

Expression and hypermethylation of JAM and EPB41L3 in cervical squamous cell carcinoma: Clinical significance and applications

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Summary. This study aimed to explore the expression and hypermethylation of EPB41L3 and JAM3 in cervical squamous cell carcinoma (CSCC) and to investigate their clinical significance. JAM3 and EPB41L3 mRNA expression was analyzed using a public database, and protein expression was detected using immunohistochemistry. The methylation status of JAM3 and EPB41L3 was detected in CSCC tissues and cervical cytological specimens using a quantitative methylation-specific PCR (qMSP). JAM3 and EPB41L3 mRNA were downregulated in CSCC. The JAM3 protein was positively detected in 39.4% of CSCC tissues and frequently expressed in those with lower FIGO stage and no lymph node metastasis. EPB41L3 was expressed in 18.9% of CSCC tissues. The hypermethylation of JAM3 was detected in 52.3% of CSCC tissues and related to higher FIGO stage and lymph node metastasis. EPB41L3 hypermethylation was detected in 72.7% of CSCC tissues and related to older ages and lymph node metastasis. In cervical cytological specimens, no methylation of JAM3 and EPB41L3 was found in normal or inflamed cervical epithelial cells. The methylation of JAM3 was detected in 0%, 8.3%, and 6.3% of ASCUS, LSIL, and HSIL samples, while EPB41L3 was detected in 12.5%, 42.9%, and 71.4%, respectively. The sensitivity of the combination of JAM3 and EPB41L3 methylation detection in ASCUS, LSIL, and HSIL was 8.3%, 15.6%, and 85.7%, respectively. The specificity of the combination of JAM3 and EPB41L3 methylation detection was 100%.

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Downregulation of JAM3 and EPB41L3 by hypermethylation was detected in CSCC. JAM3 and EPB41L3 hypermethylation are potential biomarkers for cervical cancer screening.

Key words: JAM3, EPB41L3, Hypermethylation, Cervical cancer, Screening

Introduction

Cervical cancer is the fourth most common cancer among women globally, which leads to high morbidity and mortality in low- and middle-income countries (Broutet et al., 2022). The incidence and mortality of cervical cancer vary greatly among countries. Estimated new cases in China and the United States were 111,820 and 13,740, and estimated deaths in China and the United States were 61,579 and 5830 in 2020, respectively (Xia et al., 2022). China accounts for approximately one-fifth of the world population, and its cervical cancer burden has a substantial effect on the global estimate of the current and future burden of the disease. Cervical cancer is the fifth most common cancer among women in China ranking after breast, lung, thyroid, and colorectal cancer (Dong et al., 2020; Zhao et al., 2020; Wang et al., 2022; Xia et al., 2022).

Cervical squamous cell carcinoma (CSCC) accounts for 80~90% of cervical cancers and most CSCCs are associated with high-risk human papillomaviruses (HR-HPVs) (Lu et al., 2023). The oncogenesis of human papillomaviruses in cervical cancer has been investigated for more than 30 years. Twelve high-risk HPV strains (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) have been classified by the World Health Organization (WHO) as oncogenic in CSCC (Liang et al., 2021; Wang et al., 2022). Though 90~95% of CSCCs are associated with HPV infection, a category of CSCC



with HPV-negative evidence has been found. The 5th edition of the WHO Classification of Female Genital Tumors afforded that HPV-independent CSCC accounts for 5~7% of CSCCs. Multiple factors contribute to the development of CSCC. The progression of high-grade lesions to CSCC requires the accumulation of additional, not yet completely understood, epigenetic and genetic alterations, a process that may take 20-30 years. Hypermethylation of a CpG island in the promoter region of tumor suppressor genes has been accepted as a molecular event change from a High-grade squamous intraepithelial lesion (HSIL) towards cervical cancer (Huang et al., 2020; Tawe et al., 2021; Zhao et al., 2021; Zhang et al., 2022; Shi et al., 2023). More than 70% of HPV-associated CSCCs exhibit genomic alterations in PI3K/MAPK and/or TGF-beta signaling pathways (Zhang et al., 2019; Zheng et al., 2021; Yang et al., 2022; Zhong et al., 2023). A better understanding of the pathogenesis of CSCC is critical for the development of preventive and therapeutic approaches to CSCC.

