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Original Paper

Promoter Methylation of Ezrin and its Impact on the Incidence and Prognosis of Cervical Cancer

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Key Words
Cervical cancer • Ezrin gene • Methylation • Incidence • Prognosis

Abstract
Background/Aims: Aberrant localization and over-expression of Ezrin have been reported to be implicated in cervical cancer (CC). Aberrant promoter methylation of some gene families may serve as potential diagnostic biomarkers for CC. In this study, we explored the correlation of promoter methylation of the Ezrin gene with the incidence and prognosis of CC. ***Methods:*** Cervical tissues from a total of 483 patients with CC were collected from the China-Japan Union Hospital of Jilin University. Samples were assigned into four groups accordingly to pathological diagnosis, namely the control group, the cervical intraepithelial neoplasia (CIN) I group, the CIN II-III group and the CC group. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed to detect the mRNA expression of Ezrin. Methylation-specific polymerase chain reaction (MSP) was used to detect the promoter methylation of the Ezrin gene. The Kaplan-Meier product-limit method and the log-rank analysis were used for survival analysis, the Cox regression analysis for the prognostic factors for CC, and the logistic regression analysis for the risk factors for the occurrence of CC. ***Results:*** The methylation rate of the Ezrin gene was correspondingly increased from the control, the CIN I, the CIN II-III to the CC groups. Over-expressed mRNA of Ezrin was determined in CC tissues. The mRNA expression of Ezrin was correlated with tumor size, lymphatic metastasis, pathological grade and clinical stage (FIGO). The risk factors for the occurrence of CC were the number of abortions and the promoter methylation of the Ezrin gene. Poor prognosis of CC correlated to lymphatic metastasis, higher pathological grade, higher FIGO stage and positive Ezrin promoter methylation. ***Conclusion:*** These findings indicate that promoter methylation of the Ezrin gene may play a crucial role in carcinogenesis, progression and prognosis of CC.

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Introduction

Cervical cancer (CC) is one of the most common female tumors worldwide associated with poverty, race and health disparities [1]. Invasive CC is a major reason for female cancer death, leading to about 300, 000 deaths each year [2]. Human papillomavirus (HPV) infection is regarded as a main reason for CC and the most often detected types in CC are HPV16 (especially for squamous cell carcinoma) and HPV18 (especially for adenocarcinoma) [3]. However, only a small percentage of women infected with HPV develop CC. Other factors such as water-based metalworking fluids (MWF) may also increase the occurrence of CC [4]. Besides, cytokine gene polymorphisms have been related to the development of CC [5]. A previous study showed that nuclear factor-kappaB (NF-κB), a tumor promoter and a prognostic indicator, was associated with poor prognosis of CC [6].

Recently, the Ezrin gene has been found to aberrantly express in CC and been considered as an indicator of prognosis in early-stage CC [7]. As the first member of the Ezrin-Radixin-Moesin (ERM) family, Ezrin is up-regulated in many tumors including pancreatic carcinoma, hepatocellular carcinoma, gastric cancer and breast cancer [8, 9]. Ezrin is regarded as a scaffold protein which contributes to oncogenesis by linking cytoskeletal and membrane proteins [10]. Expression change of Ezrin is an independent prognostic factor in osteosarcoma [11]. DNA methylation is usually considered as a silencing epigenetic marker in diseases like cancer [12]. For example, the methylation of Dishevelled Binding Antagonist of Beta Catenin (DACT) 1and DACT 2 was associated with the development of esophageal squamous cell carcinoma (ESCC) and was regarded as prognostic biomarkers of ESCC [13]. Methylation of DNA repair gene O-6-methylguanine-DNA methyltransferase (MGMT) promoter region has been correlated with better prognosis of glioblastoma multiforme [14]. Ezrin can promote cellular movement and motility as well as controlling cellular growth, whereby contributing to tumorigenesis, invasion, and metastasis [15]. Ezrin was previously found to be over-expressed in CC and its expression was actively involved in metastasis and poor prognosis [16]. Therefore, in the present study, we further investigate the correlation of promoter methylation of the Ezrin gene with the risk and prognosis of CC.

Materials and Methods

Ethical statement

The study protocol was approved by the Committee on the Ethics of China-Japan Union Hospital of Jilin University (No. 2009-01-15-18) and informed consent was obtained from each participant.

