Original Article

High methylation of *ZNF582* in cervical adenocarcinoma affects radiosensitivity and prognosis

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Background: Our previous study demonstrated hypermethylation of the *ZNF582* gene in cervical cancer, but its prognostic value in cervical cancer, especially in cervical adenocarcinoma (CAC), remains unclear. The present study aimed to investigate the value of *ZNF582* gene methylation for diagnosis and prediction of radiochemotherapy sensitivity and prognosis in CAC.

Methods: We first determined *ZNF582* methylation levels using quantitative methylation-specific PCR in a training set. Disease-free survival and overall survival (DFS and OS) rates were estimated using the Kaplan-Meier method. A Cox regression model was used to assess the prognostic significance of *ZNF582* gene methylation in CAC patients. Immunohistochemistry was used to test ZNF582 protein expression in CAC tissues, and an MTT assay evaluated the sensitivity of Hela cells (with or without ZNF582 transfection) to radiation and chemotherapy.

Results: The *ZNF582* gene showed a higher level of methylation in the CAC group than in the noncancer group, and patients negative for *ZNF582* methylation had worse prognoses. We also found that *ZNF582* methylation levels were reduced in concomitant chemo-radio-therapy (NCRT) patients compared with that in non-NCRT patients. Methylation-negative status was correlated with high ZNF582 protein expression, and ZNF582 protein overexpression could increase resistance to radiation and chemotherapy in Hela cells.

Conclusions: Aberrant high methylation of *ZNF582* may be a potential biomarker for CAC detection and prognosis monitoring. Overexpression of ZNF582 protein could increase CAC chemoradiotherapy resistance.

Keywords: *ZNF582*; methylation; cervical adenocarcinoma (CAC); chemoradiotherapy

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Introduction

Cervical cancer ranks fourth in terms of both incidence and mortality in women globally (1). Over 85% of new cases and 90% of cervical cancer deaths occur in developing countries (1,2). In China, cervical cancer is still a huge clinical burden with high incidence (7.5/100,000) and mortality (3.4/100,000) rates (3). In terms of the tissue type affected, cervical adenocarcinoma (CAC) is the second most common to squamous cell carcinoma (SCC). The histology of adenocarcinoma is an independent significant risk factor for recurrence and survival (4,5). Both CAC and SCC are HPV-related cancers (6). Although early screening and the use of HPV vaccine have greatly decreased the incidence of cervical cancer in some countries and regions, the frequency of CAC has increased in many countries, particularly in younger women, and it now accounts for 20–25% of cervical cancer in developed countries (7). About 2/3 (60.3%) of CAC patients are younger than 50 years old, of which more than half (57.38%) are younger than 40 (8). Existing screening methods such as cytology-based tests are not ideal for CAC early detection because of low sensitivity and low reproducibility (9-11). The increased frequency and poor prognosis of CAC call for new biomarkers to help early diagnosis, treatment, and prognosis monitoring, and to enable individualized treatment and services.

Our understanding of DNA methylation patterns as potential biomarkers for diagnosis, prognosis, personalized therapy, and disease management is just starting to emerge. As a biomarker to detect cervical SCC, DNA methylation has several advantages over conventional cytology-based tests. Our previous studies showed that the *ZNF582* methylation test improves on current cervical cancer screening efficacy and reduces unnecessary referral for colposcopy and biopsy by up to 60% (12-14), but how it performs in CAC is unclear. Whether DNA methylation can predict clinical outcome of CAC is not known. To investigate this problem, we conducted a series of follow-up investigations based on our previous studies, to investigate whether methylation of *ZNF582* might contribute to the early diagnosis, radiochemotherapy sensitivity and prognosis prediction in CAC.

Methods

Study design and specimen collection

This was a retrospective clinical investigation studying methylation of *ZNF582* in CAC. We enrolled 232 patients from Hunan Cancer Hospital, the Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, between 2013 and 2015. Among these patients, 202 were diagnosed with adenocarcinoma, 22 were diagnosed with adenosquamous carcinoma, and 30 were non-cancer specimens (inflammation cases). After evaluation, 13 subjects were excluded (six specimens lacked sufficient clinical information and seven had very low DNA concentration). This left 219 to be subjected to gene methylation studies (*Table 1*). We tracked the prognoses of these patients.

