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Association of *C8orf4* expression with its methylation status, aberrant β-catenin expression, and the development of cervical squamous cell carcinoma

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Abstract

Chromosome 8 open reading frame 4 (C8orf4) is an activator of Wnt signaling pathway, and participates in the tumorigenesis and progression of many tumors. The expression levels of C8orf4 and β-catenin were assessed via immunohistochemical staining in 100 cervical squamous cell carcinoma (CSCC) tissues, 50 high-grade squamous intraepithelial lesions (HSILs), 50 low-grade squamous intraepithelial lesions (LSILs), and 50 normal cervical tissues. Bisulfite sequencing polymerase chain reaction analysis was used to examine the methylation status of the *C8orf4* locus in CSCC and normal cervical tissues. The expression rates of C8orf4 and β-catenin were significantly higher in CSCCs or HSILs than in LSILs or normal cervical tissues ($P < .05$). *C8orf4* expression was positively correlated with the poor differentiation of CSCCs ($P = .009$), and with aberrant expression of β-catenin in CSCCs ($P = .002$) and squamous intraepithelial lesions ($P < .001$). The methylation rate of *C8orf4* in CSCCs was significantly lower than that in normal cervical tissues ($P = .001$). The Cancer Genome Atlas genomics data also confirmed that the mRNA expression of *C8orf4* was positively associated with the copy number alteration of *C8orf4* (correlation coefficient = 0.213, $P < .001$), and negatively correlated with the methylation level of *C8orf4* (correlation coefficient = −0.408, $P < .001$). In conclusion, the expressions of C8orf4 and β-catenin were synergistically increased in CSCCs and HSILs and higher than those in LSILs and normal cervical tissues. The methylation level of *C8orf4* is decreased in CSCCs and is responsible for the increased expression of *C8orf4*.

Abbreviations: BSP = bisulfite sequencing PCR, C8orf4 = chromosome 8 open reading frame 4, CSCC = cervical squamous cell carcinoma, FIGO = the International Federation of Gynecology and Obstetrics, HSIL = high-grade squamous intraepithelial lesion, LSIL = low-grade squamous intraepithelial lesion, PCR = polymerase chain reaction, SIL = squamous intraepithelial lesions, TCF/LEF = T-cell factor/lymphoid enhancer factor, TCGA = The Cancer Genome Atlas.

Keywords: β-catenin, chromosome 8 open reading frame 4, cervical cancer, Wnt

1. Introduction

Cervical cancer is the fourth leading cause of cancer-related death among women worldwide. More than 560,000 new cases of cervical cancer were detected, and more than 310,000 people died of cervical cancer in 2018.[1] Cervical squamous intraepithelial lesions (SIL) are a group of precancerous lesions closely associated to cervical cancer, reflecting the continuous process of the occurrence and development of cervical cancer. Many signaling pathways participate in this process and promote the transformation of cervical cancer. Wnt signaling pathway is one of the key pathways involved in cervical cancer transformation. The aberrant activation of Wnt signaling contributes to tumor initiation, progression, invasion, and therapeutic resistance of cervical cancer.[2]

Chromosome 8 open reading frame 4 (C8orf4, also named thyroid cancer 1) is a positive regulator of the Wnt signaling pathway.[3] C8orf4 interacts with Chibby via its transient helical structure, and, in turn, releases β-catenin from Chibby.[3–5] Free β-catenin then forms a complex with transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family, leading to the activation of Wnt target genes.[6] Besides regulation of the Wnt signaling pathway, C8orf4 promotes the G1 to S-phase transition of the cell cycle by regulating the mitogen-activated extracellular signal-regulated kinase1/2 signaling pathway.[7] C8orf4 is also a target gene of nuclear factor-κB, and serves as an endothelial inflammatory regulator enhancing nuclear factor-κB activity.[8] C8orf4 can be upregulated by transforming growth factor β pathway and the Interleukin-1β/tumor necrosis factor-α and fibroblast growth factor receptor 2 pathways.[9–11] C8orf4 also serves as a novel heat shock response regulator and a novel hematopoietic regulator in mice.[12,13]

