PLAC1 is an independent predictor of poor survival, and promotes cell proliferation and invasion in cervical cancer

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Abstract. Placenta-specific protein 1 (PLAC1) is inversely associated with survival in several types of cancer. However, whether PLAC1 is involved in the progression of cervical cancer (CC) remains to be elucidated. Therefore, the present study aimed to evaluate the prognostic role of PLAC1 in CC by determining the relationship between clinicopathological factors, *PLAC1* gene expression and survival prognosis using univariate and multivariate Cox proportional-hazards regression analyses. Similarly, Kaplan-Meier curves were evaluated with the log-rank test. Subsequently, gene set enrichment analysis was performed to compare the high- and low-PLAC1 expression phenotypes. Functional studies were further conducted in PLAC1-overexpressing HeLa cells and PLAC1-silenced MS751 cells, and western blotting was performed to determine whether PLAC1 promoted CC progression via epithelial-mesenchymal transition (EMT). The findings demonstrated that high expression of PLAC1 was associated with American Joint Committee on Cancer metastasis pathological score and suggested a poor overall survival. 'mTOR complex 1 signaling', 'interferon α response' and 'hypoxia' were differentially enriched in the high-PLAC1 phenotype. Furthermore, PLAC1 promoted the invasion of CC cells *in vitro*. E-cadherin expression was decreased in the PLAC1-overexpressing cells, accompanied by increased expression of the mesenchymal markers, Vimentin, MMP2 and Slug, and the opposite effects were observed in PLAC1-silenced cells. Taken together, the present results indicated that high expression of PLAC1 was associated with poor survival and PLAC1 promoted metastasis via EMT in CC.

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Introduction

Cervical cancer (CC) is the fourth most common type of cancer among female patients worldwide (1) and is a major health problem, particularly in developing countries (2). The introduction of a specific human papillomavirus vaccine into clinical practice has led to a gradual reduction in the incidence of CC (3). Over the past two decades, the exploration of the molecular mechanisms underlying tumor development has increased (4). Although significant progress has been achieved, potential prognostic and diagnostic biomarkers must be identified to facilitate the management of patients with CC.

Cancer/testis antigens (CTAs) are a class of tumor-related antigens with restrictive expression patterns that are mostly located on the X chromosome or restricted to cancer and reproductive tissues, and represent a set of extensively researched targets of tumor immunotherapy (5). Placenta-specific protein 1 (PLAC1) is a member of the CTAs; the gene encoding *PLAC1* is on the X chromosome (Xq26.3) near the hypoxanthine-guanine phosphoribosyl transferase gene (6). PLAC1 is a small secreted cell-adhesion protein, which has a predicted amino terminal transmembrane domain and a divisible signal peptide of 23 amino acids (6). PLAC1 is normally expressed in placental trophoblasts and the testis, but exhibits extremely low expression in other normal tissues (7,8). In recent decades, PLAC1 has been found to be localized on the surface of tumor cells and be approachable to antibodies (9). High expression levels of PLAC1 have been detected in a broad variety of solid tumors, including liver, digestive tract, mammary, prostatic, ovarian and uterine tumors (10-12). Devor *et al* (13) reported that PLAC1 was also expressed in human papillomavirus-positive CC, including in four common pathological types. Furthermore, Wang *et al* (8) revealed that PLAC1, which was elicited by Epstein-Barr virus and expressed in human tumor cells, was an important CTA. Moreover, PLAC1-knockout mice have been reported to show considerable placental defects, with placental layers revealing aberrant differentiation (14). Considering the inherent invasive ability of the placental trophoblast, it has been hypothesized that tumor cells may use the placental mechanisms to facilitate invasion (15). Understanding this process may lead to the development of methods for blocking PLAC1

expression in tumor cells. Currently, on account of its immuno-genicity and carcinogenicity, PLAC1 is on the National Cancer Institute list of 'cancer antigens', which may act as a target for developing vaccines to prevent breast carcinoma (16).

The present study investigated the role of PLAC1 in the proliferation and invasion of CC cells, and proposed a hypothesis that PLAC1 may be an important oncogenic and prognostic factor in CC.

Materials and methods

Data preprocessing and survival analysis. RNA-sequencing (RNA-seq) expression data and clinicopathological information (including survival data) from patients with CC were derived from The Cancer Genome Atlas (TCGA) portal (https://portal.gdc.cancer.gov/). The methods used for sample acquisition, RNA extraction and sequencing were previously described by TCGA Research Network (17). Survival curves were estimated using Kaplan-Meier methods and compared by the log-rank test.

Gene set enrichment analysis (GSEA). The aim of GSEA is to determine the different expression levels of a predefined gene set in two different phenotypes (18). In the present study, GSEA was conducted to examine the significance of survival differences between 'high-PLAC1' and 'low-PLAC1' expression groups based on hallmark collections of gene sets in the Molecular Signatures Database (19) using the official Java GSEA tool (18) (version: 4.1.0, number of permutations=1,000), the two groups were divided by the median expression level. This method identifies genes with large phenotype correlations showing the greatest contribution of enrichment scores in multiple gene sets within a molecular signatures database collection.

Immunohistochemistry (IHC) analysis. The tissue microarray (HUteS154Su01) containing patient clinical information was obtained from Shanghai Outdo Biotech Company and was used to detect the expression levels of PLAC1 in 101 cervical tumor tissues, 35 non-tumor tissues and 18 high-grade squamous intraepithelial lesions (HSILs). The tissue microarray experiment was completed by Shanghai Outdo Biotech Company. The slide was blocked in 10% goat serum (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Antibody against PLAC1 (1:100; cat. no. sc-365919, Santa Cruz Biotechnology Inc) was added and incubated at 4°C overnight. The ready-to-use horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. K5007; Dako; Agilent Technologies, Inc.) was added and incubated for 30 min at room temperature. Slides were visualized under a light microscope (Olympus Corporation; cat. no. CX31) at x400 magnification. Semi-quantification of protein expression was determined according to the percentage of positive cells (N) as follows: 0, ≤5; 1, 5<N≤25; 2, 25<N≤50; 3, 50<N≤75 and 4, N>75%. Staining intensity ranged from 0-3 (0 negative; 1, weak; 2, moderate; 3, strong). , The two scores were multiplied together to calculate the total score, which ranged from 0-12. All IHC data were interpreted by the same qualified pathologist for consistency.

Cell culture. The human CC cell lines CaSki, MS751, C-33A and HeLa, were obtained from the American Type Culture Collection. All the cells were cultured in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (HyClone; Cytiva). All cells were incubated in 5% CO_2 at 37°C.