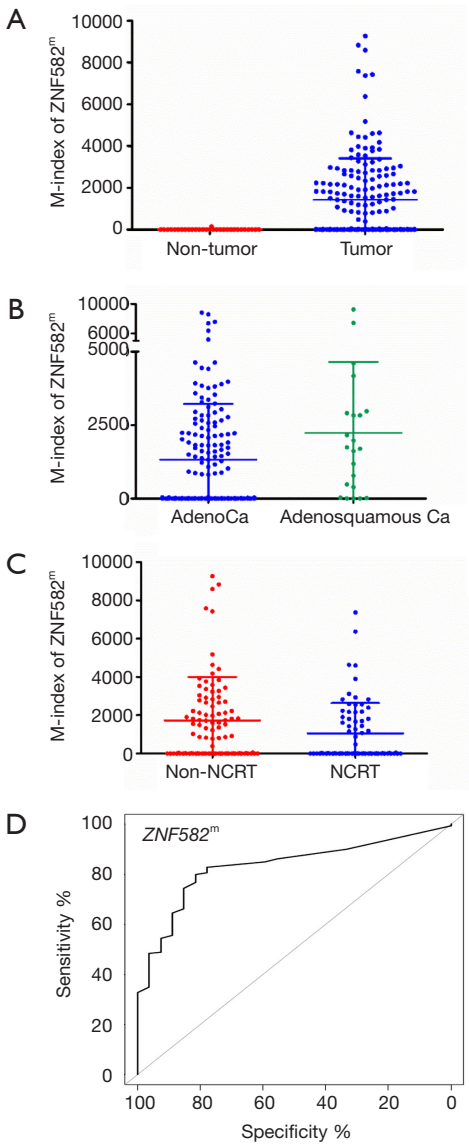
Table 1 Characteristics of subjects

Characteristics	Numbers	%
Specimen (N=219)		
Adenocarcinoma	167	76.3
Adenosquamous carcinoma	22	10.0
Inflammation	30	13.7
Cancer group (N=189)		
Age		
<50	96	50.8
≥50	93	49.2
FIGO stage		
< IIB	155	82.0
≥ IIB	29	15.3
Unknown	5	2.6
Differentiation		
Well	17	9.0
Moderate	113	59.8
Poorly	50	26.5
Unknown	9	4.8
NCRT		
With	75	39.7
Without	100	52.9
Unknown	14	7.4

FIGO, International Federation of Gynecology and Obstetrics; NCRT, neoadjuvant radio (chemo-) therapy.

DNA preparation, bisulfite conversion, and quantitative methylation-specific polymerase chain reaction (qMSP)

All the methylation tests were performed in an ISO17025-



	ZNF582^{m}	
	Non-tumor	tumor
Total	30	189
Mean	5.6±4.9	1423±142
P-value	<0.001***	

	ZNF582^{m}	
	AdenoCa	Adenosquamous Ca
Total	167	22
Mean	1317±145	2231±515
P-value	0.04*	

	ZNF582^{m}	
	Non-NCRT	NCRT
Total	100	75
Mean	1734±226	1057±185
P-value	0.094	

	ZNF582^{m}
	Adenocarcinoma Detection
Sensitivity	60.00%
Specificity	92.59%
AUC	83.13%
P-value	<0.001***

Figure 1 (A) Methylation-Index (M-Index) of *ZNF582* gene methylation between the cervical cancer group (167 adenocarcinoma and 22 adenosquamous carcinoma) and the non-cancer group (30 inflamed tissues); P<0.001***. (B) M-Index of *ZNF582* gene methylation between adenocarcinoma (n=167) and adenosquamous carcinoma (n=22); P=0.04*. Each dot represents the results from one patient. (C) *ZNF582* methylation level showing a reduced tendency in patients compared to non-patients, P=0.029*. (D) The area under the ROC curve for the *ZNF582* gene methylation assay was calculated for detecting cervical adenocarcinoma. The sensitivity and specificity of *ZNF582* methylation were 60% and 92.59%, respectively, with a cutoff value of delta CP =11. The AUC was 83.13%.