Study subjects and grouping

Cervical tissue specimens were collected from patients in Department of Gynecology and Obstetrics in the China-Japan Union Hospital of Jilin University between February 2010 and January 2013. Tissues from a total of 483 case were collected and were assigned into four groups, namely 137 cases in the control group (mean age, 49.63 ± 5.26 years), 117 cases in the cervical intraepithelial neoplasia (CIN) I group (mean age, 48.54 ± 5.11 years), 121 cases in the CIN II-III group (mean age, 48.11 ± 4.50 years) and 108 cases in the CC group (mean age, 49.72 ± 5.25 years).

Inclusion/exclusion criteria and sample collection

Sample collection strictly adhered to the inclusion criteria and the exclusion criteria. The CIN I, the CIN II-III and the CC groups included patients: (1) with complete clinical and pathological data; (2) with definite pathological results of CIN or CC; (3) who did not receive preoperative chemotherapy or radiotherapy. Patients were excluded from the CIN I, the CIN II-III and the CC groups if: (1) received cervical treatment within 3 months before operation, including vaginal medication, cryotherapy, and laser treatment; (2) were pregnant; (3) diagnosed with other gynecological malignancies, such as endometrial cancer, ovarian cancer and uterine sarcoma; and (4) diagnosed with other tumors, such as gastric cancer, colorectal cancer and breast cancer. The control group comprised cases: (1) who received total hysterectomy for benign uterine

lesions; and (2) with no atypical squamous cells or cancer cells detected by Thinprep cytologic test (TCT). The exclusion criteria of the control group followed those of the CIN I, the CIN II-III and the CC groups. Collected samples were snap-frozen in Trizol and preserved at -80°C.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Frozen tissues were taken out from -80°C (100 mg/sample) and grinded according to the instructions for the Luna® Universal One-Step RT-qPCR Kit (New England Biolabs, Inc, US). The primers were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The reaction conditions for Ezrin were pre-denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 67°C for 45 s and renaturation at 72°C for 45 s; and extension at 72°C for 10 min. The samples were stored at -20°C. The reaction conditions for β-actin were pre-denaturation at 94°C for 5 min; 27 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 45 s and renaturation at 72°C for 45 s; and extension at 72°C for 10 min. The IS 8900 gel imaging scanner was used to determine the gray values of paired samples of Ezrin and β-actin. The gray value ratio of the Ezrin and β-actin bands was considered as the mRNA expression of Ezrin. A higher ratio indicated higher mRNA expression of Ezrin.

Methylation-specific polymerase chain reaction (MSP)

The DNA in cervical tissues was extracted by phenol/chloroform extraction method and the qualified sample was stored at -80°C. According to the sequence of the Ezrin gene (Genebank Locus EF184645), the promoter is located in 1064bp-1459bp. Based on the requirements of RT-qPCR primers in literature, the methylated (M) and unmethylated (U) primers of the Ezrin gene were designed by the Premier Primer 5.0 software (Premier, Canada). The primer sequences (Table 1) were synthetized by a commercial supplier (Beijing SBS Genetech Co., Ltd., Beijing, China). DNA modified by the methylation kit (Methylation-gold kitDNA, ZYMO-RESEARCH, US) was selected as the template DNA. The methylated and unmethylated primers were used to amplify the template DNA. The PCR amplification system (50 μL) included 5 μL of 10 × PCR buffer treated with sodium bisulfite, 4 μL of deoxy-ribonucleoside triphosphates (dNTPs), 0.5 μL of TaqDNA polymerase, 2.5 μL of upstream primer, 2.5 μL of downstream primer and 35.5 μL of water. The methylated and unmethylated primers were used for PCR amplification. The reaction conditions for methylated primers were as follows: pre-denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 45 s, renaturation at 72°C for 45 s; extension at 72°C for 7 min and finally stored at 4°C. Amplified products of a volume of 5 μL were used for electrophoresis on 2% agarose gel at 75 V for 20 min. The results were observed and recorded by the UV transilluminator and the band of 153 bp was considered as positive band. The reaction conditions for unmethylated primers were as follows: pre-denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 45 s, and renaturation at 72°C for 45 s; extension at 72°C for 7min and finally stored at 4°C. Amplified products of a volume of 5 μL were used for electrophoresis on 2% agarose gel at 75 V for 20 min. The results were observed and recorded by the UV transilluminator and the band of 153 bp was considered as positive band.

