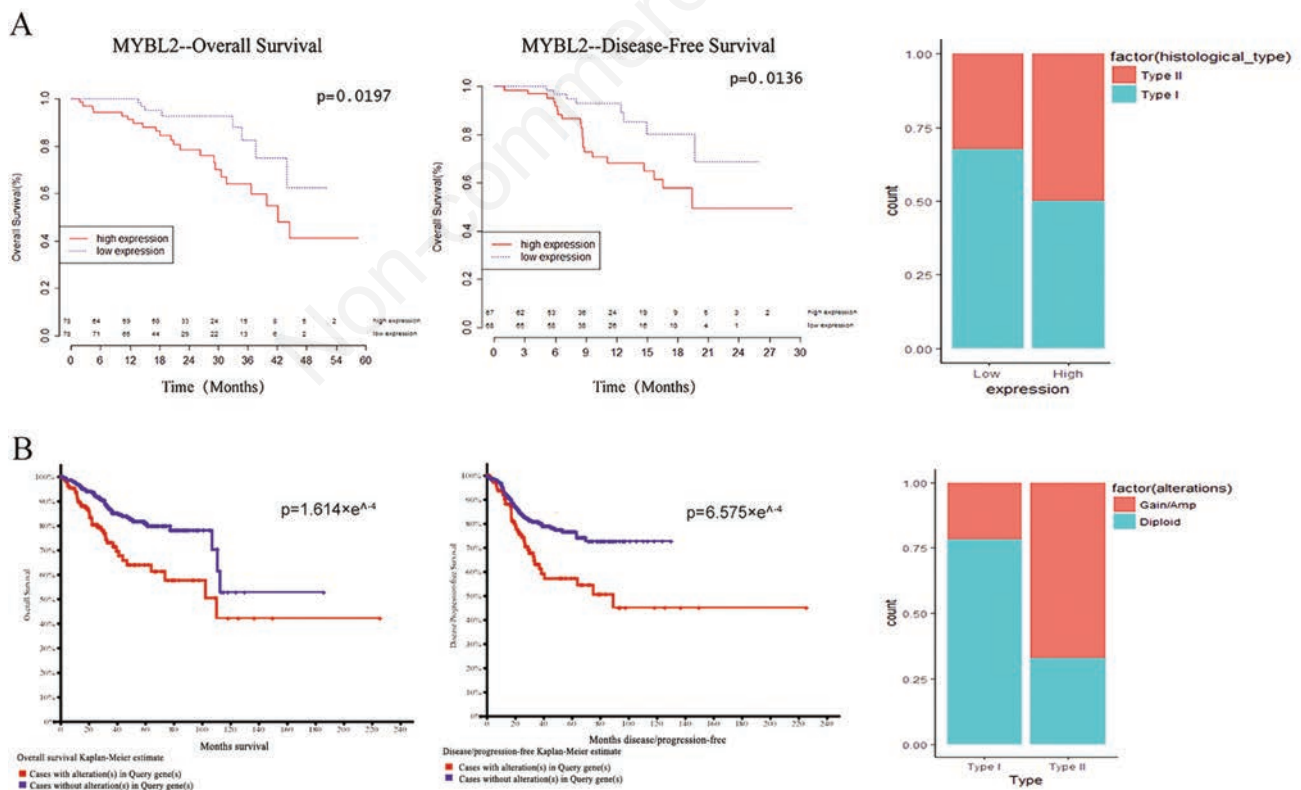


Figure 2. The association between *MYBL2* overexpression and amplification and clinical data analysis. A) The overexpression of *MYBL2* affects the OS and DFS, pathological type and pathological stage of patients with EC. B) CNA of the *MYBL2* leads to a significant deterioration in the prognosis of EC patients, and CNA is more significant in type II EC than in type I EC.