certified laboratory (iStat Biomedical Co., Ltd., New Taipei City, Taiwan). Briefly, paraffin-embedded cervical tissues were deparaffinized and genomic DNA (gDNA) samples were then extracted and bisulfite-converted with the use of an EpiGene™ nucleic acid extraction kit and an EpiGene™ bisulfite conversion kit (iStat Biomedical Co., Ltd., New Taipei City, Taiwan). Quantitative methylation-specific PCR (qMSP) was later performed to determine the methylation level of *ZNF582* (*ZNF582*^{m}) using the TaqMan Probe system in a Light Cycler LC480 system (Roche Applied Science, Penzberg, Germany). Specific primers and probes for qMSP were published in our previous study (13).

Hypermethylation (positive) was inferred where the delta Cp value was smaller than 11 (the cut-off value; *Figure 1*). The registered CaSki and A375 cancer cell lines were used as methylated and unmethylated controls to ensure the quality of the bisulfite conversion and qPCR processes.

Transfection of ZNF582

Hela cells were transfected with 20 μg of ZNF582 expression vectors (Vigene biosciences, NM_144690) using ViaFect Transfection Reagent (Promega Corporation).

Western blotting, immunohistochemistry, and immunofluorescence assays

Crude cellular protein was loaded onto an SDS-PAGE gel for protein separation and subsequently transferred to nitrocellulose membranes. After incubation in blocking solution (5% nonfat milk), the membranes were probed using primary antibodies against ZNF582 (Biorbyt, 1:1,000 dilution) overnight at 4 ℃. They were detected using a Bio-Rad imaging system after incubation with horseradish peroxidase-conjugated secondary antibodies for 1.5 h. For immunohistochemistry and immunofluorescence, the slides were treated with the primary antibody against ZNF582 (Biorbyt, 1:200 dilution) at room temperature for 1 h after hydration and blocking, and then incubated with the secondary antibody for 10 min and examined using a light microscope or fluorescence microscope.

Statistical analysis

The cutoff values for defining the methylation status of the *ZNF582* gene were generated from data gathered from 204 subjects, of which 30 were non-cancer controls and 174 were cancer patients. In this study, ROC curve analysis was used to determine which M-index of the target gene *ZNF582* would have the highest accuracy for discerning worse cases of CAC from milder cases. An optimal cut-off value for the biomarkers was determined by the Youden index (15) from the correlations between M-index and case severity. The relationship between *ZNF582* methylation and clinical pathological parameters of patients was analyzed using the Mann-Whitney and Dunnett tests. The data were analyzed using Graphpad Prism 5. The Kaplan-Meier method was used to estimate disease-free survival and overall survival (DFS and OS). We calculated DFS from treatment to the date of the first relapse at any site or death from any cause, whichever occurred first, and OS to death from any cause, and calculated hazard ratios (HRs) with multivariate Cox regression analysis.