Follow-up

Follow-ups ended on December 29, 2016. If patients were still alive by the end of follow-up, censored data were collected for analysis. Follow-ups were conducted via invited outpatient visits, telephone interviews or referring to medical records. The survival time of patients was recorded by using the overall survival (OS) for prognosis.

Statistics

Data were presented as mean values ± standard deviations (SD). The Statistical Program for Social Sciences (SPSS) 20.0 software (SPSS, IBM, West Grove, PA, USA) was used for data analysis. The one-way analysis of variance (ANOVA) and the Kruskal-Wallis test were used to compare values among groups followed by the Student's t-test or the Mann-Whitney U-test to compare values between groups. The Tukey-

Table 1. Primer sequences for MSP and RT-qPCR. Note: MSP, methylation specific polymerase chain reaction; RT-qPCR, reverse transcription quantitative polymerase chain reaction

Primer	Sequence
Ezrin-M	5'-CTGGC AGCCC CGGGA AGTT-3' 5'-CCAGG ACAGC CAGCG CGAG-3'
Ezrin-U	5'-TTGGT AGTTT TGGGA AGTT-3' 5'-CCAAAACAAC CAACA CAAA-3'
Ezrin (upstream)	5'-GATGATGCGCGAGAAGGAGGAGTT-3'
Ezrin (downstream)	5'-GGGGCGGGGGTGCTGTCAT-3'
β-actin (upstream)	5'-GGCTACAGCTTCACCACCAC-3'
β-actin (downstream)	5'-CGGACTCGTCATACTCCT-3'

Kramer test or the Steel test was also used depending on the distribution of data. The Pearson correlation analysis was carried out for the analysis of the correlation of two variable quantities. The Kaplan-Meier product-limit method was used for survival analysis. Comparison of survival time was conducted by the Log-rank analysis. The Cox regression analysis was used to analyze the prognostic factors. All tests were two-tailed, with the significance level set to $P < 0.05$.

Results

Age of menarche, menstrual cycle and the number of abortions correlate to the occurrence of CC

Baseline clinical data of the four groups were compared to assess the risk factors of CC. Significant differences in the age of menarche, menstrual cycle and the number of abortions were seen among the control, the CIN I, the CIN II-III and the CC groups (all $p < 0.05$) (Table 2). These results demonstrated that CC correlated with age of menarche, menstrual cycle and the number of abortions. Insignificant differences in age, marital history, smoking and drinking habits, menstrual period and fertility were noted among the control, the CIN I, the CIN II-III and the CC groups (all $p > 0.05$).

Over-expressed Ezrin mRNA is associated with the development of CC

RT-qPCR was applied to determine the mRNA expression of Ezrin. mRNA expression of Ezrin was different among the control, the CIN I, the CIN II-III and the CC groups. The mRNA expression of Ezrin in the CC group was significantly higher than that in the control, the CIN I and the CIN II-III groups (all $p < 0.05$) (Fig. 1). The mRNA expression of Ezrin was not associated with age, pathological type and focus type of the patients (all $p > 0.05$). However, the mRNA

Table 2. Clinical data of the control, CIN I, CIN II-III and CC groups. The one-way analysis of variance (ANOVA) was used to compare values among groups. Note: CIN, cervical intraepithelial neoplasia; CC, cervical cancer

Characteristics	control	CIN I	CIN II-III	CC	p values
Case	137	117	121	108	
Age (years)	49.6 ± 5.3	48.54 ± 5.1	48.1 ± 4.5	49.7 ± 5.3	0.352
Married (years)	23.2 ± 1.9	22.9 ± 1.8	23.1 ± 2.2	23.3 ± 1.7	0.433
Smoking (n, %)	10 (7.3)	5 (4.3)	11 (9.1)	9 (8.3)	0.505
Drinking (n, %)	22 (16.1)	16 (13.7)	16 (13.2)	17 (15.7)	0.906
Age of menarche (years)					
< 14 (n, %)	42 (30.7)	42 (35.9)	50 (41.3)	53 (49.1)	0.025
≥ 14 (n, %)	95 (69.3)	75 (64.1)	71 (58.7)	55 (50.9)	
Period (days)					
< 5 (n, %)	9 (6.6)	5 (4.3)	10 (8.3)	8 (7.4)	
≤ 5 < 7(n, %)	108 (78.8)	102 (87.2)	102 (84.3)	91 (84.3)	0.382
≥ 7 (n, %)	20 (14.6)	10 (8.5)	9 (7.4)	9 (8.3)	
Menstrual cycle (days)					
< 26 (n, %)	12 (8.8)	5 (4.3)	6 (5.0)	3 (2.8)	
≤ 26 < 30 (n, %)	96 (70.1)	75 (64.1)	92 (76.0)	87 (80.6)	0.032
≥ 30 (n, %)	29 (21.2)	37 (31.6)	23 (19.0)	18 (16.7)	
Abortion (time)					
< 1 (n, %)	32 (23.4)	28 (23.9)	31 (25.6)	12 (11.1)	
1-2 (n, %)	82 (59.9)	68 (58.1)	56 (46.3)	55 (50.9)	0.001
≥ 3 (n, %)	23 (16.8)	21 (17.9)	34 (28.1)	41 (38.0)	
Children (NO.)					
≤ 1 (n, %)	59 (43.1)	51 (43.6)	62 (51.2)	41 (38.0)	0.238
≥ 2 (n, %)	78 (56.9)	66 (56.4)	59 (48.8)	67 (62.0)	