FGF10. The remaining DEGs were highly expressed, including *ZNF695*, *SKA3*, *MYBL2*, *TROAP*, *CCNB2*, and *CDC25C* (Figure 1A and 1B). In addition, the DEGs between EC tissues and nontumor tissues were assessed in the GEO data set after normalization and analysis. The results showed that *ASPA*, *MYBL2*, *TROAP*, *CCNB2*, and *CDC25C* were the most significantly altered genes ($p < 0.05$). We verified that *MYBL2* was highly expressed in EC by assessing these two different data sets. Moreover, copy number variation data of these top 10 significant DEGs from the TCGA were analyzed by GISTIC2.0 online software (Table S1). Among them, *MYBL2* had obvious CNA in EC ($p < 0.001$), which caused the expression of *MYBL2* mRNA to significantly increase ($p < 0.05$; Figure 1C). Therefore, *MYBL2* may be involved in EC occurrence through CNA.

We also used clinical experiments to identify *MYBL2* expression in EC tissue *via* IHC. Thirty EC tissue samples and 30 nontumor tissue samples were collected. The IHC results revealed that *MYBL2* was overexpressed in tumor tissues (Figure S1).

MYBL2 overexpression and amplification were related to the clinicopathological characteristics of EC

To explore the relationship between *MYBL2* and the clinicopathological features and prognosis of EC, we analyzed the clinical data, expression profiles, and CNA data in the TCGA. Cox regression analysis showed that the high expression of *MYBL2* was positively correlated with the pathological type ($p = 0.034$) and pathological stage ($p < 0.001$) of EC (Table 1). We also performed overall survival (OS) and disease-free survival (DFS) analyses of *MYBL2*, and they were significantly different in EC patients with *MYBL2* overexpression ($p < 0.05$, Table S2). The EC patients with high expression of *MYBL2*, especially those with high-grade and type II EC, had a worse prognosis ($p < 0.05$) than those with low expression (Figure 2A). Interestingly, the CNA of *MYBL2* also led to a significant deterioration in the prognosis of EC patients, and this effect was more significant in type II EC than in other types (Figure 2B). Thus, overexpression of *MYBL2* and CNA may be prognostic factors for EC.

MYBL2 knockdown inhibited EC cell proliferation

To further investigate the biological effect of *MYBL2* on the

proliferation of EC cells, we chose two EC cell lines (HEC-1-B and AN3CA) and designed two shRNAs to silence *MYBL2* expression. Quantitative RT-PCR and Western blotting (WB) were used for assessment. qRT-PCR and WB confirmed that sh-*MYBL2* can significantly down-regulate the expression of *MYBL2* in EC cell lines (Figure 3 A,B). Moreover, EdU and Cell Counting Kit-8 (CCK-8) data analysis also showed that knockdown of *MYBL2* inhibited the proliferation of the two EC cell lines, especially HEC-1-B cells with sh-*MYBL2*-2, which had a 32% reduction in the expression level of *MYBL2* compared to that in control cells (Figure 3 C,D). Similarly, compared with negative control cells (sh-NC), colony formation was decreased after knockdown ($p = 0.0012$; Figure 3E). Together, these results indicate that *MYBL2* may play an important role in regulating the proliferation of EC cells.

Downregulation of MYBL2 inhibits the expression of genes involved in cell proliferation

To explore the molecular mechanism of *MYBL2* expression in EC, we used RNA-seq to analyze the effect of *MYBL2* silencing on the transcriptome of EC cell models. The expression of a total of 16,238 genes was detected in EC cells treated with sh-NC and sh-*MYBL2* (RPKM > 0 in any group). Comparison and screening of sh-NC and sh-*MYBL2* groups revealed 1,367 DEGs ($p < 0.05$), accounting for 8.42% of the total expressed genes; these DEGs included 644 upregulated genes ($\log_{2}FC > 0$) and 723 downregulated genes ($\log_{2}FC < 0$; Figure 4A). To deeply understand the biological significance of *MYBL2* silencing on the gene expression regulation of EC cells, the research team conducted pathway enrichment analysis based on 1,367 DEGs. WebGestalt analysis showed that these DEGs were enriched in 20 functional pathways (FDR < 0.05). We selected the top 10 enriched pathways for further study. Among them, *MYBL2* was closely related to the two pathways of the cell cycle and apoptosis (Figure 4B). Further analysis of cell cycle-related genes revealed that 22 genes were related to the cell cycle (Figure 4C). Consistent with these results, downregulation of *MYBL2* could induce EC cell apoptosis, inhibit cell cycle progression, and block most cells in the G_1 phase (Figure 4 D,E). These results indicated that *MYBL2* silencing can inhibit the proliferation and growth of EC cells by blocking cell cycle progression.