In the United States, the incidence and mortality of cervical cancer have been declining due to the wide-scale implementation of cytological screening programs (Buskwofie et al., 2020). The Papanicolaou test has been used in cytological screening programs for cervical cancer since the 1970s in the United States. Molecular testing for HPV DNA has been approved by the US Food and Drug Administration (FDA) as an adjunct to cytology or as a concomitant test (Liang et al., 2021; Nieminen et al., 2004). At present, various tests can be used as screening methods to prevent cervical cancer in women. The screening of cervical cancer includes three approaches: the cytological method (Liquid-based cytology, and Dual staining of p16 and Ki-67), visual inspection (naked eye, and magnified by colposcope or camera), and molecular test (high-risk HPV DNA/mRNA, DNA methylation, protein biomarkers). Hypermethylation of CpG islands in the promoter regions of tumor suppressor genes appears to be important for malignant progression and has been recognized as a molecular change from HSIL towards cervical cancer, as this molecular feature is common in HSILs but rarely seen in Low-grade Squamous Intraepithelial Lesions (LSIL). Increasing research on DNA methylation detection has been used in cervical cancer diagnosis and screening (Bhat et al., 2021; Li et al., 2021; Zhang et al., 2021; Banila et al., 2022).

Junctional adhesion molecules (JAMs) include members JAM1, JAM2, JAM3, JAM4, ESAM, and CAR, belonging to a subfamily that directly affects tight junction functions in epithelial cells. JAM3, also known as JAM-C, is selectively expressed in a variety of human tissues. The expression and role of JAM3 in human cancer were contradictory in published papers. For example, JAM3 mRNA was significantly elevated in human renal cancer cells compared with renal tubular epithelial cells. The knockdown of JAM3 in renal cancer cells leads to the inhibition of renal cancer cell migration and promotes its apoptosis (Li et al., 2018). JAM3 was

downregulated in colorectal cancer compared with normal mucosa. The depletion of JAM3 by siRNA in a colon cancer cell line promoted the clonogenicity and migration capability of cancer cells and suppressed the apoptosis and cell-cycle arrest of cancer cells (Zhou et al., 2019). Erythrocyte membrane protein band 4.1-like 3 (EPB41L3) is a member of the protein 4.1 family, a skeletal protein that is extensively expressed in various human tissues (Jiang and Newsham, 2006). EPB41L3 has been reported as a tumor suppressor in prostate, gastric, ovarian, and colorectal cancer (Bernkopf and Williams, 2008; Dafou et al., 2010; Li et al., 2011; He and Shu, 2020; Mo et al., 2020; Son et al., 2020). Hypermethylation of EPB41L3 was associated with oropharyngeal cancer and oral squamous cell carcinoma (Khongsti et al., 2019; Giuliano et al., 2020; Dickey et al., 2022). A few studies on JAM3 and EPB41L3 have been reported in cervical cancer (Brentnall et al., 2015; Huisman et al., 2015; Yin et al., 2015; Kelly et al., 2018; Hou et al., 2020; Gu et al., 2022), however, detailed expression, methylation status of JAM3 and EPB41L3, and their clinical significance in cervical cancer are not well investigated.

Materials and methods

Bioinformatics databases

The expression levels of JAM3 and EPB41L3 mRNA in cervical cancer and normal cervical mucosa were analyzed using the public database GEPIA (Gene Expression Profiling Interactive Analysis). The relationship between the disease-free survival (DFS) of patients and different expression levels of JAM3 and EPB41L3; the low and high methylation status of JAM3 and EPB41L3 were analyzed using GEO (Gene Expression Omnibus) and MEXPRESS (<https://mexpress.be/>) databases.