Results

Performance of ZNF582 methylation as a biomarker of CAC

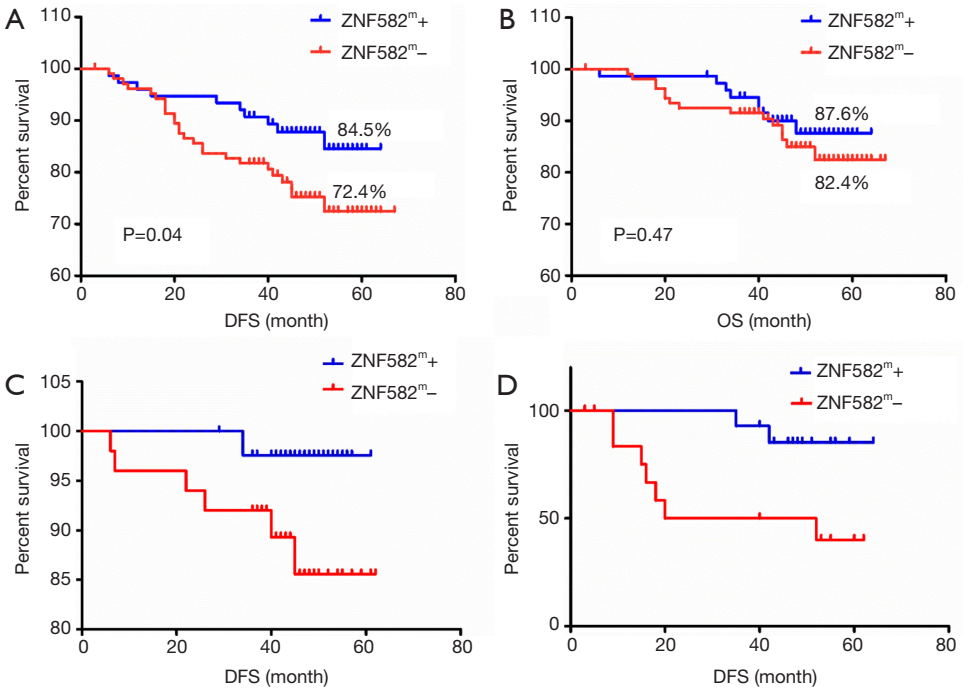
Among our 219 cervical specimens, 189 were cancer tissues (167 adenocarcinoma and 22 adenosquamous carcinoma), and 30 were non-cancer tissues (inflammations) (*Table 1*). The *ZNF582* methylation index was significantly higher in the cancer group than in the non-cancer groups (*Figure 2A*). The methylation index was also higher in the adenosine squamous carcinoma group than in the adenocarcinoma group (*Figure 2B*). Among the cancer patients, 75 were subjected to neoadjuvant chemoradiotherapy (NCRT) while 100 were not treated (*Table 1*). The results showed that *ZNF582* methylation level was reduced in the NCRT group compared with that in the non-NCRT patients (*Figure 2C*).

To determine the clinical application of a *ZNF582* methylation assay, and to obtain a suitable cutoff value for this assay, we calculated ROC curves and area under the curve (AUC) values. The sensitivity and specificity of *ZNF582* were 60.00% and 92.59% respectively. The cutoff value of delta Cp was 11, and the AUC of *ZNF582* was 83.13% (*Figure 2D*). Methylation level of *ZNF582* was independent of differentiation, depth of invasion, tumor size, and FIGO stage (*Figure S1*).

ZNF582^{m}-positive CAC patients have better prognosis

We further investigated whether the methylation level of *ZNF582* influenced prognosis in CAC patients. After 5 years' follow-up, we collated 188 patients' prognostic information. Characteristics of *ZNF582*^{m}-positive and *ZNF582*^{m}-negative patients are shown in *Table 2*. Negative status for *ZNF582* methylation was found to be an independent predictor of recurrence and survival [P=0.03, HR =2.826, 95% confidence interval (CI): 1.125–7.099; *Table 3*].

Five-year DFS rates were significantly higher in *ZNF582*^{m}-positive patients than in *ZNF582*^{m}-negative patients (84.5% *vs.* 72.4%; P=0.04) (*Figure 2A*), but there was no obvious difference in OS between these two groups (*Figure 2B*). For early stage (< IIB stage) patients without NCRT, *ZNF582*^{m}-positive status was also associated with



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	Cervical adenocarcinoma (<IIB stage without NCRT)	
	ZNF582^{m}+	*ZNF582*^{m}–
Total	42	51
5-year DFS	97.6%	85.6%
P-value	0.07	

F

	Cervical adenocarcinoma (≥IIB stage)	
	ZNF582^{m}+	*ZNF582*^{m}–
Total	15	14
5-year DFS	85.7%	33.3%
P-value	0.03*	