Table 1. Association of MYBL2 expression levels with clinicopathologic characteristics in EC.

Factors	MYBL2 protein expression				p
	Low (n=68)		High (n=72)		
	Count	%	Count	%	
Age (years)					
>60	17	12.10	18	12.90	1
≤60	51	36.40	54	38.60	
Menopause status*					0.829
Peri/Pre	5	3.60	6	4.30	
Post	63	45.00	66	47.10	
Histological type					0.034
Type I	46	32.90	26	25.70	
Type II	22	15.70	36	25.70	
Clinical stage					0.283
Type I	42	30.00	38	27.10	
Type II-IV	26	18.60	34	24.30	
Histologic Grade					<0.001
G1-G2	23	16.40	5	3.60	
G3-G4	45	32.10	67	47.90	

*Peri, 6-12 months since last menstrual period (LMP); Pre, <6 months since LMP and no prior bilateral ovariectomy and not on estrogen replacement; Post, prior bilateral ovariectomy or >12 months since LMP with no prior hysterectomy.

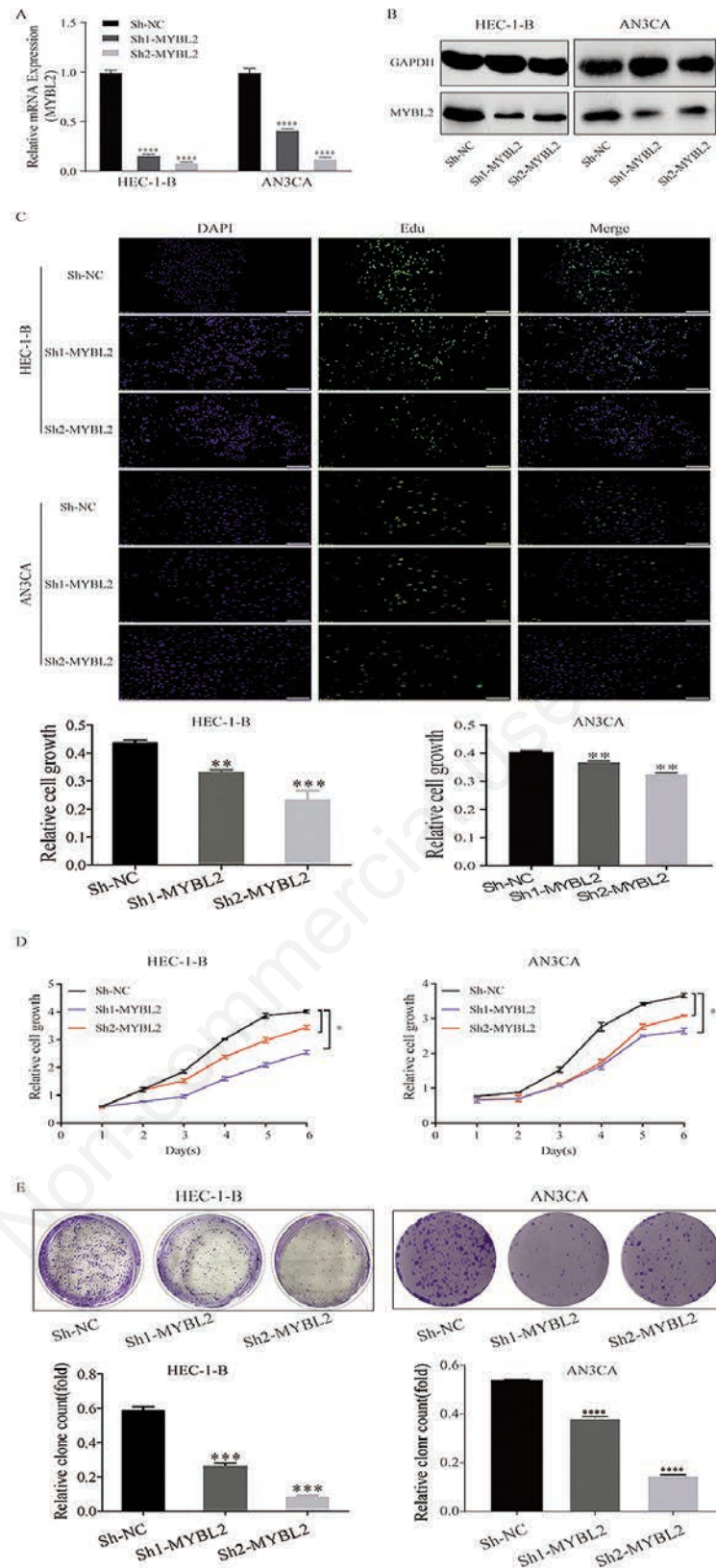


Figure 3. Cell proliferation assay in *MYBL2* knockdown EC cell lines. A) The capability of different shRNAs to downregulate *MYBL2* expression. B) The ability of different shRNAs to inhibit the expression of *MYBL2* protein. C) The cell proliferation rate in HEC-1-B and AN3CA cells at 48 h after *MYBL2* knockdown; after transfecting the two cell lines with *MYBL2* shRNA, *MYBL2* expression was downregulated, and the cell proliferation of HEC-1-B and AN3CA cells was inhibited dramatically compared to that of the sh-NC cells. D) CCK-8 cell growth curve. E) Knockdown of *MYBL2* significantly reduced the colony forming ability of HEC-1-B and AN3CA cell lines. ** $p < 0.01$; *** $p < 0.001$.