RNA silencing and cDNA overexpression. Lentivirus harboring two short hairpin RNAs (shRNAs) targeting PLAC1 gene (PLAC1 shRNA1 and PLAC1 shRNA2) or *PLAC1* cDNA were designed and produced by OriGene Technologies, Inc. shRNA targeting firefly luciferase (shLuc) was used as a negative control for RNA silencing, and empty vector was used as a negative control for cDNA overexpression. HeLa cells at 70-80% confluence were exposed to the lentivirus with *PLAC1* cDNA and empty vector (MOI=10), MS751 cells at 70-80% confluence were exposed to the lentivirus harboring two shRNA (PLAC1 shRNA1 and PLAC1 shRNA2) and shLuc (MOI=10). Subsequently, 1 μg/ml puromycin was used for selection for 10 days. The established cells were then incubated at 37°C and 5% CO_2.

Western blot analysis. The cells were harvested using an enzymatic digestion method, then ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% EDTA and 0.5% NP-40) was added and incubated for 20 min at 4°C. Centrifugation was performed at 13,000 x g at 4°C for 15 min. Total protein concentration was determined using a BCA Protein Quantification kit. Samples (30 μg total proteins) were separated via SDS-PAGE on a 10% gel and subsequently transferred to a PVDF membrane. Membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and incubated with the following primary antibodies overnight at 4°C: PLAC1 (1:500; cat. no. sc-365919; Santa Cruz Biotechnology, Inc.), Slug (1:1,000; cat. no. 9585; Cell Signaling Technology), Snail (1:1,000; cat. no. 3879; Cell Signaling Technology), E-cadherin (1:1,000; cat. no. 3195; Cell Signaling Technology), Vimentin (1:1,000; cat. no. 5741; Cell Signaling Technology) and MMP2 (1:1,000; cat. no. 4022; Cell Signaling Technology) and β-actin (1:10,000; cat. no. MABT825; Sigma-Aldrich; Merck KGaA). After secondary antibody incubation for 1 h at room temperature (both 1:3,000; HRP-conjugated anti-rabbit and anti-mouse; cat. nos. 7074 and 7076, respectively; Cell Signaling Technology, Inc.), protein expression was determined with the Pierce ECL system (Thermo Fisher Scientific, Inc.). The protein band density was determined using ImageJ (version: 1.53; National Institutes of Health).

Wound healing assay. The established HeLa cells and MS751 cells were seeded into 6-well plates at 80-90% confluence and grown to a confluent monolayer overnight, then a scratch was made with a 200 μl pipette tip. DMEM without FBS was used for cell culture at 37°C. The cell migratory ability was evaluated by recording the wound area at 48 h and as percentage of initial wound area at 0 h. The images were visualized under a light microscope. Experiments were performed in triplicate.

Cell migration and invasion assays. The *in vitro* cell migration and invasion assays were conducted using cell culture chambers (24-well, pore size, 8 μm; Corning, Inc.). For the invasion assay, the upper chamber was pre-coated with 60 μl Matrigel (BD Biosciences) at 37°C for 1 h. Cells were collected and resuspended in serum-free medium. Subsequently, cells

$(2x10^4$/well in migration assay or $5x10^4$/well in invasion assay) were plated in the upper chamber. DMEM containing 10% FBS was added to the lower chamber. All samples were incubated for 24 or 36 h at 37°C for the cell migration or cell invasion assay, respectively. In the lower surface of the chamber membrane, migrating or invasive cells were fixed with 4% formaldehyde for 15 min at room temperature and stained with 0.1% crystal violet solution (Sigma-Aldrich; Merck KGaA). All experiments were performed in triplicate, and numbers of migrated or invaded cells in five random fields were visualized under a light microscope.

Ethynyl-2'-deoxyuridine (EdU) cell proliferation assay. Detection of EdU incorporation into the DNA was performed with an EdU Apollo DNA *in vitro* kit (Guangzhou RiboBio Co., Ltd.). According to the manufacturer's instructions, 50 μM EdU was used for incorporation. The established cells were seeded in 96-well plates and incubated overnight at 37°C. The supernatant was removed by aspiration, and the attached cells were fixed with 100 μl fixing buffer (4% polyformaldehyde in PBS) for 30 min at room temperature. Following incubation with 2 mg/ml glycine for 10 min at room temperature, the cells were washed with 1X PBS. The cells were treated with 100 μl/well permeabilization buffer (1X PBS containing 0.5% Triton X-100) for 10 min at room temperature and incubated with 100 μl 1X Apollo solution for 30 min at room temperature in the dark. Subsequently, cells were incubated with 100 μl 1X Hoechst 33342 solution for 30 min at room temperature in the dark. Finally, the cells were observed under a fluorescence microscope.

Colony formation assay. The established HeLa cells and MS751 cells were plated in 6-well plates at the density of 1,000 cells per well and cultured in DMEM supplemented with 10% FBS under the condition of 5% CO_2 at 37°C for 10 days to form colonies consisting of ≥50 cells. Subsequently, 100% methanol was used to fix cells for 30 min at room temperature and 0.1% crystal violet solution was used to stain the cells for 20 min at room temperature. The number of colonies was calculated using ImageJ (version no. 1.53) (20). All experiments were performed in triplicate, and representative results are shown.

Statistical analysis. Statistical analysis was performed using R software (version: 4.0.2) (21). Univariate Cox regression analysis was performed to evaluate survival according to PLAC1 expression and clinicopathological parameters, with median PLAC1 expression used as the boundary. Differences in overall survival between the 'high-PLAC1' and 'low-PLAC1' groups (based on median expression value of PLAC1) were analyzed via the Kaplan-Meier method, with the P-value computed using the 'survival' package in R software (22). Multivariate Cox analysis was performed to evaluate various parameters associated with the hazard ratio, including age, BMI, number of pregnancies, histology, clinical stage and PLAC1 expression using the 'survival' package in R software. In multivariate Cox analysis, PLAC1 expression was converted into a categorical variable based on the median value to interpret relative risk of PLAC1 expression in CC. Unpaired Student's t, χ^2 or Fisher's exact test was performed to compare variables between groups. For multiple comparisons, one-way ANOVA and Tukey's test were used. The Shapiro-Wilk test was performed for the assessment of data normality. Non-normally distributed data were analyzed using Mann-Whitney U test. Normally distributed data were presented as the mean ± SEM and non-normally distributed data are presented as the median with quartile values (Q1, Q3). P<0.05 was considered to indicate a statistically significant difference.