Figure 2 Kaplan-Meier estimation of 5-year disease-free survival (DFS) rate according to *ZNF582* methylation status in 188 patients. Methylation status of the *ZNF582* gene was defined as the cutoff. The 5-year disease free survival (DFS) rate (A) and overall survival rate (B) of *ZNF582*^{m}-positive patients and *ZNF582*^{m}-negative patients. (C,E) The 5-year DFS rates of *ZNF582*^{m}-positive and ZNF582^{m}-negative patients below stage IIB were 97.6% and 85.6%, respectively; P=0.07. (D,F) The 5-year DFS rate was 85.7% for ZNF582^{m}-positive patients compared with 33.3% for *ZNF582*^{m}-negative patients where cancer stage was ≥ IIB; P=0.03*.

better prognosis, with an impressive 5-year DFS rate of 97.6% (*Figure 2C*). *ZNF582*^{m}-negative patients with late-stage cancers (stage ≥ IIB) showed a very low 5-year DFS rate of 33.3% (*Figure 2D*). Characteristics of *ZNF582*^{m}-positive and *ZNF582*^{m}-negative patients are shown in *Tables S1* and *S2*, respectively. The results indicated that *ZNF582*^{m}-positive patients have better prognosis.

ZNF582^{m}-negative status was correlated with high ZNF582 protein expression, and ZNF582 overexpression could increase radiation and chemotherapy resistance in Hela cells

To investigate the mechanism by which *ZNF582* methylation affects the prognosis of CAC, we tested the association between *ZNF582* protein expression and gene methylation in CAC tissues. We found that negative *ZNF582* gene methylation status was correlated with high ZNF582 protein expression, and positive *ZNF582* gene methylation status was correlated with low ZNF582 protein expression (*Figure 3A*). Further in vitro studies found that overexpression of ZNF582 protein in HeLa cells (*Figure 3B,C*) could increase their resistance to radiation (*Figure 3D*) and cisplatin treatment (*Figure 3E*).

Discussion

Several recent studies have found an increase in the incidence of CAC, particularly among younger women; for example in the United States (16,17), Sweden (18), and

Table 2 Characteristics of ZNF582^{m}-positive and ZNF582^{m}-negative subjects

Characteristics	Patients (n=188)	ZNF582^{m}+ (n=77)	ZNF582^{m}- (n=111)	P value
Age		48.4±9.3	49.4±9.7	
FIGO stages				0.27
< IIB	155	60 (78%)	95 (86%)	
≥ IIB	29	15 (19%)	14 (13%)	
Unknown	4	2 (3%)	2 (1%)	
Tumor size (cm)				0.81
<4	83	33 (43%)	50 (45%)	
≥4	77	32 (41%)	45 (41%)	
Unknown	28	12 (16%)	16 (14%)	
Deep of invasion				0.93
<1/2	63	24 (31%)	39 (35%)	
≥1/2	98	38 (49%)	60 (54%)	
Unknown	27	15 (20%)	12 (11%)	
Differentiation				0.34
Well/moderate	126	50 (65%)	76 (68%)	
Poorly	49	24 (31%)	25 (23%)	
Unknown	13	3 (4%)	10 (9%)	
NCRT				0.13
With	75	24 (31%)	51 (46%)	
Without	99	44 (57%)	55 (50%)	
Unknown	14	9 (12%)	5 (4%)	

FIGO, International Federation of Gynecology and Obstetrics; NCRT, neoadjuvant radio (chemo-) therapy.

Table 3 Multivariate Cox regression analysis of DFS in cervical cancer

Variable	5-year DFS		5-year OS	
	HR (95% CI)	P value	HR (95% CI)	P value
Age	1.016 (0.970–1.064)	0.51	0.984 (0.931–1.041)	0.578
FIGO stages	2.120 (0.893–5.057)	0.09	3.632 (1.372–9.612)	0.009
Tumor size	1.832 (0.811–4.141)	0.15	1.885 (0.667–5.329)	0.232
Deep of invasion	2.141 (0.796–5.759)	0.13	4.012 (0.875–18.390)	0.074
NCRT	0.598 (0.259–1.381)	0.23	0.375 (0.115–1.224)	0.104
ZNF582^{m}	2.826 (1.125–7.099)	0.03	2.039 (0.700–5.938)	0.191

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; NCRT, neoadjuvant radio (chemo-) therapy.