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C8orf4 was originally detected to be overexpressed in thyroid cancer.[14,15] Then, accumulating reports showed that *C8orf4* acts as an oncogene, and its overexpression was correlated with the progression of many cancers, including thyroid cancer,[14] lung cancer,[16,17] gastric cancer,[4] breast cancer,[11] ovarian carcinomas,[18] oral tongue squamous cell carcinomas,[19] and hematological malignancies.[20,21] However, a recent report indicated that C8orf4 suppressed Notch2 signaling and negatively regulated self-renewal of liver cancer stem cells.[22] So, the role of C8orf4 in cancers is still unclear and needs further investigation.

The expression and role of C8orf4 in cervical cancer was unclear till now. In this study, we examined the expression levels of C8orf4 and β-catenin in cervical cancer, SILs and normal cervical tissues, and investigated the correlations among C8orf4, β-catenin, and the development of cervical cancers. We also examined the methylation status of C8orf4 in cervical cancer and normal cervical tissues, and analyzed its role in cervical cancer.

2. Materials

2.1. Patients and tissue samples

A total of 100 cervical squamous cell carcinoma (CSCC) tissues, 50 high-grade squamous intraepithelial lesions (HSILs), 50 low-grade squamous intraepithelial lesions (LSILs), and 50 normal cervical tissue samples were obtained randomly from patients who underwent surgery or biopsy at Shenyang Women's and Children's Hospital and the First Hospital of China Medical University between 2016 and 2018. Patients included in the study ranged from 19 to 70 years of age (mean: 39 years). The histological diagnosis and grade of differentiation were assessed by examination of hematoxylin and eosin-stained sections. The 100 CSCC tissues were classified as well (n = 54), moderately (n = 33), or poorly (n = 13) differentiated tumors according to the classification system of the World Health Organization. None of the CSCC patients exhibited lymphatic metastasis. The tumor stage was classified as stage Ia, Ib, and IIa (n = 23, 59, and 18, respectively) according to the International Federation of Gynecology and Obstetrics (FIGO) staging system (2009). Thirty fresh CSCC samples and corresponding normal cervical tissues were also obtained from patients who underwent surgery at the First Hospital of China Medical University in 2017 and stored at −70°C immediately after resection. These samples were used for DNA extraction. The study was conducted according to the regulations stipulated by the institutional review boards at the China Medical University and Shenyang Women's and Children's Hospital.

2.2. Immunohistochemistry

Specimens were fixed in 10% neutral-buffered formalin for 24 hours and embedded in paraffin blocks. Tissue blocks were cut into 4-μm sections. These sections were deparaffinized, rehydrated, and processed by pressure cooking in a citrate buffer (pH 6) for 1.5 min. Then, the sections were incubated with polyclonal rabbit anti-C8orf4 antibody (1:200; ab133885, Abcam, Cambridge, MA) and monoclonal mouse anti-β-catenin antibody (1:200; 610154; BD Transduction Laboratories, Lexington, KY) at 4°C overnight. The streptavidin–peroxidase method was used to detect the staining by antibodies. Some slides were stained in the absence of primary antibodies and served as negative controls.

2.3. Evaluation of immunostaining

Two investigators evaluated the immunostained sections without knowing the clinical data of enrolled patients. Five representative views per slide were examined, and 100 tumor cells were observed per view at 400× magnification. The positivity of each case was obtained by calculating the percentage of positively stained cells. Positive rate of each case was scored as follows: 1 (1% to 25%), 2 (26% to 50%), 3 (51% to 75%), and 4 (76% to 100%). The intensity of immunostaining was scored as 0, 1, 2, or 3 in case of negative, weak, moderate, or strong, respectively. Scores from each sample were multiplied to give a final score ranging from 0 to 12, and the cases were categorized based on scores as having low (≤4), moderate (>4 and ≤8), or high (>8) expression, respectively.