Results

PLAC1 expression and its association with clinicopathological variables. A total of 304 primary CC cases from TCGA database, including both clinical information and RNA-seq data, were analyzed. The median mRNA expression was 37.57 (range, 0-1059.43) for *PLAC1*. The patient characteristics of TCGA cohort are presented in Table I. The median age of the patients with CC was 46 years (age range, 20-88 years). In total, 28% of the patients had a history of smoking. Clinical stages I, II, III and IV comprised 53, 23, 15 and 7% of the cases (297/304), respectively. The pathological types included cervical squamous cell carcinoma, cervical adenocarcinoma, cervical endocervical mucinous adenocarcinoma and cervical adenosquamous carcinoma, accounting for 83%, 10%, 5% and 2% of the cases, respectively. The median follow-up time for surviving patients with CC was 21.0 months (follow-up time range, 0-210.51 months).

The expression levels of PLAC1 in CC tissues and normal cervix tissues were subsequently compared, and the result revealed that PLAC1 was highly expressed in CC tissues (Fig. 1A-E). The expression of PLAC1 was significantly higher in all pathological types of cervical cancer than that in normal cervical tissue. Consistent with this, higher PLAC1 was also observed in cervical squamous cell carcinoma samples. However, the increase of PLAC1 expression was not statistically significance in other pathological types of cervical cancer,including endocervical mucinous adenocarcinoma, cervical adenosquamous carcinoma and cervical adenocarcinoma. According to the median expression levels of PLAC1 (37.57), cases were divided into 'high-PLAC1' and 'low-PLAC1' expression groups. χ^2 or Fisher's exact test results showed that the two groups had statistically significant differences in the following characteristics: Number of pregnancies, number of live births and American Joint Committee on Cancer metastasis pathological score (Table I).

GSEA based on PLAC1 expression. Based on the median expression value of PLAC1, all subjects were split into two groups, 'high-PLAC1' and 'low-PLAC1' expression groups, and GSEA was performed. Enrichment results in the 'high-PLAC1' phenotype group showed that 33/50 gene sets were upregulated, 24 gene sets were significant at a false discovery rate (FDR) <25% and 15 gene sets at FDR <5% (Table II), 14 gene sets were significantly enriched at nominal P-value <1% and 16 gene sets were significantly enriched at nominal P-value <5% (data not shown). The normalized enrichment score of three gene sets was >2.0, including 'mTOR complex (mTORC)1 signaling', 'interferon α response' and 'hypoxia' (Fig. 2A-C). Enrichment results in the
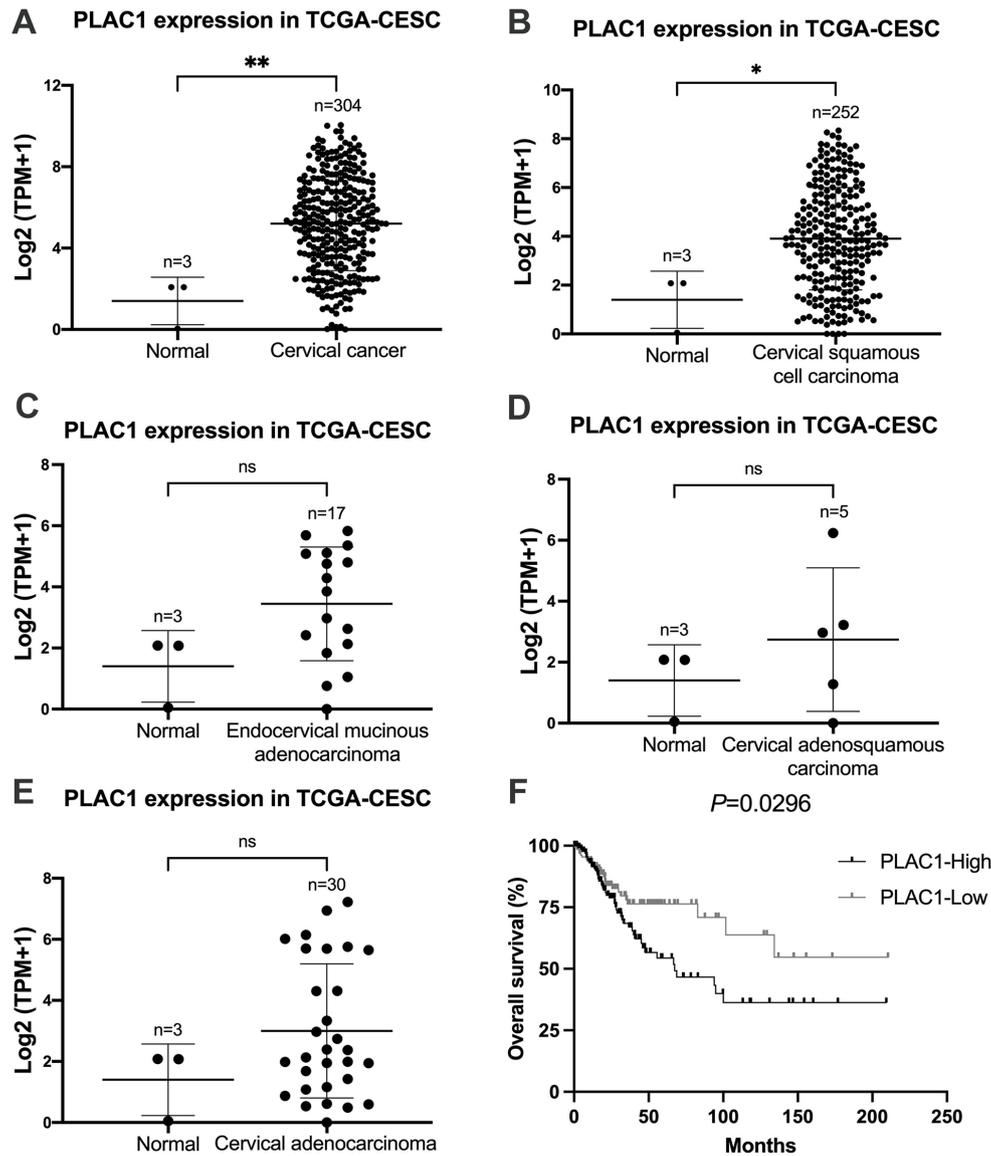
Figure 1. PLAC1 expression is significantly upregulated in CC and high PLAC1 expression is associated with poor survival. (A) PLAC1 expression was upregulated in CC tissues compared with in normal cervical tissues, as revealed using TCGA-CESC dataset. (B) PLAC1 expression was upregulated in cervical squamous cell carcinoma tissues compared with that in normal cervical tissues, as revealed using TCGA-CESC dataset. In other pathological types of CC, (C) endocervical mucinous adenocarcinoma, (D) adenosquamous carcinoma and (E) cervical adenocarcinoma, PLAC1 expression was upregulated, but did not reach a statistically significant level. Data are presented as the mean ± SEM. *P<0.05, **P<0.01. (F) Kaplan-Meier survival analysis showed that CC cases with PLAC1-high expression had a worse prognosis than those with PLAC1-low expression (P=0.0296). CC, cervical cancer; TCGA-CESC, The Cancer Genome Atlas Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma; PLAC1, placenta-specific protein 1; ns, not significant; TPM, transcripts per million.