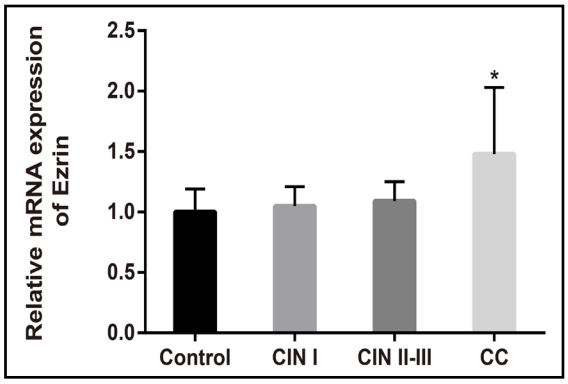

Fig. 1. Increased Ezrin mRNA expression is observed in CC tissues. RT-qPCR was applied to determine the mRNA expression of Ezrin. The mRNA expression of Ezrin in the CC group was significantly higher than that in the control group but not than that in the CIN I and the CIN II-III groups. *, p<0.05, compared with the control group; CIN, cervical intraepithelial neoplasia; CC, cervical cancer; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

expression of Ezrin was correlated with lymphatic metastasis, pathological grade, clinical stage (FIGO) and tumor size (all $p < 0.05$) (Table 3). These findings indicated that over-expression of Ezrin mRNA might be involved in the development of CC.

Table 3. Relationship between mRNA expression of Ezrin and clinical pathological features of patients with CC. The one-way analysis of variance (ANOVA) was used to compare values among groups followed by the Student's t-test or the Mann-Whitney U-test to compare values between groups. Note: SD, standard deviation; CC, cervical cancer

Clinical parameters	Items	n	mean ± SD	p values
Age	< 50	53	1.56 ± 0.53	0.142
	≥ 50	55	1.40 ± 0.57	
Lymphatic metastasis	No	60	1.38 ± 0.55	0.041
	Yes	48	1.60 ± 0.53	
Pathological type	Squamous cancer	57	1.53 ± 0.53	0.334
	Glandular cancer + adenosquamous cancer	51	1.43 ± 0.58	
Pathological grade	High	17	1.15 ± 0.51	0.008
	Moderately	42	1.45 ± 0.59	
	Poorly	49	1.62 ± 0.48	
FIGO stage	I b stage	43	1.34 ± 0.58	0.022
	II a stage	35	1.49 ± 0.52	
	II b stage	30	1.67 ± 0.50	
Tumor size	≤ 4 cm	66	1.39 ± 0.58	0.044
	> 4 cm	42	1.61 ± 0.49	
Focus type	Exogenous	22	1.54 ± 0.40	0.239
	Endogenous	30	1.34 ± 0.69	
	Ulcerative	27	1.43 ± 0.50	
	Rigid tube	29	1.62 ± 0.51	