2.4. DNA extraction and bisulfite sequencing polymerase chain reaction analysis

We analyzed the gene sequence of *C8orf4* as described previously.[16] The CpG island of *C8orf4* was located closer to the 5′ end of the exon, near the start codon. The extraction of genomic DNA and bisulfite conversion of DNA was performed using the tissue/cell DNA extraction reagent kit (Bioteke, Beijing, China) and the EZ DNA Methylation kit (Zymo Research, Beijing, China) according to the manufacturer's instructions. The primers used for bisulfite sequencing PCR (BSP) of *C8orf4* were as follows: forward, 5′-GGAGTTGAATTTCGGAAGAT-3′; reverse, 5′-ATTACCCACGACTTTCTTAC-3′ (product length: 144 bp). The bisulfite-treated DNA was amplified for 30 cycles: 95°C for 5 min, followed by cycling at 95°C for 10 s, 52°C for 20 s, and 72°C for 30 s, with a final step at 4°C for 5 min. Polymerase

Table 1

The expressions of chromosome 8 open reading frame 4 and β-catenin in cervical lesions and normal cervical tissues.

		C8orf4 expression				Abnormal β-catenin expression			
Category	n	Low	Moderate	High	*P*	Low	Moderate	High	*P*
Age					.189				.06
≤39	142	54	54	34		52	49	41	
>39	108	50	31	27		43	39	26	
Lesion					<.001				<.001
Normal	50	27	20	3		27	19	4	
LSIL	50	30	15	5		27	19	4	
HSIL	50	12	15	23		10	17	23	
CSCC	100	35	35	30		31	33	36	

C8orf4 = chromosome 8 open reading frame 4, CSCC = cervical squamous cell carcinomas, HSIL = high-grade squamous intraepithelial lesion, LSIL = low-grade squamous intraepithelial lesions.