Clinical specimens

One hundred and thirty-two formalin-fixed and paraffin-embedded tissue blocks (FFPE) of CSCCs surgically resected at the Women's Hospital of Nanjing Medical University, Nanjing Maternity and Child Health Care Hospital, between January 2020 and December 2022, were studied. The median age of the patients was 50 years, with a range of 30 to 73 years. Liquid-based cytology samples include 30 normal (or with inflammation), 12 ASCUS (Atypical Squamous Cells of Undetermined Significance), 32 LSIL, and 7 HSIL, which were collected from the Pathology Department, Nanjing Maternity and Child Health Care Hospital in 2022. The median age of the patients was 46 years, with a range of 23 to 70 years. The 5th edition of the World Health Organization Classification of Female Genital Tumors was used for pathological evaluation of cervical cancer. Hematoxylin and eosin (H&E) staining sections were examined to confirm CSCC. The international

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Bethesda classification for cytology (2014) was used for pathological evaluation of Liquid-based cytology samples. Histological features of all specimens were confirmed by two pathologists (Dr. B Yang and JD Wang). This study was approved by the ethics committee of the Women's Hospital of Nanjing Medical University, Nanjing Maternity and Child Health Care Hospital, China.

Immunohistochemistry

FFPE tissues were sectioned at 4- μ m thicknesses and then processed for immunohistochemical staining as we described previously (Liu et al., 2022a). Briefly, sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked by incubation with 0.3 % H₂O₂ for 10 min at room temperature. Antigen retrieval was performed by autoclaving the sections in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min. The sections were incubated at 4°C overnight with a polyclonal anti-EPB41L3 antibody (Abcam, Discovery Drive, Cambridge, CB20AX, UK) and a polyclonal anti-JAM3 antibody (Invitrogen, Carlsbad, CA, USA) diluted at 1:500. After washing, the sections were incubated with a secondary antibody (Dako REAL EnVision Detection System; Dako, Denmark) for 30 min at room temperature. Finally, the color was developed with 3,3'-diaminobenzidine (DAB), and the nuclei were lightly counterstained with hematoxylin. The stained slides were evaluated independently by two pathologists and any differences were resolved by discussion. Immunohistochemical staining of cancer cells was assessed according to the staining intensity of positive cells. EPB41L3 and JAM3 expression were assessed for intensity as follows: 0=no staining, 1=weak staining, 2=moderate staining, and 3=strong staining. Scores of 0 and 1 were defined as negative, while scores of 2 and 3 were defined as positive staining.

Hypermethylation detection

The detection of JAM3 and EPB41L3 hypermethylation was carried out as we reported previously as follows (Liu et al., 2022b). For FFPE samples, 6 μ m thick sections were cut from paraffin blocks by using a microtome with disposable blades. Five sections were placed into 1.5 mL microcentrifuge tubes for DNA extraction. Deparaffinization of FFPE tissues was performed by adding 1 mL xylene to the sample and vortexing. Centrifuge at room temperature, then add 1 mL ethanol (95-100%) to the pellet, and mix by vortexing. Centrifuge at room temperature. Air-dry the pellet at room temperature to remove all residual ethanol. For Liquid-based cytology samples, centrifuge at room temperature and then digest. DNA extraction was performed using the QIAmp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Resuspend the pellet in

Buffer ATL, add 20 μ l proteinase K and mix by vortexing. Incubate at 56°C until the sample has been completely lysed, then at 90°C for 1 h. The final elution volume for each sample was 30 μ l. A NanoDrop spectrophotometer was used to quantify DNA concentration and quality.

qMSP (quantitative Methylation Specific PCR)