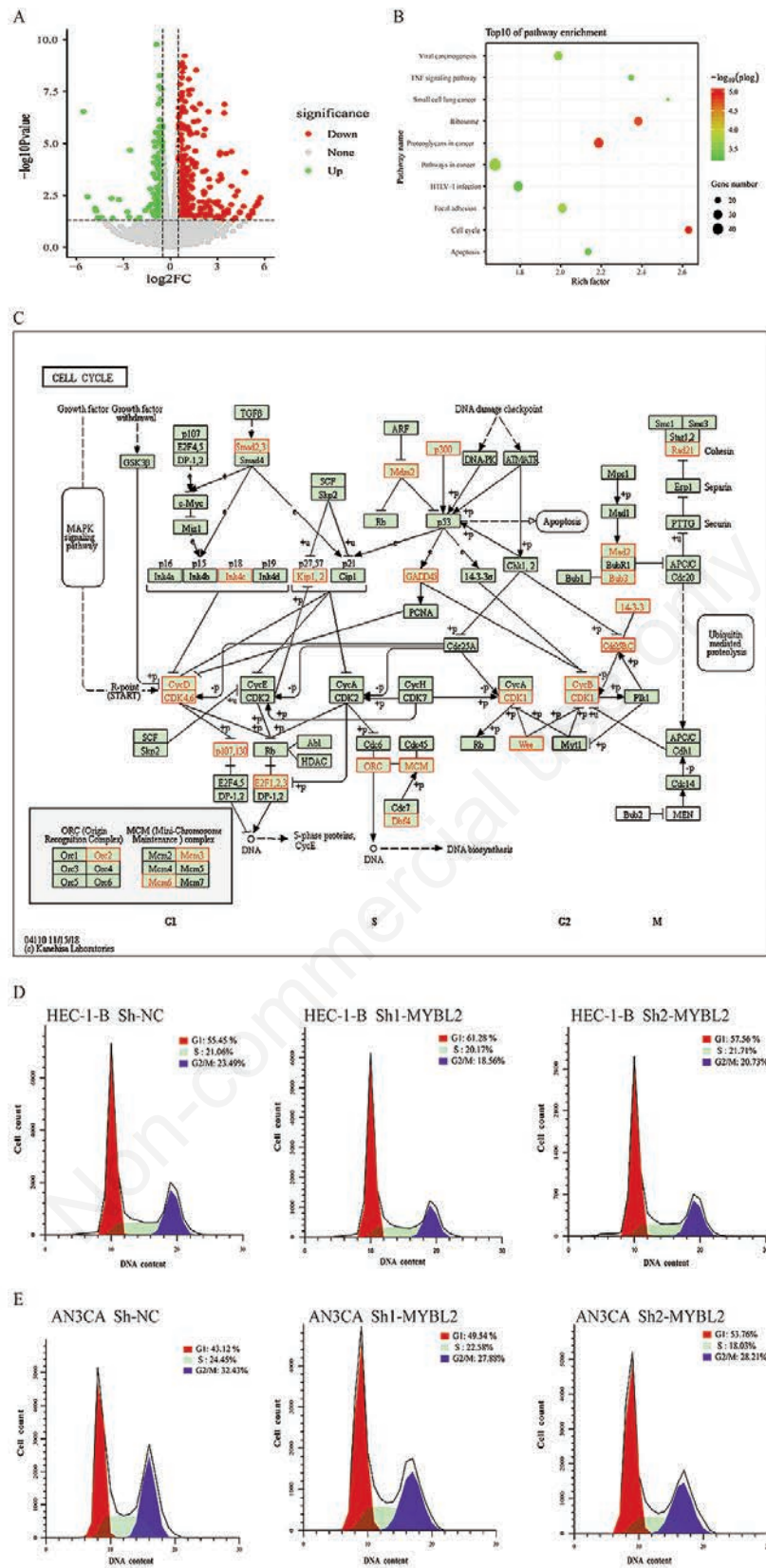


Figure 4. Pathway enrichment analysis of DEGs in *MYBL2* knockdown EC cells. A) Analysis of DEGs in EC cell untreated and treated groups. Volcano plots were used to summarize the DEGs. B) Pathway enrichment analysis of the top 10 DEGs; the negative log₁₀ Q value from pathway enrichment analysis is plotted, and the pathways are sorted based on the p-value of DEGs and enrichment based on the core DEG data set. C) Heat map of genes related to the cell cycle. The heat map showed that after knocking out *MYBL2*, 22 genes in EC cells were significantly differentially expressed. D-E) Cell cycle analysis of EC cells 48 h after transfection with the indicated plasmids.

Discussion

In our study, based on the transcriptome and survival data of EC in the TCGA and GEO databases. After screening, it was determined that the DEGs were *ASPA*, *MYBL2*, *TROAP*, *CCNB2* and *CDC25C*. *MYBL2* is highly expressed in EC (Figure 1B). IHC results also verified the high expression of *MYBL2* in EC tissues (Figure S1). We used the Phenotype database to analyze the survival of the above five candidate genes. The results showed that only the high expression of *MYBL2* was positively correlated with the poor prognosis of EC patients (Figure 2A; Table S2). *MYBL2* has obvious CNAs in EC ($p < 0.001$), and CNAs significantly increased the expression of *MYBL2* gene mRNA level ($p < 0.05$) (Figure 1C; Table S1). On the other hand, the clinical data of TCGA shows that the overexpression of *MYBL2* in EC is related to clinicopathological characteristics (such as poor survival, high pathological type and pathological stage) (Table 1). In addition, through the data in cBio, we found that the high expression of *MYBL2* is closely related to its CNAs, and CNAs is closely related to clinical indicators (Figure 2B). In summary, *MYBL2* does have an impact on EC, so the target gene *MYBL2* was finally screened out. But the exploration of its mechanism will require further experimental verification.