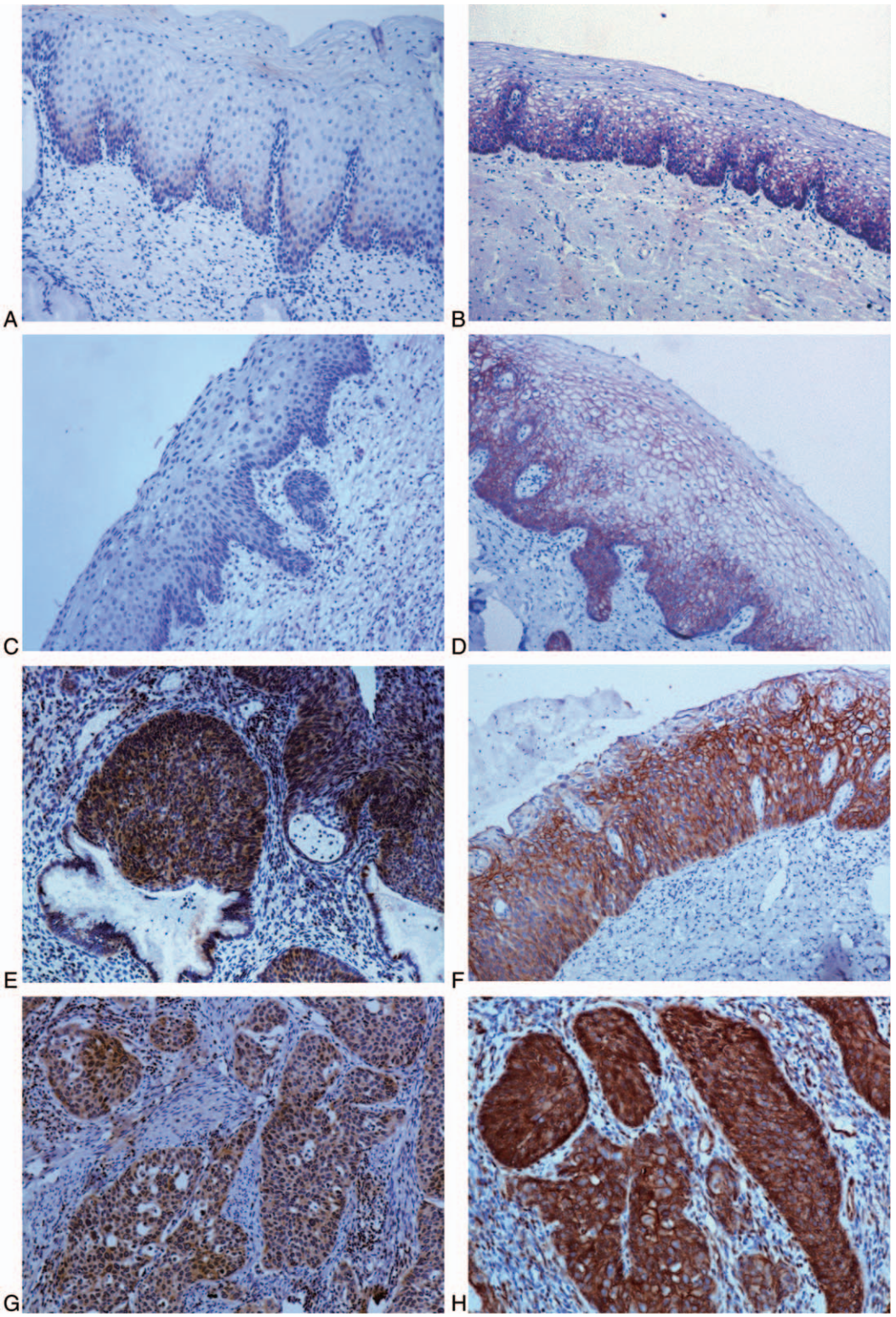

Figure 1. Expressions of C8orf4 and β-catenin in normal cervical epithelia, squamous intraepithelial lesions, and cervical squamous cell carcinomas. The expression of C8orf4 was weak in normal cervical epithelia (A) and low-grade squamous intraepithelial lesion (C), but significantly strong in high-grade squamous intraepithelial lesions (E) and cervical squamous cell carcinomas (G). The expression of β-catenin was primarily at the membrane of cells in normal cervical epithelia (B) and low-grade squamous intraepithelial lesion (D), the cytoplasmic expression of β-catenin was weak. But, in high-grade squamous intraepithelial lesion (F) and cervical squamous cell carcinoma (H), the expression of β-catenin was significantly strong in the cytoplasm. (Original magnification, 100×; streptavidin–peroxidase immunohistochemistry method). C8orf4 = chromosome 8 open reading frame 4.

chain reaction (PCR) products were electrophoresed on 1.5% agarose gels, and then, observed using a Bio-Imaging system (UVP, Upland, CA). Purified PCR products were used for sequencing.

2.5. Statistical analysis

Pearson's chi-squared and likelihood ratio tests were used to examine correlations among the expression of C8orf4, β-catenin, and cervical lesions. Pearson's and Spearman's correlation tests were used to assay correlations between expression levels of C8orf4 and β-catenin. Nonparametric Wilcoxon signed ranks test was used to compare the *C8orf4* methylation status of cervical cancers with that of the corresponding normal cervical tissues. Statistical significance was established at $P < .05$.

3. Results

3.1. Expression levels of C8orf4 in different tissues and its association with differentiation of CSCCs

The expression of C8orf4 was primarily localized in cytoplasm. As summarized in Table 1, the high and moderate expression rates of C8orf4 were 30.0% (30/100) and 35.0% (35/100) in CSCCs, respectively, which were significantly higher than those in LSILs (10.0% and 30.0%, $P = .005$) and normal cervical tissues (6.0% and 40.0%, $P = .003$). Similarly, in HISLs, the high and moderate expression rates of C8orf4 were 46.0% (23/50) and 30.0% (15/50), which were also significantly higher than those in LSILs ($P < .001$) and normal cervical tissues ($P < .001$) (Fig. 1). Furthermore, enhanced *C8orf4* expression was positively correlated with the poor differentiation of CSCCs ($P = .009$), but was not correlated with the FIGO stage of CSCCs ($P = .107$) (Table 2).