Bisulfite modification of genomic DNA was performed using the EZ DNA Methylation-Gold Kit (Catalog No. D5005, ZYMO Research Corp. 17062 Murphy Ave. Irvine, CA92614, USA) according to the manufacturer's instructions. Hypermethylation analysis of EPB41L3 and JAM3 was subjected to MethyLight technology (qMSP), which is a sensitive, fluorescence-based real-time PCR technique. The NCBI Reference Sequence of JAM3 is NC_000011.10. The following primers were used: JAM3-qMSP-F: 5'-GGACGTTTCG GTAGTTGGATC-3', JAM3-qMSP-R: 5'-CTAAAAA AAACCCACCCGAA-3', JAM3-qMSP-Probe: Rox-5'-AAACCCCGCCCGAAAAAAACCC-3'-BHQ2. PCR product length is 164 bp. The EPB41L3 reference sequence is NC_000018.10. The following primers were used: EPB41L3-qMSP-F: 5'-GAATTTAAGTCG GATTAGGAGGTC-3'; EPB41L3-qMSP-R: 5'-ATAC TATACGCTACAACGACGACG-3'; EPB41L3-qMSP-Probe: FAM-5'-ACTCCGACACGAACGCCCGC-3'-BHQ1. PCR product length is 140 bp. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as an internal control for unmethylated human DNA and bisulfite modification. The NCBI Reference Sequence for GAPDH is NC_000012.12. The following primers were used: GAPDH-qMSP-F: 5'-TGGATATTGTTGT TATTAATGATT-3'; GAPDH-qMSP-R: 5'-AAATATAA AAAA ACTACCCATCAACC-3'; GAPDH-qMSP-Probe: VIC-5'-TCCTCCACACCAACTTTAAACT CACCA-3'-BHQ1; PCR product length is 124 bp.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS version 13.0 for Windows, IBM, Armonk, NY, USA). The relationship between hypermethylation of JAM3 and EPB41L3 and clinicopathological parameters in cervical cancer was analyzed by Spearman's rank correlation test. A Kaplan-Meier curve associated with the log-rank test was used for survival analysis. Statistical significance was set at $P < 0.05$.

Results

Downregulation of JAM3 and EPB41L3 in CSCC

The mRNA expression levels of JAM3 and EPB41L3 in cervical mucosa and cervical cancer were analyzed using the public database GEPIA. There were 306 cervical cancer specimens and 13 normal cervical

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mucosa specimens involved in this study. A significantly higher expression of JAM3 and EPB41L3 mRNA was found in normal mucosa compared with cervical cancer ($P<0.05$, Fig. 1).

The expression of JAM3 and EPB41L3 protein was checked using immunohistochemistry. JAM3 and EPB41L3 proteins were located in the cytoplasm of normal cervical mucosa cells or cervical cancer cells (Fig. 2). The positive expression of JAM3 and EPB41L3 protein was detected in all normal cervical mucosa cells. JAM3 protein was positively detected in 52 (30 cases with staining score 2+ and 22 with 3+) out of 132 cervical cancer specimens (39.4%), negatively detected in 80 (23 cases with staining score 1+ and 57 with 0) out of 132 cervical cancer specimens (60.6%). EPB41L3 protein was positively detected in 25 (10 cases with staining score 2+ and 15 with 3+) out of 132 cervical cancer specimens (18.9%), negatively detected in 107 (12 cases with staining score 1+ and 95 with 0) out of 132 cervical cancer specimens (81.1%). The relationship between the expression of JAM3 and EPB41L3 protein and clinicopathological parameters was statistically analyzed in Table 1. Positive expression of JAM3 was associated with lower FIGO stage and no lymph node metastasis. No significance was found with EPB41L3 protein expression.

Hypermethylation of JAM3 and EPB41L3

Methylated JAM3 (69/132, 52.3%) and EPB41L3 (81/132, 61.4%) DNAs were detected in CSCC specimens. The relationship between the methylation status of JAM3 and EPB41L3 and clinicopathological parameters of CSCC was analyzed in Table 1. Methylated JAM3 DNA was frequently detected in higher FIGO stages and greater lymph node metastasis. Methylation of EPB41L3 was detected in older patients and more lymph node metastasis.