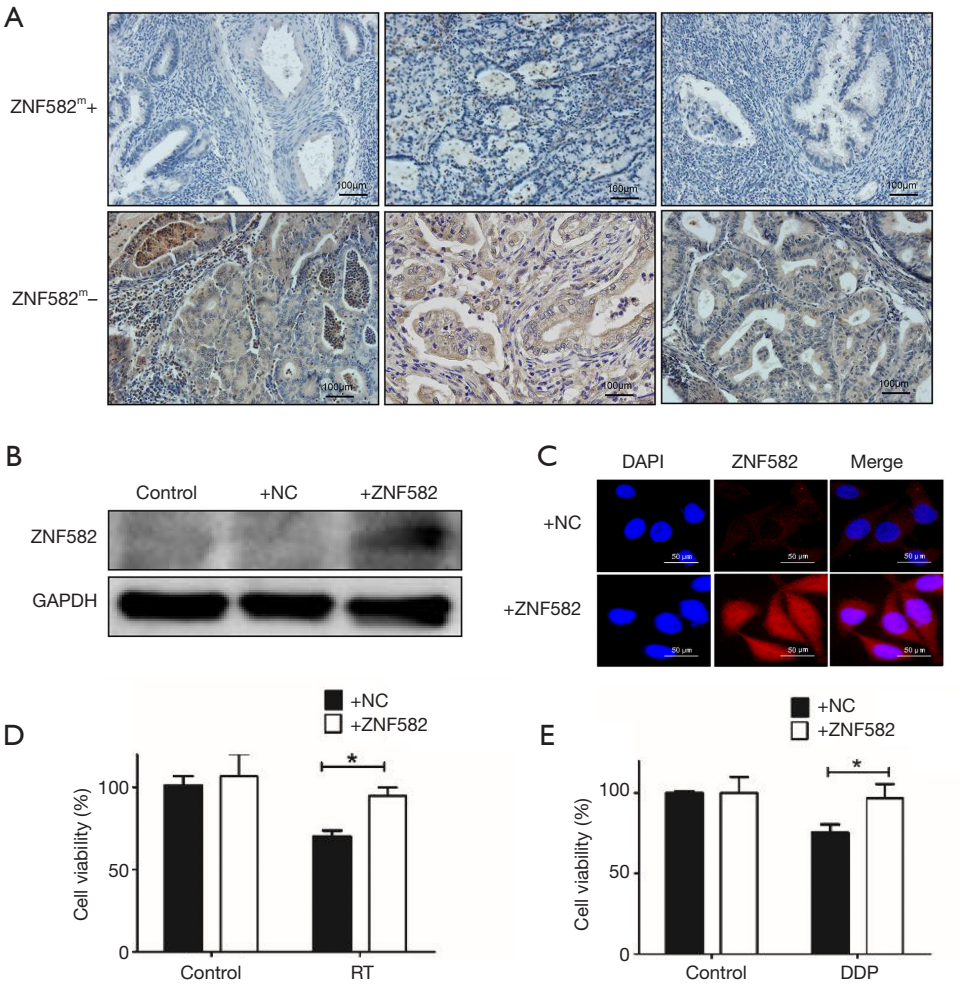

Figure 3 (A) Representative immunohistochemical staining of ZNF582 protein expression in three different cases of *ZNF582*^{m}-positive and *ZNF582*^{m}-negative tissues, respectively. (B) Western blot analysis of ZNF582 protein expression in Hela cells (control), in Hela cells transfected with vector control (NC), and in Hela cells transfected with ZNF582 (+ ZNF582). (C) As revealed by confocal microscopy, ZNF582 was overexpressed after transfection with ZNF582 (+ ZNF582) compared to vector control (NC). Viabilities of Hela cells after 4GY radiation treatment (RT) (D) or cisplatin treatment (E) for 48 h with ZNF582 or vector transfection. Data are represented as mean ± SD. *, P<0.05.