3.2. Abnormal expression of β-catenin in different tissues, and its correlation with poor differentiation and advanced FIGO stage of CSCCs

The high and moderate abnormal expression rates of β-catenin were 36.0% (36/100) and 33.0% (33/100) in CSCCs, respectively, which were significantly higher than those in LSILs (8.0% and 38.0%, $P = .001$) and normal cervical tissues (8.0% and 38.0%, $P = .001$). Meanwhile, in HISLs, the high and moderate abnormal expression rates of β-catenin were 46.0% (23/50) and 34.0% (17/50), which were significantly higher than those in LSILs ($P < .001$) and normal cervical tissues ($P < .001$) (Table 1) (Fig. 1). Furthermore, the abnormal expression of β-catenin was positively correlated with the poor differentiation ($P = .004$) and advanced FIGO stage of CSCCs ($P < .001$) (Table 2).

3.3. Methylation levels of C8orf4 in CSCCs were lower than those in corresponding normal cervical tissues

As observed after BSP and sequencing analysis, the methylation rate of *C8orf4* in CSCCs (73.10% ± 2.32%) were significantly lower than that in normal cervical tissues (84.76% ± 1.84%) ($P = .001$, n = 30) (Fig. 2). But, the methylation status of *C8orf4* was not correlated with the differentiation (correlation coefficient = −0.111, $P = .559$) and FIGO stage of CSCCs (correlation coefficient = −0.043, $P = .820$).

3.4. The correlations between C8orf4 and β-catenin in SILs and CSCCs

Spearman correlation analysis showed that C8orf4 expression was correlated with the aberrant expression of β-catenin in CSCCs (correlation coefficient = 0.310, $P = .002$) and SILs (correlation coefficient = 0.346, $P < .001$) (Table 3). By analyzing The Cancer Genome Atlas (TCGA) genomics data of cervical cancers, which were obtained from cBioPortal database,[23,24] we disclosed that the mRNA expression of *C8orf4* was positively correlated with the copy number alteration of *C8orf4* gene (correlation coefficient = 0.213, $P < .001$), and negatively correlated with the methylation level of *C8orf4* (correlation coefficient = −0.408, $P < .001$) and methylation level of *β-catenin* (correlation coefficient = −0.121, $P = .038$). Moreover, the methylation level of *C8orf4* was positively correlated with the methylation level of *β-catenin* (correlation coefficient = 0.122, $P = .037$), but negatively correlated with mRNA expression of β-catenin (correlation coefficient = −0.223, $P < .001$) (supplementary Table, http://links.lww.com/MD/D152).

Table 2

The relationships between the expressions of chromosome 8 open reading frame 4 and β-catenin and clinicopathological factors in cervical squamous cell carcinomas.

		C8orf4 expression			Abnormal β-catenin expression		
Factor	n	Low	Moderate-high	*P*	Low	Moderate-high	*P*
Age				.817			.536
≤39	53	18	35		15	38	
>39	47	17	30		16	31	
Differentiation				.009			.004
Well	54	26	28		24	30	
Moderate	33	6	27		6	27	
Poor	13	3	10		1	12	
FIGO stage				.107			<.001
Ia	23	12	11		15	8	
Ib	59	19	40		13	46	
IIa	18	4	14		3	15	

C8orf4 = chromosome 8 open reading frame 4, FIGO = the International Federation of Gynecology and Obstetrics.