The methylation status of a CpG island in the promoter region of EPB41L3 and JAM3 was checked in cervical cytological specimens, including normal or inflamed cervical epithelial cells, ASCUS, LSIL, and HSIL. As shown in Table 2, no methylation of either

EPB41L3 or JAM3 was found in normal mucosa or inflamed cervical epithelial cells. The methylation of JAM3 was detected in 0/12 (0%), 2/32 (6.3%), and 3/7 (42.9%) of ASCUS, LSIL, and HSIL, respectively. The methylation of EPB41L3 was detected in 1/12 (8.3%), 4/32 (12.5%), and 5/7 (71.4%) of ASCUS, LSIL, and HSIL, respectively.

Survival analysis

The DFS of cervical cancer patients with different mRNA expression and methylation status of a CpG island in the promoter region of EPB41L3 and JAM3 was analyzed. As shown in Figure 3, high expression of JAM3 was associated with longer DFS of cervical cancer patients ($P<0.05$). No significant relationship was found between EPB41L3 expression and DFS of cervical cancer patients. No significant relationship was found between the methylation status of EPB41L3 and

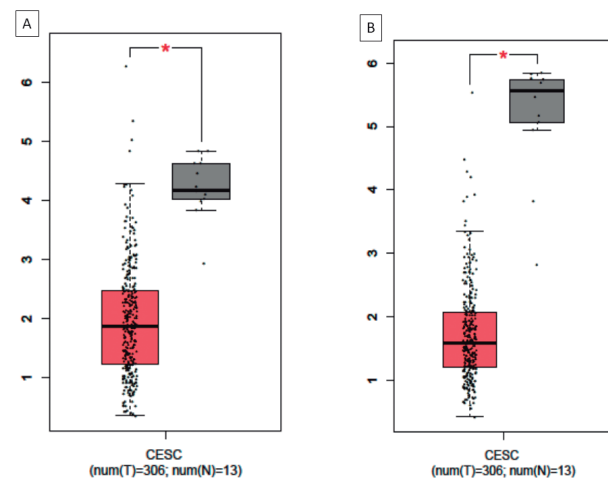


Fig. 1. Analysis of JAM3 and EPB41L3 mRNA expression in a public database. **A.** JAM3 mRNA was downregulated in cervical cancer tissues ($P<0.05$). **B.** EPB41L3 mRNA was downregulated in cervical cancer tissues ($P<0.05$).

Table 1. Association of expression and hypermethylation of JAM3 and EPH41L3 with clinicopathological parameters in CSCC.

Parameter	No.	JAM3 protein		P value	JAM3 methylation		P value	EPB41L3 protein		P value	EPB41L3 methylation		P value
		-	+		M	U		-	+		M	U	
Age (years)													
<50	57	34	23	0.859	27	30	0.380	47	10	0.824	45	12	<0.001
≥50	75	46	29		42	33		60	15		36	39	
FIGO stage													
I+II	111	62	49	0.014	52	58	0.008	87	24	0.124	69	42	0.807
III+IV	21	18	3		17	4		20	1		12	9	
Lymph node metastasis													
no	117	67	50	0.047	57	60	0.028	93	24	0.301	68	49	0.027
yes	15	13	2		12	3		14	1		13	2	