'low' phenotype showed that 17/50 gene sets were upregulated and no gene sets were significantly enriched at a FDR <25% (data not shown). The heat map of the top 50 features for each phenotype is shown in Fig. 2D.

PLAC1 expression in CC tissues and cell lines. The expression levels of PLAC1 in CC tissues, HSILs and normal exocervix were assessed via IHC analysis (Fig. 3A-F). A tissue microarray, comprising 101 CC tissues, 35 paired or unpaired non-cancer cervical tissues and 18 HSILs, was analyzed. CC samples and HSILs exhibited significantly higher expression levels of PLAC1 compared with those in normal tissues (Fig. 3G and H). Moreover, PLAC1 expression was detected in 100% (20/20) of CC samples, compared with in 15% (3/20) of normal tissue (Data not shown). The staining

intensity of PLAC1 in CC tissues was more intense than that in normal tissues. A similar trend was observed in the HSILs (Data not shown). To the best of our knowledge, PLAC1 is expressed at low levels in normal cervical cells (23). Protein expression levels of PLAC1 in CC lines, including CaSki, MS751, C-33A and HeLa, were notably upregulated (Fig. 4A). Among these, the expression of PLAC1 in MS751 cells was the highest and the expression of PLAC1 in HeLa cells was relatively low. Therefore, MS751 and HeLa cells were selected for further study.

High PLAC1 expression is associated with poor survival in CC. A total of 304 CC cases with PLAC1 expression data and clinical information across all patient characteristics were derived from TCGA. Three of these cases contained

Table I. Patient characteristics of The Cancer Genome Atlas cohort.

Characteristic	Total	PLAC1 expression level Low	High	χ^2	P-value
Age at diagnosis, years				0.000	>0.999
<46	145	72	73		
≥46	159	80	79		
BMI				0.096	0.757
<24	81	42	39		
≥24	178	87	91		
Menopausal status				0.514	0.774
Premenopausal	124	62	62		
Perimenopausal	28	13	15		
Postmenopausal	82	44	38		
Pregnancies count total				5.188	0.023
<5	191	105	86		
≥5	73	28	45		
Pregnancies count live birth				4.760	0.029
<5	220	116	104		
≥5	40	13	27		
Smoking status				0.405	0.524
Yes	86	46	40		
No	218	106	112		
Smoking years				0.000	1.000
<19	43	23	20		
≥19	43	23	20		
Smoking pack years				2.616	0.106
<10	32	13	19		
≥10	54	33	21		
ECOG score				-	0.693[a]
0	79	40	39		
1	83	37	46		
2	9	5	4		
4	1	-	1		
Histology				-	0.250[a]
Cervical squamous cell carcinoma	252	121	131		
Cervical adenocarcinoma	30	20	10		
Cervical endocervical mucinous adenocarcinoma	17	8	9		
Cervical adenosquamous carcinoma	5	3	2		
HPV types positive				-	0.580[a]
HPV16	12	5	7		
HPV18	3	1	2		
Others	7	1	6		
Clinical stage				1.709	0.635
I	161	85	76		
II	69	32	37		
III	45	21	24		
IV	22	13	9		
AJCC tumor pathological score				-	0.553[a]
T_X	17	6	11		
Tis	1	0	1		
T_1	140	72	68		
T_2	71	40	31		
T_3	20	9	11		
T_4	10	6	4		

Table I. Continued.

Characteristic	Total	PLAC1 expression level		χ^2	P-value
		Low	High		
AJCC metastasis pathological score				7.167	0.028
M_X	128	56	72		
M_0	115	67	48		
M_1	11	8	3		
AJCC nodes pathological score				3.764	0.152
N_X	66	29	37		
N_0	133	76	57		
N_1	60	28	32		

[a]Fisher's exact test. ECOG, electrocorticogram; HPV, human papillomavirus; AJCC, American Joint Committee on Cancer; PLAC1, placenta-specific protein 1.
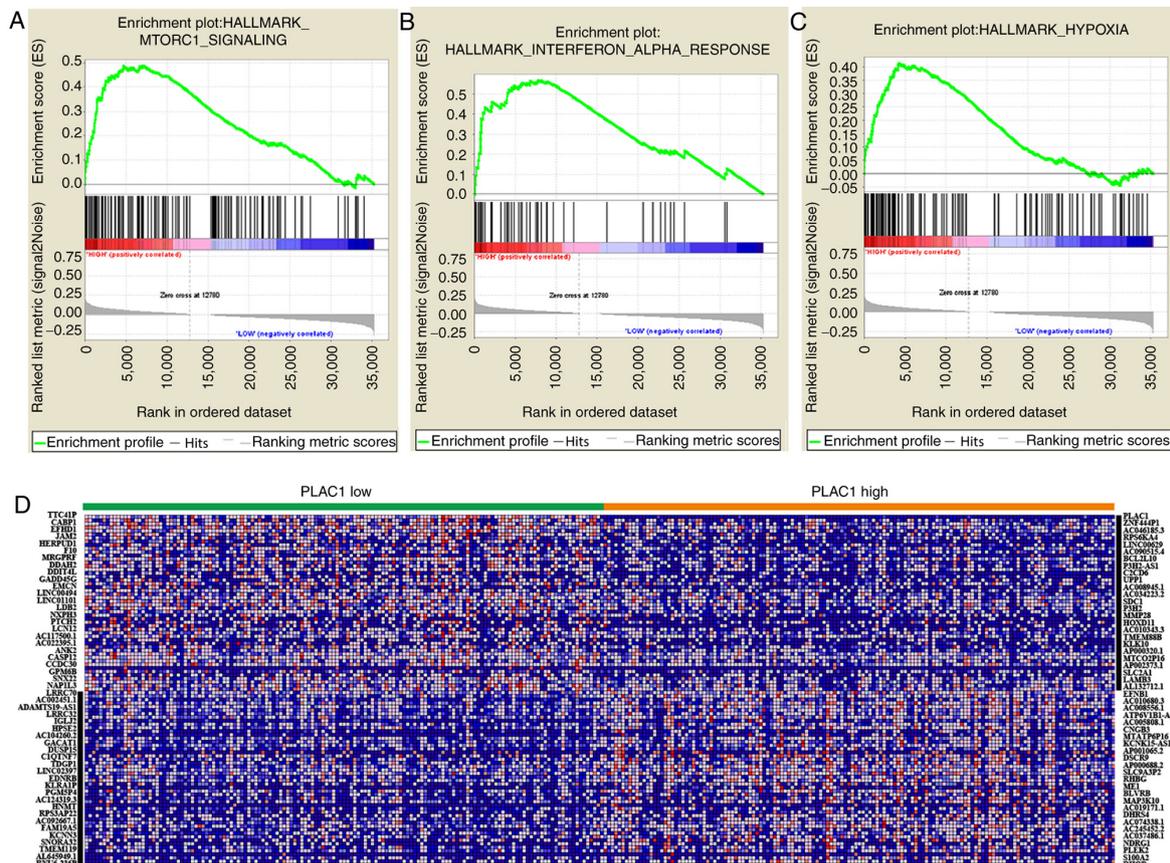
Figure 2. Gene set enrichment analysis. (A) 'mTORC1 signaling', (B) 'interferon α response' and (C) 'hypoxia' were differentially enriched in the 'high-PLAC1' expression phenotype. (D) Heat map of the top 50 features for each phenotype. PLAC1, placenta-specific protein 1; mTORC1, mTOR complex 1.