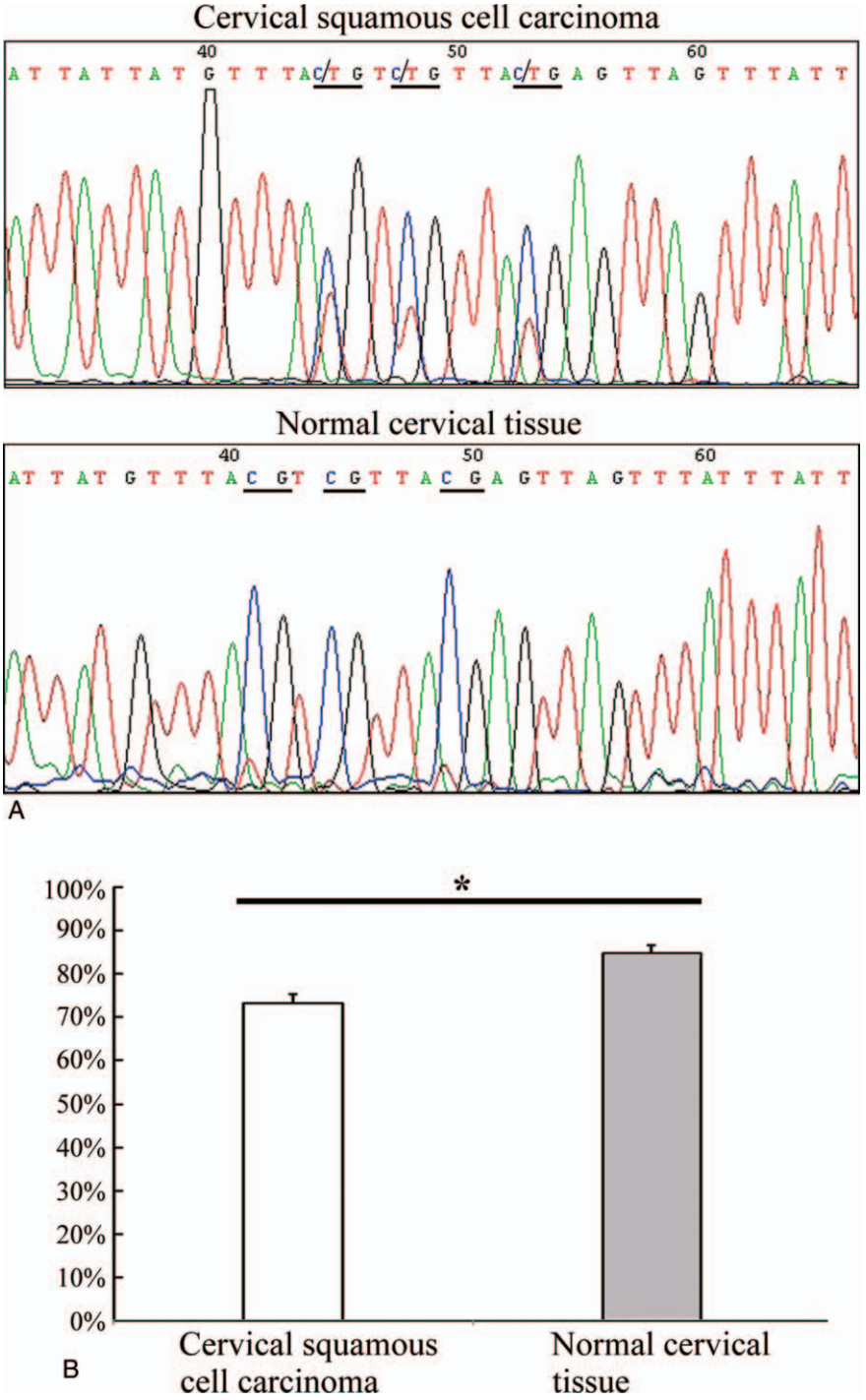

Figure 2. Bisulfite sequencing PCR analysis of cervical squamous cell carcinomas and corresponding normal cervical tissues. (A) In a representative case of cervical squamous cell carcinoma, the CpG sites (underlined) were semi-methylated, whereas their corresponding sites in normal cervical tissue were completely methylated (underlined). (B) The methylation rate of *C8orf4* gene in cervical squamous cell carcinomas (73.10% ± 2.32%) were significantly lower than that in normal cervical tissues (84.76% ± 1.84%) ($P = .001$, n = 30). C8orf4 = chromosome 8 open reading frame 4, PCR = polymerase chain reaction, $^{*}P < .05$.