Italy (19). In the current study, we demonstrated a higher *ZNF582* methylation level in cervical CAC compared with that in non-cancerous inflamed tissue (*Figure 1A*). Our data showed that *ZNF582* methylation level reached 60.00% sensitivity and 92.59% specificity for the detection of CAC (*Figure 1D*), suggesting that the *ZNF582* gene is appropriate for CAC detection. We also showed that *ZNF582* methylation could contribute to differential diagnosis between adenocarcinoma and adenosine carcinoma (*Figure 1B*). Our third major finding was that *ZNF582* methylation level was significantly reduced in patients who received NCRT compared with those who did not (*Figure 1C*). A good monitoring tool has always been crucial for understanding cancer treatment efficacy. Further study of *ZNF582* methylation before and after NCRT, and investigation of other possible applications of *ZNF582* methylation for monitoring cancer treatment efficacy, are warranted.

It is well known that CAC is radio-resistant and chemo-resistant, and its prognosis is worse than cervical SCC. In China, the prognosis of CAC is poor even for patients with early-stage disease (stage IB–IIB) (20), highlighting

the need for closer attention to CAC. Our results showed the potential of *ZNF582* methylation level as a biomarker for monitoring CAC treatment and prognosis, and this has not been considered elsewhere to our knowledge. *ZNF582* methylation-positive patients have better prognosis in CAC (*Figure 2A*), suggesting that *ZNF582* gene methylation has potential for 5-year DFS outcome prediction, in addition to being a prospective screening biomarker for early stage (< IIB) disease. Overall, our results suggest that *ZNF582* methylation may play an important role in the molecular pathogenesis, clinical cancer progression, and prognosis of CAC.

Gene methylation is a regulatory epigenetic modification, regulated by DNA methyltransferase (DNMT) (21,22). Methylation can contribute to cancer development by modifying DNA base pairing patterns, resulting in stable mismatches that lead to carcinogenesis (23). Aberrant DNA methylation is associated with different types of cancer, such as cervical cancer (14,24,25), colorectal cancer (26), and lung cancer (27). Gene methylation can function as a negative regulator of gene expression, and have tumor-suppressive or oncogenic effects, potentially playing important roles in cancer progression. Prior to this study, the relationship between *ZNF582* gene methylation and protein expression was unclear. We showed, for the first time, that *ZNF582* gene hypermethylation correlates with low protein expression, and that negative *ZNF582* gene methylation correlates with high protein expression (*Figure 3A*). Furthermore, we found that overexpression of ZNF582 protein increases resistance of Hela cells to radiation and chemotherapy (*Figure 3D,E*). Resistance to radiotherapy and chemotherapy is the main cause of poor prognosis in patients with CAC. These data may explain why *ZNF582*^{m}-negative patients have a poor prognosis.

In our previous study, we reported 100% detection of *ZNF582* methylation in SCC tissue (13), but in this study we found lower efficiency in CAC tissues. One possible reason for this is that HPV 16, which causes SCC, is much more prevalent than HPV 18, which causes CAC (28,29), and 20–40% of CAC is not associated with HPV infection (30). The second reason is that the sensitivity and accuracy of gene methylation detection in paraffin samples are not as good as in fresh-frozen specimens (31). For clinical applications, cervical scrapings rather than cervical tissues are more logical and feasible for methylation analysis and cancer detection. Whether the degree of methylation in cervical scrapings is consistent with cervical tissues requires further study.

In conclusion, this study suggests that *ZNF582* methylation level may be useful as a biomarker for CAC detection. In addition, *ZNF582* methylation may prove to be an important prognostic marker for CAC and may be a potential tool for monitoring the sensitivity of radiation and chemotherapy treatment.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by the Hunan Cancer Hospital ethics committee {project number: 2015[01]} and the Chinese Clinical Trial Registry (registration number: ChiCTR1800018931).