matched paracancerous samples. A total of three normal cervical samples were excluded from the survival analysis. It was demonstrated that high expression of PLAC1 was a risk factor for the poor prognosis of patients with CC (Fig. 1F). Similarly, the clinical data contained in the tissue microarray also suggested that patients with high expression of PLAC1 had a poor survival (Fig. 3I).

Univariate Cox analysis suggested that four characteristics were associated with prognosis, including BMI, clinical stage, American Joint Committee on Cancer tumor pathological score and PLAC1 expression (as a continuous independent variable) (Table III). Multivariate Cox analysis suggested age (hazard ratio=1.02; P=0.047), clinical stage (hazard ratio=1.68; P<0.001) and PLAC1 expression (hazard ratio=1.92; P=0.032) were associated with the prognosis of patients with CC (Table III). Similarly, PLAC1 expression as a binary variable (according to median expression value of 37.57) was associated with poor prognostic clinicopathological characteristics (Table III). Age,

Table II. Gene sets enriched in 'high-PLAC1' phenotype (FDR<0.05).

Gene set	Size	NES	P-value	FDR	Rank at max
MTORC1 SIGNALING	132	2.485	<0.001	<0.001	7,108
INTERFERON ALPHA RESPONSE	48	2.381	<0.001	<0.001	7,782
HYPOXIA	127	2.130	<0.001	0.001	4,222
MYC TARGETS V1	135	1.993	<0.001	0.002	7,970
E2F TARGETS	128	1.983	<0.001	0.002	8,358
GLYCOLYSIS	130	1.968	<0.001	0.002	5,077
P53 PATHWAY	126	1.949	<0.001	0.002	5,817
TNFA SIGNALING VIA NFKB	121	1.919	<0.001	0.001	7,875
PI3K AKT MTOR SIGNALING	66	1.899	<0.001	0.001	7,224
MYC TARGETS V2	32	1.832	<0.001	0.004	8,037
REACTIVE OXIGEN SPECIES PATHWAY	33	1.800	0.009	0.004	6,849
INTERFERON GAMMA RESPONSE	124	1.783	<0.001	0.005	7,379
APOPTOSIS	100	1.537	<0.001	0.026	7,310
ESTROGEN RESPONSE LATE	117	1.509	<0.001	0.029	4,559
CHOLESTEROL HOMEOSTASIS	49	1.474	0.021	0.038	6,553

FDR, false discovery rate; NES, normalized enrichment score; PLAC1, placenta-specific protein 1
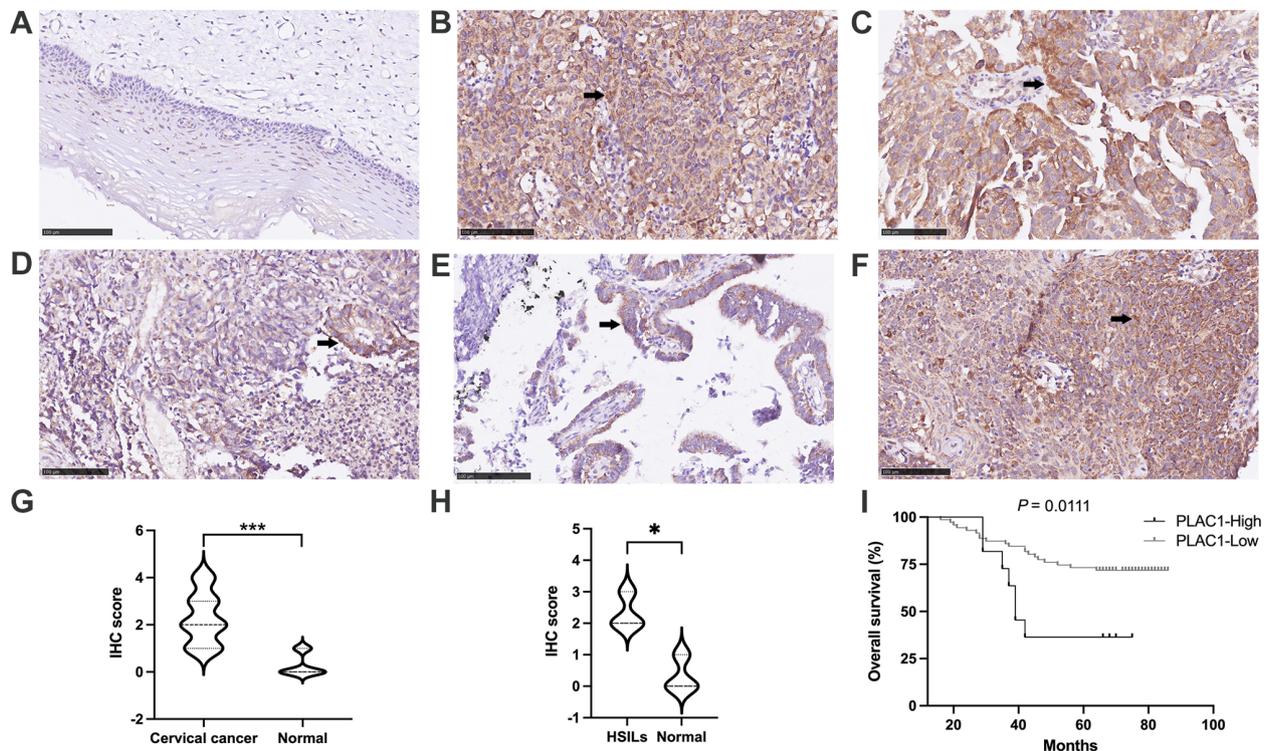
Figure 3. Immunohistochemical evaluation of PLAC1 in (A) normal exocervix, (B) cervical squamous cell carcinoma, (C) cervical adenocarcinoma (D) cervical adenosquamous carcinoma, (E) small cell neuroendocrine cervical cancer and (F) high-grade squamous intraepithelial lesions. Black arrows show PLAC1 expression. IHC score values of PLAC1 were significantly higher in (G) CC and (H) HSILs than those in normal tissues. Bars indicate the median and interquartile range. *P<0.05, ***P<0.001. (I) Patients with high expression of PLAC1 had poor overall survival (P=0.0111). Magnification, x200. PLAC1, placenta-specific protein 1; IHC, immunohistochemistry.