4. Discussion

Wnt signaling pathway is involved in the multistep process of cervical carcinogenesis, including tumor initiation, progression, and invasion, suggesting its role as a potential biomarker or therapeutic target.[2,25,26] Activation of Wnt signaling pathway promotes cell growth and tumorigenicity, and inhibits apoptosis of cervical cancer cells.[27,28] Downregulating the activity of Wnt signaling pathway suppresses cell proliferation and tumor formation of cervical cancer.[29] As a member of Wnt signaling pathway, the aberrant expression of β-catenin has been reported in many tumors, and its expression is correlated with the proliferation and invasion of tumor cells.[30–34] Previous studies on cervical cancers also indicate that the aberrant expression of β-catenin promotes cell migration, invasion, and epithelial–

Table 3

The correlation of chromosome 8 open reading frame 4 and β-catenin in cervical squamous intraepithelial lesions and cervical squamous cell carcinomas.

Abnormal β-catenin expression	n	C8orf4 expression			Correlation coefficient	*P*
		Low	Moderate	High		
Cervical squamous intraepithelial lesions					0.346	<.001
Low	37	22	8	7		
Moderate	36	16	12	8		
High	27	4	10	13		
Cervical squamous cell carcinomas					0.310	.002
Low	31	13	12	6		
Moderate	33	15	14	4		
High	36	7	9	20		

C8orf4 = chromosome 8 open reading frame 4.

mesenchymal transition, and is associated with poor cancer-specific survival and overall recurrence rate.[32,33,35] Our results showed that aberrant expression of β-catenin was significantly enhanced with increase in grade of SILs and CSCCs, and was correlated with the FIGO stage of CSCCs, which confirms that aberrant expression of β-catenin promotes the carcinogenesis and progression of CSCCs.

C8orf4 is a novel activator of β-catenin and Wnt signaling pathway.[3–5] Recent studies have indicated that C8orf4 is overexpressed and correlated with the development of many cancers.[4,11,14,16–21] But the expression and role of C8orf4 in CSCC and SIL was unclear till now. Our study demonstrated that the expression level of C8orf4 in CSCCs and HSILs was significantly higher than that in LSILs and normal cervical tissues, which was similar to the trend of β-catenin expression. Moreover, the expression of C8orf4 was positively correlated with the aberrant β-catenin expression and poor differentiation of CSCCs. So, C8orf4 plays a synergistic role with β-catenin and enhances the activation of Wnt signaling pathway, and, in turn, promotes the carcinogenesis and development of CSCCs. To investigate the underlying mechanisms of upregulation of *C8orf4* expression in CSCCs, we, for the first time, examined the methylation level of *C8orf4* in CSCCs and corresponding normal cervical tissues. The results revealed that the methylation level of *C8orf4* in CSCCs was much lower than that in normal cervical tissues, which indicated that reduction of methylation level was one of the key reasons for increased expression of *C8orf4*. We also found the reduced methylation level and increased expression of C8orf4 in lung cancer tissues previously, which supported this view.[16] But we did not find any correlation between the methylation levels of *C8orf4* and clinicopathological factors, which might be due to the small sample size. By analyzing the TCGA genomics data obtained from cBioPortal database, it was also confirmed that the mRNA expression of *C8orf4* was negatively correlated with its methylation level, and positively correlated with the copy number alteration of *C8orf4*. In addition, mutation in *C8orf4* was not found in 607 cases of TCGA genomic data.[23,24] So, reduction of methylation level and copy number alteration are considered to be the key causes for increased expression of C8orf4 in CSCCs.

In conclusion, the expressions of C8orf4 and β-catenin were synergistically increased in CSCCs and HSILs and higher than those in LSILs and normal cervical tissues, which promoted the carcinogenesis and development of CSCCs. The methylation level of *C8orf4* is decreased in CSCCs and is responsible for the increased expression of *C8orf4*.

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