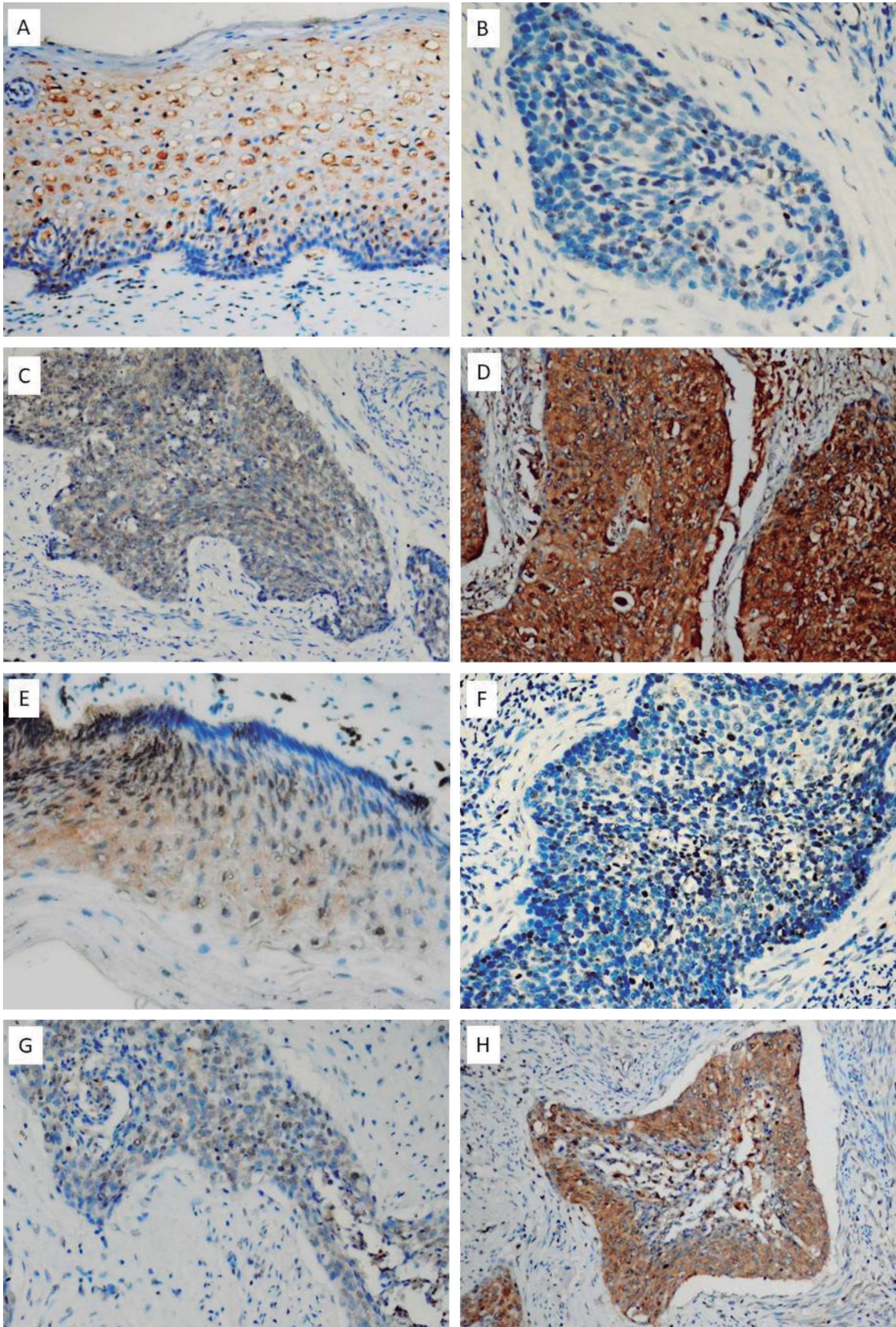


Fig. 2. Expression of JAM3 and EPB41L3 in cervical normal mucosa and cancers was detected using IHC. **A.** positive expression of JAM3 in cervical normal mucosa. **B.** negative expression of JAM3 in cervical cancer. **C.** weak expression of JAM3 in cervical cancer. **D.** strong expression of JAM3 in cervical cancer. **E.** positive expression of EPB41L3 in cervical normal mucosa. **F.** negative expression of EPB41L3 in cervical cancer. **G.** weak expression of EPB41L3 in cervical cancer. **H.** strong expression of EPB41L3 in cervical cancer. x 400.