The proto-oncogene *MYBL2*, also known as *B-MYB*, is a member of the Myb family of transcription factors, which also includes *A-MYB* and *C-MYB*. *C-MYB* was first identified as highly expressed in hematopoietic stem cells¹⁶ and in the brain¹⁷ and colon.¹⁸ *A-MYB* is mainly expressed in the testis, with extremely low expression in the ovary, spleen and brain.¹⁹ *MYBL2* is usually expressed in rapidly dividing cells, especially in adult hematopoietic and embryonic stem cells and is involved in the proliferation, differentiation and apoptosis of various cells.²⁰ Studies have confirmed that *MYBL2* is highly expressed in malignant tumors such as colorectal cancer, pancreatic ductal adenocarcinoma and lung cancer.²¹ Previous research also indicates that *MYBL2* is associated with the prognosis of many tumors.²² Possible mechanisms of *MYBL2* dysregulation in cancer were assumed. Moreover, the amplification of chr20q13 can cause changes in *MYBL2* expression, and the amplification of this chromosomal region has been reported in breast and ovarian cancers.^{23,24} Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer and can lead to an increase in *MYBL2*. These findings suggest that *MYBL2* plays a vital role in the occurrence and development of various tumors. However, the expression of *MYBL2* and its biological functions in EC have not been studied. Previous reports prompted us to explore the role of *MYBL2* in the progression of EC.

MYBL2 is widely expressed in all proliferating cells, and its expression level is related to the cell proliferation capacity.²⁵ Liang and colleagues found that after knocking down *MYBL2*, the proportion of cells in the G₂M phase increased, and cell cycle arrest tended to occur. Overexpression of *MYBL2* increased the proportion of S-phase cells. The same results were observed in liver cancer and breast cancer cell lines, and the mechanism may be related to the downregulation of *CCNB1*, *CCNA2*, *CDC2*, and Plk1 protein after inhibition of *MYBL2* protein.²⁶ The downregulation of *MYBL2* leads to the inhibition of cell cycle progression, and *MYBL2*, together with *MuvB* and *FoxM1*, regulates the expression of several key proteins required for the G₂M transition.²⁷ Therefore, we speculated that the transcription factor *MYBL2* can promote cell cycle progression to S phase and M phase by affecting the transcription of important genes related to the cell cycle, which could promote the proliferation of cancer cells. Studies have shown that *MYBL2* knockout in mice indicates that the colony formation of blastocysts is blocked. These results indicated that *MYBL2* is an essential gene that regulates the cell cycle and cell

survival.²⁸ Previous studies have shown that the mechanism of oncogenesis is related to the cell cycle.⁷ These data demonstrate the important role of *MYBL2* in promoting cell proliferation. Nevertheless, the mechanism of *MYBL2* in EC is still unknown.

Therefore, we established a cell model of *MYBL2* down-regulation to explore the role of *MYBL2* in EC. We selected two EC cell lines (HEC-1-B and AN3CA) and constructed two shRNAs to down-regulate *MYBL2* expression. *MYBL2* mRNA and protein expression levels were significantly down-regulated in sh-*MYBL2* groups (Figure 3 A,B). Moreover, EdU and CCK-8 data analysis also showed that sh-*MYBL2* inhibited the proliferation of the two EC cell lines (Figure 3C and 3D). Similarly, colony formation was significantly reduced ($p = 0.0012$, Figure 3E). Consistent with these results, pathway enrichment analysis based on RNAseq data shows that *MYBL2* is closely related to the two pathways of cell cycle and apoptosis, and can activate gene expression related to cell proliferation (Figure 4 B,C). These results indicate that *MYBL2* may play an important role in regulating the proliferation of EC cells.

Based on our research, we innovatively found that the *MYBL2* gene had CNA in EC, which affects *MYBL2* expression. Analysis of clinical data showed that patients with *MYBL2* overexpression have a poor prognosis. In addition, the results of the cell proliferation assay also demonstrated that *MYBL2* plays a vital role in the proliferation of EC cells. We provided evidence that *MYBL2* may contribute to the progression of EC by regulating the cell cycle and promoting cell proliferation. Therefore, *MYBL2* may be used as a new biomarker for the prognostication and treatment of EC patients.

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