BMI, number of pregnancies, histology, clinical stage and PLAC1 expression were incorporated in the multivariate Cox model. All six variables were consistent with the proportional hazard assumption (P-value of each component was <0.05, and Global Schoenfeld test P>0.05).

PLAC1 promotes cell proliferation, migration and invasion in CC. Since the expression of PLAC1 is associated with metastasis and cell proliferation in CC, the impact of overexpression or knockdown of *PLAC1* on proliferation and invasion in CC cell lines was examined. PLAC1-overexpressing HeLa cells

Table III. Overall survival and associations with clinicopathological characteristics determined using Cox regression analysis.

	Univariate Cox analysis			Multivariate Cox analysis		
Characteristic	HR	95% CI	P-value	HR	95% CI	P-value
Age at diagnosis, years	1.02	1.00-1.03	0.078	1.02	1.00-1.05	0.047
BMI	0.95	0.91-1.00	0.033	0.99	0.94-1.03	0.533
Pregnancies count total	1.03	0.94-1.12	0.594	0.98	0.88-1.09	0.692
Pregnancies count live birth	1.05	0.94-1.17	0.407	-	-	-
Menopausal status	1.08	0.81-1.43	0.612	-	-	-
Smoking years	0.98	0.92-1.05	0.635	-	-	-
Smoking pack years	1.01	0.98-1.04	0.565	-	-	-
ECOG score	1.32	0.76-2.30	0.329	-	-	-
Histology	1.11	0.75-1.64	0.603	1.25	0.81-1.93	0.308
HPV types positive	1.37	0.60-3.12	0.455	-	-	-
Clinical stage	1.47	1.18-1.83	<0.001	1.68	1.27-2.23	<0.001
AJCC tumor pathological score	1.34	1.00-1.78	0.047	-	-	-
AJCC metastasis pathological score	0.77	0.47-1.25	0.295	-	-	-
AJCC nodes pathological score	0.78	0.52-1.17	0.233	-	-	-
PLAC1 expression	1.70	1.05-2.74	0.031[a]	1.92	1.06-3.51	0.032[b]

[a]As a continuous variable. [b]As a categorical variable. ECOG, electrocorticogram; HPV, human papillomavirus; AJCC, American Joint Committee on Cancer; PLAC1, placenta-specific protein 1; HR, hazard ratio; CI, confidence interval.

and PLAC1-silenced MS751 cells with shRNA2 (Fig. 4B) were employed for functional studies. Colony formation and EdU incorporation assays were used to investigate the effect of PLAC1 on cell proliferation. The results demonstrated that overexpression of PLAC1 significantly promoted cell proliferation, whereas PLAC1 silencing inhibited proliferation (Fig. 4C-E). Furthermore, as confirmed by wound healing assay (Fig. 4F and G), the motility of cells was significantly increased in PLAC1-overexpressing group and decreased in PLAC1-silenced group compared with that in control group. Transwell migration and invasion assays were performed to estimate the effect of PLAC1 on cell migration and invasion. The results revealed that overexpression of PLAC1 facilitated cell migration and invasion (Fig. 4H), whereas PLAC1 silencing in MS751 cells suppressed migration and invasion (Fig. 4I). These results suggested that PLAC1 may exert a critical role in regulating cell proliferation and invasion in CC.

PLAC1 enhances invasion via the epithelial-mesenchymal transition (EMT) signaling pathway. Since EMT is an essential process in the pathogenesis of metastasis in tumor cells (24), studies were subsequently conducted to investigate whether EMT was a causative factor for PLAC1-induced changes in cell motility. Compared with control cells, the expression of E-cadherin, one of most common epithelial markers, was decreased in the PLAC1-overexpressing HeLa cells, accompanied by an increase in the expression of the mesenchymal markers, Slug, MMP2 and Vimentin (Fig. 5A and B).

As Slug and Snail are transcriptional regulators that have been substantiated as crucial EMT regulators through inhibiting the epithelial phenotype-related genes and activating

mesenchymal markers (25), we then evaluated whether E-cadherin, Snail and Slug were affected in the MS751 cells infected with PLAC1-shRNA. The results showed that silencing of PLAC1 inhibited the expression of Slug and Snail, but upregulated E-cadherin (Fig. 5C and D), suggesting that decreased expression of PLAC1 may increase the expression of E-cadherin by suppressing Slug and Snail. Mesenchymal marker Vimentin was downregulated in PLAC1-silenced cells. Taken together, these findings indicated that PLAC1 may serve a crucial role in carcinogenesis by activating the EMT-related signaling pathways.

Discussion

The present study analyzed the clinical information and RNA-seq data from 304 patients with CC in TCGA database. Verification studies using IHC confirmed that the expression levels of PLAC1 were significantly increased in CC tissues and HSILs. It was identified that high PLAC1 expression indicated a poor prognosis. Furthermore, multivariate survival analysis revealed that high PLAC1 expression was a poor prognostic parameter for overall survival. Functional studies demonstrated that overexpression of PLAC1 promoted cell proliferation and motility. Collectively, these data indicated that PLAC1 may be a prognostic marker and therapeutic target in CC.