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JAM3 and DFS of cervical cancer patients.

Discussion

Though JAM3 and EPB41L3 have been reported to be involved in different cancers, their expression and clinicopathological significance in CSCC are still not well studied. In this study, we explored JAM3 and EPB41L3 mRNA and protein expression in CSCC and found that both are downregulated. The relationship between JAM3 and EPB41L3 protein expression and clinicopathological parameters was statistically analyzed. JAM3 expression is related to FIGO stage and lymph node metastasis. We detected hypermethylation of JAM3 and EPB41L3 in CSCC, which is a major mechanism leading to the downregulation of these genes in CSCC. Next, we assessed JAM3 and EPB41L3

hypermethylation in cervical cellular samples and found that the combination of JAM3 and EPB41L3 could reach a high sensitivity for HSIL detection.

Despite the conventional Pap smear drastically decreasing overall cervical cancer incidence and mortality rates in high-income countries, screening with cytology is resource-intensive and prone to poor reproducibility with a wide-ranging sensitivity of 43% to 96% (Liang et al., 2021). The low sensitivity of cytology in detecting cervical cancer and HSIL in various settings has led to the evaluation of alternative screening approaches. In China, cytology-based screening is not the first choice due to the large population, associated complicated technology and infrastructure for cytology testing, and geographical and socioeconomic inequities. Molecular testing for HPV DNA is an alternative cervical cancer screening method accepted in Europe.

Table 2. Hypermethylation of JAM3 and EPB41L3 in normal, ASCUS, LSIL, and HSIL cervical cell specimens.

	No	JAM3		EPB41L3		JAM3 and/or EPB41L3	JAM3 and EPB41L3
		methylation	no methylation	methylation	no methylation	methylation	no methylation
Normal	30	0 (0.0%)	30 (100.0%)	0 (0.0%)	30 (100.0%)	0 (0.0%)	30 (100.0%)
ASCUS	12	0 (0.0%)	12 (100.0%)	1 (8.3%)	11 (91.7%)	1 (8.3%)	11 (91.7%)
LSIL	32	2 (6.3%)	30 (93.7%)	4 (12.5%)	28 (87.5%)	5 (15.6%)	27 (84.4%)
HSIL	7	3 (42.9%)	4 (57.1%)	5 (71.4%)	2 (28.6%)	6 (85.7%)	1 (14.3%)