Promoter methylation of the Ezrin gene may contribute to the development of CC

To assess whether Ezrin promoter methylation is related to CC, PCR products of the control, the CIN I, the CIN II-III and the CC groups were detected by MSP. Our results showed that the M primers were negative and the U primers were positive in the control group, indicating that methylation was absent in normal tissues. In the CC group, the M primers were positive and the U primers were negative, showing that methylation occurred in the CC group (Fig. 2A). In the CIN I and the CIN II-III groups, the M and the U primers were both positive, indicating that partial methylation occurred in both groups (Fig. 2B). The methylation proportions were 0, 18%, 29% and 86%, respectively in the control, the CIN I, the CIN II-III and the CC groups, and the difference was statistically significant (all $p < 0.05$). Significant difference of the methylation proportions was seen among the CIN I, the CIN II-III and the CC groups (Control <CIN I < CIN II and III < CC for amplified products of methylated primers, and Control > CIN I > CIN II and III > CC for amplified products of unmethylated primers, all $p < 0.05$) (Table 4). Thus, methylation of the

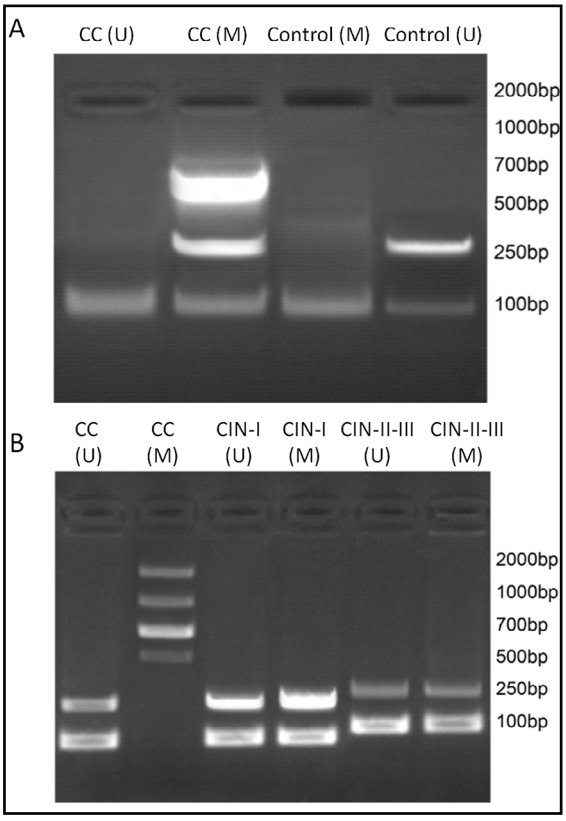

Fig. 2. Ezrin promoter methylation correlates to the occurrence of CC. The M primers are negative and the U primers were positive in the control group while the M primers are positive and U primers are negative in the CC group (2A). The M and the U primers are both positive in the CIN I and the CIN II-III groups (2B). M, amplified products of methylated primers; U, amplified products of unmethylated primers; CIN, cervical intraepithelial neoplasia; CC, cervical cancer.

Ezrin promoter is correlated with the development and progression of CC.

Independent risk factors for CC includes the number of abortions and Ezrin promoter methylation

The logistic regression analysis was conducted to assess the risk factors for the occurrence of CC. The input method was used for the binary logistic regression analysis with CC as the dependent variable and the age of menarche, menstrual cycle, the number of abortions and the Ezrin promoter methylation as independent variables. We showed that the number of abortions and Ezrin promoter methylation were independent risk factors for CC (all $p < 0.05$) (Table 5). Thus, the occurrence of CC has two risk factors, including the number of abortions and the Ezrin promoter methylation.

Table 4. The methylation rates of Ezrin gene among the normal, CIN I, CIN II-III and CC groups. The one-way analysis of variance (ANOVA) was used to compare values among groups. Note: M, amplified products of methylated primers; U, amplified products of unmethylated primers

Gene	normal (n = 137)	CIN I (n = 117)	CIN II-III (n = 121)	CC (n = 108)	p values
Ezrin					
M n, (%)	0 (0.0%)	21 (17.9%)	35 (28.9%)	65 (60.2%)	< 0.001
U n, (%)	137 (100%)	96 (82.1%)	86 (71.1%)	43 (39.8%)	

Table 5. Logistic regression analysis for the risk factors of CC. The Cox regression analysis was used to analyze the prognostic factors of CC. Note: CI, confidence interval; Exp, exposed; B, regression coefficient; S.E., standard error; CC, cervical cancer

Factors	B	SE	Wald	p	95.0% CI for Exp (B)
Ezrin methylation	5.015	0.498	101.267	≤ 0.001	150.696 (56.739-400.243)
The number of abortions	2.672	0.42	40.446	≤ 0.001	14.463 (6.349-32.949)
Menstrual cycle	-0.157	0.326	0.231	0.631	0.855 (0.452-1.619)
Age of menarche	-1.189	0.608	3.822	0.051	0.305 (0.092-1.003)