Previous studies examining PLAC1 in other tumor types suggested that higher relative expression of PLAC1 had an association with poor survival (26,27). Inhibition of PLAC1 in breast cancer cells markedly affected motility, migration and invasion, and induced a G_1-S cell cycle block that almost completely suppressed proliferation (10). Additionally, patients with gastric adenocarcinoma exhibiting relatively low
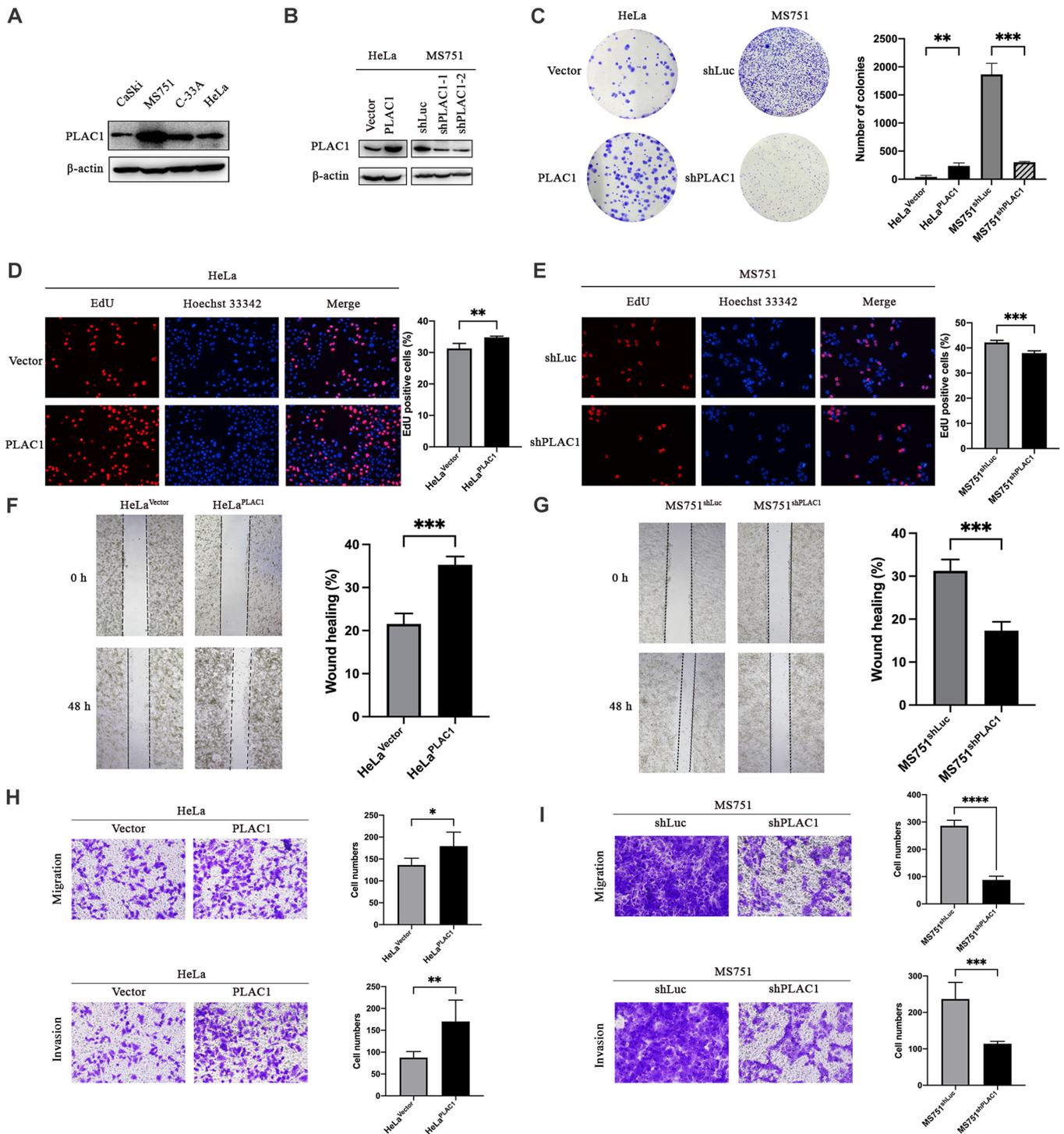
Figure 4. PLAC1 promotes proliferation and motility in CC. (A) Expression of PLAC1 in cervical cancer cell lines CaSki, MS751, C-33A and HeLa. (B) PLAC1 was upregulated in PLAC1-overexpressing HeLa cells and downregulated in PLAC1-silenced MS751 cells. (C) Cell proliferation determined using colony formation assay. (D and E) Cell proliferation measured using EdU incorporation assay. Magnification, x200. (F and G) Cell motility examined using wound healing assay. Magnification, x40. (H and I) Cell motility detected using Transwell assay. Magnification, x200. Data are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. PLAC1, placenta-specific protein 1; sh, short hairpin; EdU, ethynyl-2'-deoxyuridine.

PLAC1 expression had a longer survival time compared with those exhibiting higher PLAC1 expression (26). Furthermore, numerous studies related to hepatocellular carcinoma (11), colorectal cancer (28), ovarian cancer (29), uterine cancer (30) and prostate cancer (31) have reported that PLAC1 expression was positively correlated with clinical parameters, including clinical stage, grade and survival outcome. All these previous reports showed similar results as those obtained in the present study, as high PLAC1 expression levels indicated poor prognosis in CC.