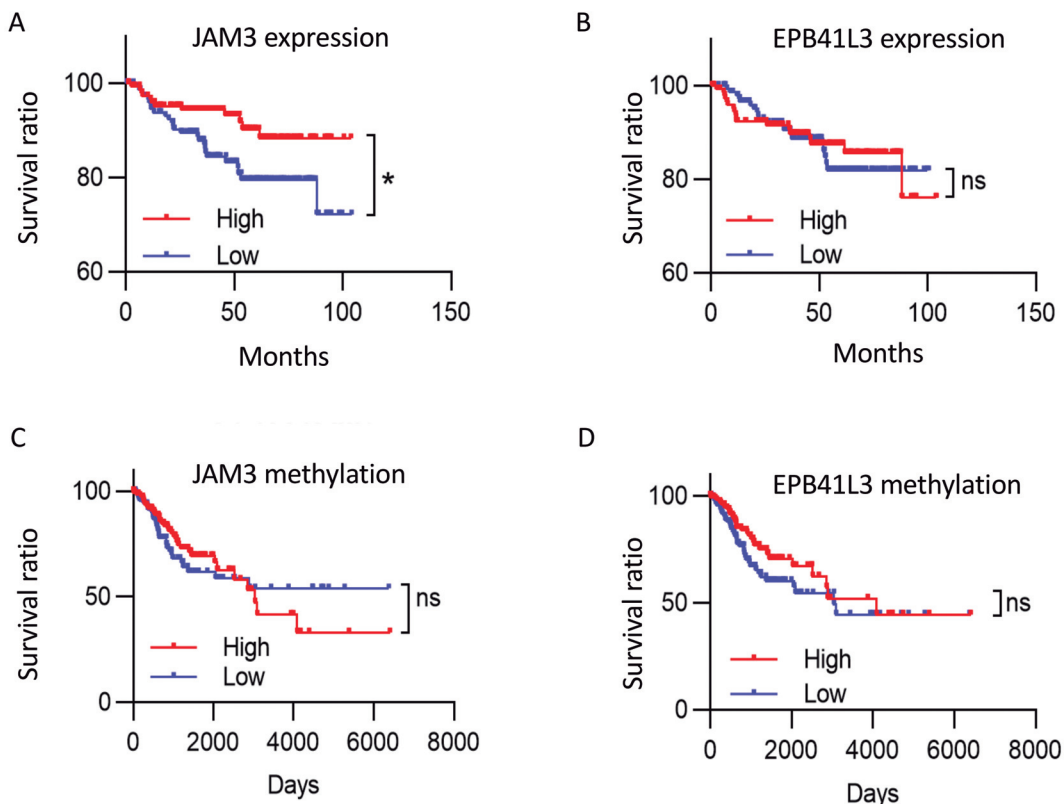


Fig. 3. Survival analysis in cervical cancer patients. High expression of JAM3 was associated with longer disease-free survival (DFS) of cervical cancer patients ($P < 0.05$) (A). No significant relationship was found between EPB41L3 expression and DFS of cervical cancer patients (B). No significant relationship was found between the methylation status of JAM3 (C) and EPB41L3 (D) and DFS of cervical cancer patients.

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The HPV DNA test has greater reproducibility and high throughput benefits. In the USA, this test has been approved as an adjunct to cytology (reflex testing) or as a concomitant test (co-test). There are several concerns about stand-alone HPV testing: lowered specificity, safety of extended screening intervals, testing in women under 30 years of age, and observations of HPV test-negative carcinomas. The high incidence of transient HPV infections affords the HPV test limited specificity, especially among young women. Several screening tests are under evaluation; they contain DNA methylation, protein biomarkers, and automated visual evaluation of digital images (Hu et al., 2021). DNA hypermethylation of a CpG island in the promoter region of a tumor suppressor gene is an epigenetic event that can be involved in the early phase of carcinogenesis. DNA methylation occurs at each stage of cervical cancer. The accumulation of DNA methylation of tumor suppressor genes can promote the progression of high-grade cervical intraepithelial neoplasia (CIN) to invasive cervical cancer (Yin et al., 2015). Several DNA methylation-based epigenetic markers including hypermethylation of tumor suppressor genes and HPV genes have been tested. The DNA methylation of CADM1, MAL, FAM19A4, and has-miR124-2 combined in a panel may reach 70% sensitivity for the detection of high-grade CIN with high specificity (Salta et al., 2021). DNA methylation-based cervical screening is a promising triage strategy due to its good performance, enabling fully automated molecular testing, and the possibility of promoting self-collection strategies which might increase screening compliance. Our data suggest that combining EPB41L3 and JAM3 hypermethylation is a potential biomarker for cervical cancer screening.

In summary, EPB41L3 and JAM3 are down-regulated in CSCC. Hypermethylation of EPB41L3 and JAM3 was detected in cervical intraepithelial neoplasia, especially in HSIL, but not in normal cervical mucosa and inflammation, which is a potential biomarker for cervical cancer screening.

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Conflict of interest statement. The authors declare that there are no conflicts of interest.

Ethics approval statement. This study was approved by the ethics committee of the Women's Hospital of Nanjing Medical University, Nanjing Maternity and Child Health Care Hospital, China.

Data availability statement. The data and material are available on reasonable request.

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