Poor survival of CC is associated with higher FIGO stage, larger tumor size, lymphatic metastasis and Ezrin promoter methylation

To evaluate the survival of CC, the log-rank analysis was used to compare survival rate among the control, the CIN I, the CIN II-III and the CC groups. The median survival time of all patients with CC was 27 months. The survival rate was 63.64% in 3 years. Significant differences were found in the survival rates of patients with different FIGO stage, Ezrin methylation, tumor size and lymphatic metastasis (all $p < 0.05$) (Fig. 3). The survival rate decreased with higher FIGO stage, larger tumor size, lymphatic metastasis or Ezrin promoter methylation. These results suggest that higher FIGO stage, larger tumor size lymphatic metastasis and Ezrin promoter methylation are associated with poor survival of CC. No significant differences in survival rate were seen among patients with different age, pathological grade, pathological type and focus type (all $p > 0.05$).

Lymphatic metastasis, pathological grade, FIGO stage and Ezrin promoter methylation associated with poor prognosis of CC

The Cox regression model was used for multivariate analysis of risk factors for prognosis of CC. The tumor size, lymphatic metastasis, pathological grade, FIGO stage, and methylation of Ezrin gene were included for analysis of the Cox regression model. We found that tumor size ($p > 0.05$) was not an independent prognostic factor for patients with CC while lymphatic metastasis ($p = 0.033$), pathological grade ($p = 0.045$), FIGO stage ($p = 0.034$) and Ezrin methylation ($p = 0.006$) were independent prognostic factors (Table 5). Therefore, poor prognosis of CC correlates to lymphatic metastasis, pathological grade, FIGO stage and Ezrin promoter methylation (Table 6).

Discussion

In the present study, we explored the correlation of promoter methylation of the Ezrin gene with the risk and prognosis of CC. We found that CC correlated with age of menarche, menstrual cycle and the number of abortions, while not with age, marital history, smoking and drinking habits, menstrual period and fertility. We also found that the mRNA expression and