Based on the GSEA, 33 gene sets exhibited significant differences in the 'high-PLAC1' phenotype group; however, no gene set enrichment exhibited significant differences in the 'low-PLAC1' phenotype group. 'mTORC1 signaling',
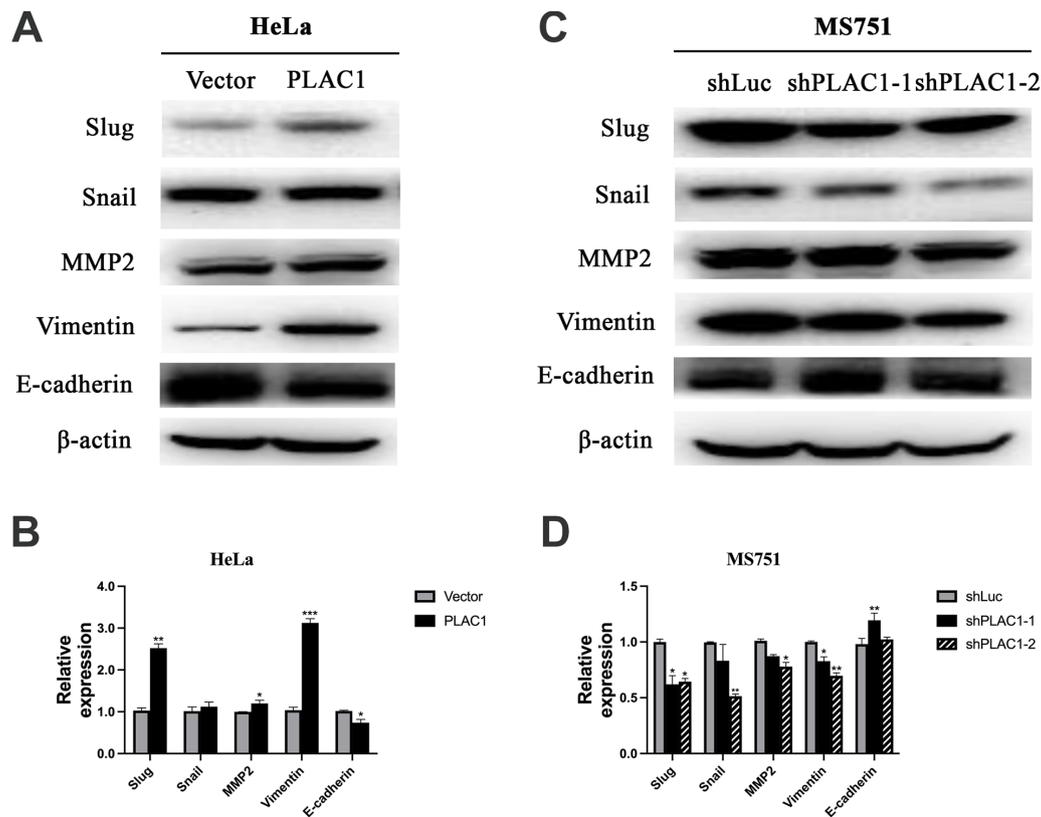
Figure 5. Overexpression of PLAC1 significantly increases the expression of EMT-associated protein and knockdown of PLAC1 significantly decreases the expression of EMT-associated protein. (A and B) Overexpression of PLAC1 in HeLa cells significantly increased the expression of Slug, MMP2 and Vimentin, but decreased the expression of E-cadherin, as detected via western blot analysis. (C and D) Knockdown of PLAC1 in MS751 cells significantly decreased the expression of Slug, Snail, MMP2, and Vimentin but increased the expression of E-cadherin, as detected via western blot analysis. The results were normalized to the expression of β-actin. *P<0.05, **P<0.01, ***P<0.001. PLAC1, placenta-specific protein 1; sh, short hairpin; EMT, epithelial-mesenchymal transition.

'interferon α response' and 'hypoxia' were differentially enriched in the 'high-PLAC1' expression phenotype. mTORC1 is sensitive to rapamycin, and is typically activated by numerous stimuli and essential signaling pathways, such as PI3K, MAPK and AMP-activated protein kinase, to control cell proliferation and survival (32). mTORC1 pathways have been reported be involved in various types of cancer (33). Kremer *et al* (34) revealed that activation of mTOR signaling led to the survival of CC cells. Moreover, microRNA-99 has been reported to inhibit CC cell proliferation and invasion by targeting the mTOR signaling pathway (35), whereas AKT inhibitors may promote cell death in CC by disrupting mTOR signaling and glucose uptake (36). Improving the anti-tumoral interferon α response can be used to treat hematological malignancies and solid tumors in cancer immunotherapy (37,38). Hypoxia, an important regulator in tumor growth (39), has been shown to be associated with poor survival and radiotherapy resistance, and numerous molecules in the hypoxia-response pathway are candidates as therapeutic targets (40,41). Furthermore, Pilch *et al* (42) reported that hypoxia stimulated the expression of tumor angiogenesis factors in CC cells and derived fibroblasts. Therefore, most gene sets enriched in the high expression group have previously been associated with tumor pathogenesis.

To investigate the carcinogenic characteristics of PLAC1 in the tumor process, we overexpressed PLAC1 in HeLa cells and used PLAC1-specific shRNA to knockdown PLAC1 in MS751 cells. Subsequently, the effects of PLAC1 on cell proliferation, migration and invasion were assessed. The present results suggested that cell proliferation was inhibited by PLAC1 silencing in MS751 cells. Furthermore, overexpression of PLAC1 in HeLa cells increased cell proliferation, which indicated a role for PLAC1 in enhancing tumor cell proliferation. Furthermore, the current results indicated that PLAC1 facilitated migration and invasion in tumor cells, as shown by the fact that PLAC1-overexpressing HeLa cells had a stronger cell motility, and PLAC1 silencing inhibited migration and invasion in MS751 cells, as determined using *in vitro* wound healing and Transwell assays.

The present study also investigated whether the changes in tumor cell migration and invasion were due to EMT. EMT is a crucial physiological process affecting the invasion and migration of cancer cells (43). Epithelial cells gain mesenchymal characteristics via dedifferentiation processes that result in the loss of epithelial characteristics. In this process, downregulation of E-cadherin, an epithelial cell adhesion marker, is considered to be an essential step (44). Vimentin, a mesenchymal marker, and the most well-studied inhibitors of E-cadherin, such as Snail, Slug, Twist, ZEB-1 and ZEB-2, which act by binding to E-boxes of E-cadherin promoter and inhibiting its transcription, actively participate in the EMT process (45). In the present study, PLAC1 silencing enhanced E-cadherin expression, along with a noticeable reduction in vimentin and MMP2 expression. Moreover, the expression

levels of Slug and Snail were downregulated, which supported the aforementioned findings. Taken together, these results suggested that PLAC1 served an oncogenic role via EMT, thus promoting metastasis in CC. Moreover, it was confirmed that PLAC1 may be a candidate oncogene for CC. Future studies will be conducted to investigate the underlying molecular mechanisms by which PLAC1 regulates EMT in CC.

In conclusion, the present study demonstrated that PLAC1 was a key regulator for CC progression by promoting cell proliferation, migration and invasion via activation of the EMT process, and high expression of PLAC1 was revealed to be a marker of poor prognosis in patients with CC. RNA-seq data from CC samples, as reported by TCGA, were analyzed; however, matched protein data were not available to support the elevation of PLAC1 transcriptome expression. Therefore, a tissue microarray was used to validate the higher expression levels of PLAC1 in CC tissues compared with in non-tumor tissues. However, the expression levels of PLAC1 were not verified in the serum of patients with CC. Thus, further studies are required to evaluate the prognostic value and feasibility of serum PLAC1 as a predictive marker in a larger, prospective cohort of patients with CC, and to elucidate the specific molecular mechanisms of PLAC1 in CC.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The results shown here are in part based upon data generated by TCGA Research Network: https://www.cancer.gov/tcga

Authors' contributions

LY and YC conceived and designed the project. RM, LZ, CS and RC completed the research work. CS and RC collected data, performed statistical analyses and drafted the manuscript. All authors read and approved the final manuscript. RM and RC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Use of the tissue microarray (HUteS154Su01) in this study has been approved by the Ethics Committee of the Shanghai Outdo Biotech Company (approval no. YB M-05-01). The tissue microarray experiment was completed by Shanghai Outdo Biotech Company.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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