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Supplementary

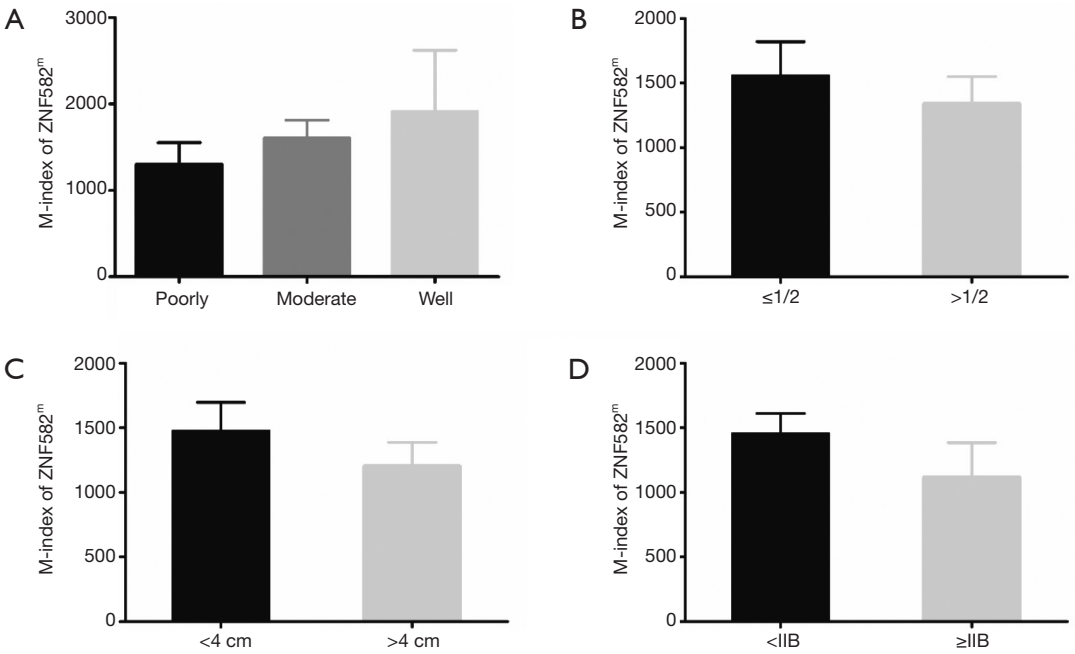

Figure S1 Methylation level of ZNF582 in different groups. Methylation level of *ZNF582* gene in cervical adenocarcinoma with (A) increasing cancer differentiation (well, moderate and poor), (B) different depth of invasion (≤1/2 and >1/2), (C) different tumor size (<4 and ≥4 cm) and (D) different FIGO stage (< IIB and ≥ IIB).

Table S1 Characteristics of ZNF582^m-positive and ZNF582^m-negative patients (<IIb stage without NCRT)

Characteristics	ZNF582^m+	ZNF582^m-	P value
N	39	53	
Age	46.5±8.7	48.3±8.3	
Tumor size (cm)			0.77
<4	26	33	
≥4	6	9	
Unknown	7	11	
Invasion			0.97
≤0.97	18	22	
>1/2	21	26	
Unknown	0	5	
Differentiation			0.49
Well/moderate	29	41	
Poorly	10	10	
Unknown	0	2	

Table S2 Characteristics of ZNF582^m-positive and ZNF582^m-negative patients (≥ IIb stage)

Characteristics	ZNF582m+	ZNF582m-	P value
N	15	14	
Age	54.5±7.9	49.9±9.1	
Tumor size (cm)			0.36
<4	2	4	
≥4	12	10	
Unknown	1	0	
Invasion			0.59
≤0.59	1	2	
>1/2	8	8	
Unknown	6	4	
Differentiation			0.93
Moderate	9	7	
Poorly	6	5	
Unknown	0	2	
NCRT			0.82
With	7	9	
Without	2	2	
Unknown	6	3	