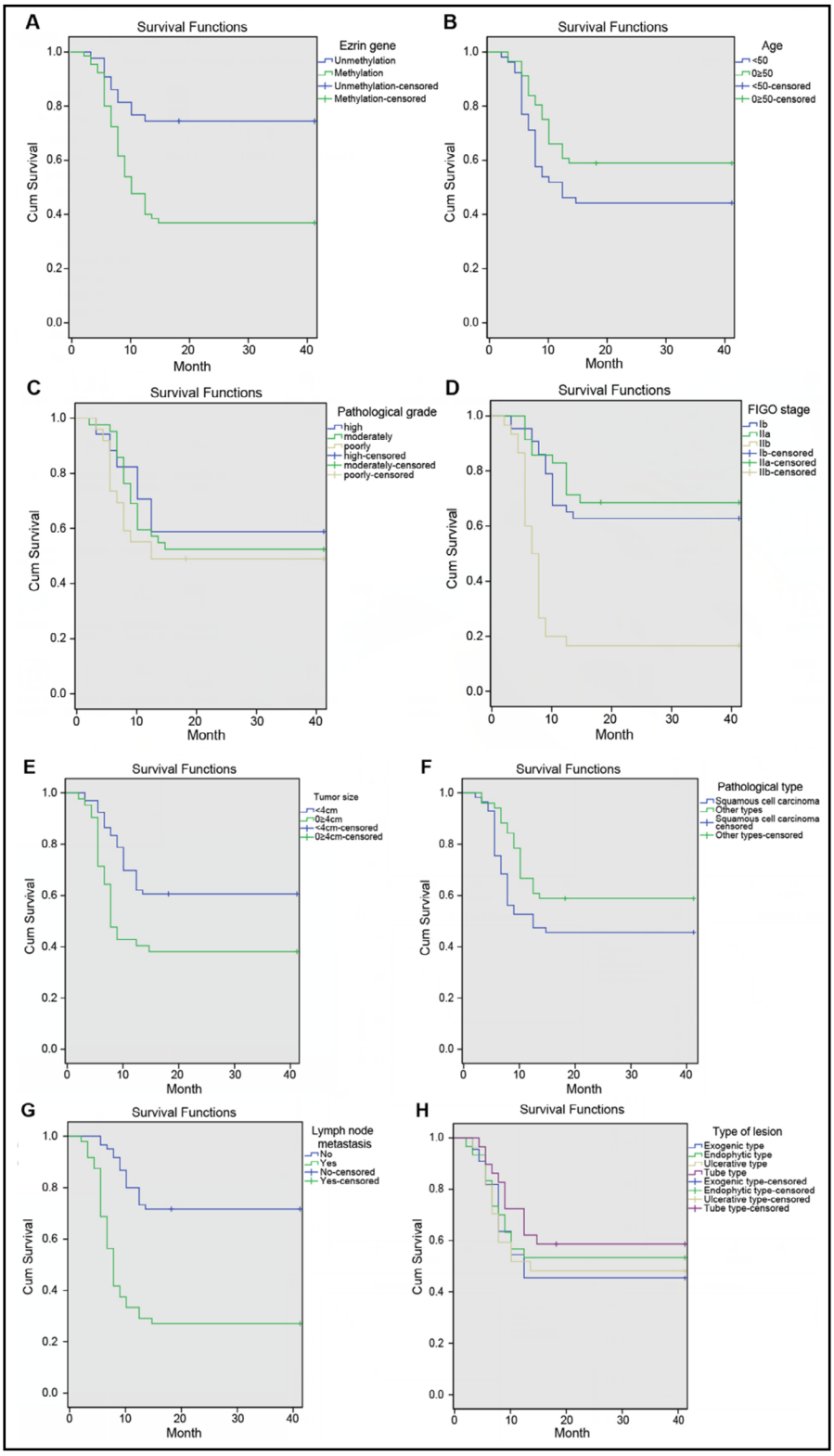

Fig. 3. Single factors of 3-year survival rate of CC. Ezrin methylation is negatively correlated with 3-year survival rate of CC (3A); age is not correlated with 3-year survival rate of CC (3B); pathological grade is not correlated with 3-year survival rate of CC (3C); FIGO stage is negatively correlated with 3-year survival rate of CC (3D); tumor size is negatively correlated with 3-year survival rate of CC (3E); pathological type is not correlated with 3-year survival rate of CC (3F); lymphatic metastasis is correlated with lower 3-year survival rate of CC (3G); focus type is not correlated with 3-year survival rate of CC (3H). CC, cervical cancer.

the promoter methylation rate of the Ezrin gene were up-regulated in CC and were related to the risk and prognosis of CC. Our findings suggest that promoter methylation of the Ezrin gene may play a crucial role in carcinogenesis, development and progression of CC.

Table 6. Cox regression analysis of prognostic factors for patients with CC. The Cox regression analysis was used to analyze the prognostic factors. Note: RC, regression coefficient; S.E., standard error; CI, confidence interval; CC, cervical cancer

Factors	RC	SE	Wald	p	95.0% CI for Exp (B)
Lymphatic metastasis	0.778	0.364	4.556	0.033	2.177 (1.066-4.446)
Pathological grade	0.544	0.271	4.027	0.045	1.723 (1.013-2.932)
FIGO stage	0.658	0.311	4.484	0.034	1.931 (1.050-3.551)
Tumor size	0.814	0.460	3.129	0.077	0.443 (0.180-1.092)
Ezrin methylation	0.983	0.358	7.566	0.006	2.673 (1.327-5.388)

CC leads to hundreds of thousands of women deaths worldwide each year [17]. The prognosis of advanced and recurrent CC is still under poor control [18]. Despite first-line therapeutic strategies for CC including radiotherapy, chemotherapy and surgery, the 5-year survival rate for patients with advanced CC is far from satisfactory [19]. DNA methylation has been associated with genetic loss of functional mutations [20]. DNA methylation is also one of the most frequent molecular alterations in human cancers like colorectal cancer [21]. For example, distinctive patterns of CpG island hypermethylation are correlated with the prognosis of CC [20]. We therefore focused on the promoter methylation of the Ezrin gene in our study.

One of our main findings was that mRNA expression of the Ezrin gene were up-regulated in CC tissues. Results from a previous study indicated the correlation of promoter methylation of the p16, the DAPK, the CDH1, and the TIMP-3 genes with the histologic type and stage of CC [22]. Moreover, another study showed that promoter methylation of SFRPs gene family was correlated with the development of CC, which could be regarded as molecular biomarker for the screening of CC [23]. The Ezrin gene was up-regulated in many tumors such as pancreatic carcinoma, hepatocellular carcinoma, gastric cancer and breast cancer, and the up-regulation of the Ezrin gene might enhance the metastatic phenotype of tumors [8, 9]. Since the Ezrin gene takes part in cell migration and cell recognition through the immune system, it may exert an direct effect on tumor progression [24]. Consistent with our results, Ezrin expression was reported to be upregulated in CC and cervical intraepithelial neoplasia tissues [7, 25]. We revealed that the mRNA expression of the Ezrin gene was markedly increased in the CC group. As a membrane-cytoskeleton crosslinking protein and tumor promoter, expression of Ezrin is known to be positively correlated with degree of malignancy in many tumors, and to be a risk factor for these cancers including breast cancer and endometrium cancer [16]. Moreover, the Ezrin gene plays a crucial role in the metastasis of tumors [26]. Ezrin also played a crucial role in the growth of cancer cells. As previously demonstrated, mRNA expression of the Ezrin gene is obviously increased in the osteosarcoma (OS) cell lines, which may be associated with the rapid proliferation of OS cells [27]. Moreover, the expression of Ezrin was significantly increased in lung cancer, indicative of its involvement in controlling the biological behavior of lung cancer [28]. Since Ezrin expression levels were closely related to metastatic tendency in various cancers, Ezrin over-expression indicated a poor prognosis of myxofibrosarcomas, which may provide a potential value in the prediction of tumor aggressiveness [29]. As compared with the late stage patients with CC and lymph node metastasis-positive patients, the equivalent early stage patients had significantly reduced expressions of Ezrin mRNA and protein [30].

Another main findings of our study was that the promoter methylation of the Ezrin gene was markedly increased in CC. Importantly, no promoter methylation of the Ezrin gene was noted at all in the control group. Interestingly, methylation of CpG islands within gene promoter regions could result in silencing of gene expression and methylation of tumor-relevant genes occurred in numerous cancers [31]. For example, methylation of the ESR1 promoter was related to higher tumor grading of patients with CC [32]. CpG promoter methylation could result in GPX3 (a possible tumor suppressor) downregulation in CC [33]. The methylation of APC1A promoter was also closely correlated with biological features in

CC and could therefore be used to predict poor prognosis of CC [34]. DNA methylation could result in carcinogenesis through silencing important tumor suppressor genes and aberrant methylation of tumor suppressor genes could be regarded as a prognostic and predictive biomarker for cancer [35]. An increase in the promotor methylation of Ezrin in CC cases would normally cause a decrease in gene transcription. However, SET and MYND domain-containing protein 3 (SMYD3), a methyltransferase, regulates transcription of EZR and LOXL2 by directly binding to the sequences of the promoter regions of these target genes in esophageal squamous cell carcinoma [36].

We also showed that FIGO stage, pathological grade, lymphatic metastasis, and promoter methylation of the Ezrin gene were independent risk factors for the prognosis of CC. Since invasion of the uterine body was related to nodal metastasis, lymphatic metastasis was an important prognostic factor for the relapse and survival of patients with invasive CC [37]. Patients without lymphatic metastasis had a decreased risk of recurrence [38], and thus lymphatic status may serve as a significant prognostic factor in patients with CC [39]. The FIGO stage has been widely used for staging CC because the recurrence rate and prognosis were directly correlated with the degree of tumor spread at the initial stageand it was considered as an important prognostic factor for the prediction of recurrence and long-term outcomes [40].

Our study has several limitations. Firstly, due to the relatively small sample size and short duration of follow-ups, our results lacked sufficient statistical power to assess the exact roles of Ezrin promoter methylation in the development and progression of CC. Secondly, because of the limitation of insufficient data, we failed to explore the correlation of Ezrin promoter methylation with other clinicopathological features, such as tumor grade and TNM stage. Lastly, the follows-up were conducted via invited outpatient visits, telephone interviews or referring to medical records. As a consequence, data collection might be insufficient due to the short follow-up period and the variable follow-up approaches. Therefore, further studies with a larger sample size and longer follow-ups are warranted for a more comprehensive understanding of promoter methylation of Ezrin in CC.

Conclusion

Our study provided evidence that patients with CC had increased mRNA expression and promoter methylation rate of the Ezrin gene; the Ezrin promoter methylation was associated with the risk and the prognosis of CC. Further studies are needed to elucidate the underlying mechanisms.

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Disclosure Statement

The authors declare to have no competing interests.

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