

Searching for the methylation sites involved in human papillomavirus type 16 and 18-positive women with cervical cancer

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Abstract. It has been reported that >90% of women with cervical cancer are human papillomavirus (HPV)-positive, with HPV16 and 18 being the most 'highest-risk' HPV genotypes. However, in numerous women, HPV infection will not progress to cervical cancer. Accordingly, more appropriate screening markers need to be explored. In the present study, genome-wide DNA methylomic differences between cervical cancer tissues with HPV-16 or HPV-18 infection and normal cervical tissues were detected by using an Illumina Human Methylation 850 K BeadChip. The Gene Ontology functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted in order to define the nearest neighbouring genes of differentiated methylation sites. Moreover, differentiated methylation sites were verified using pyrosequencing. KEGG analyses suggested that the focal adhesion pathway and pathways in cancer were highly enriched. Bioinformatics and statistical analysis indicated that the nine CpG loci had the most significant differences amongst the genes involved in these pathways. Among these, six CpG sites in the CHRM2, LAMA4, COL11A1, FGF10, IGF1 and TEK genes were highly associated with HPV-16-positive cervical cancer, as validated using pyrophosphate sequencing. Additionally, 10 significantly different CpG sites of the HPV-18-positive group were selected and verified in The

Cancer Genome Atlas, indicating their possible diagnostic roles in cervical cancer development and determination. In addition, eight hypermethylated CpG island sites that were associated with HPV-16-positive cervical cancer tissues and 10 hypermethylated CpG island sites that were associated with HPV-18-positive cervical cancer tissues were identified, highlighting their potential roles in screening and evaluating targeted therapy efficacy and prognosis. The main focus of the present study was to identify the genetic variability in HPV-16- and HPV-18-positive samples and to elucidate possible methylation biomarkers in HPV-positive women with a risk of developing cervical cancer.

Introduction

Cervical cancer is considered one of the leading causes of cancer-associated mortality among women globally (1,2). In particular, >85% of new cases and 90% of cervical cancer-related deaths occur in developing countries (3-5). Despite efforts made to improve cervical cancer therapy, the 5-year survival rate remains <50% (6,7). In China, the incidence rate of cervical cancer is estimated to be ~15.4/100,000, with a relatively high mortality rate (8-10). Due to the huge population and high rate of human papillomavirus (HPV) infection, precise diagnosis and treatment are required for cervical cancer in China.

HPV, a highly prevalent sexually transmitted virus, is a circular dsDNA virus containing six early genes (E1, E2, E4, E5, E6 and E7) and two late genes named L1 and L2 (11-16). Currently, ~200 HPV genotypes have been identified based on the nucleotide diversity of the L1 gene, and 15 of these are regarded as 'high risk', contributing to the development of cervical cancer, including HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66 and -68 (17-21). It has been reported that HPV16 is the 'highest-risk' HPV genotype with HPV-18 being second, according to their oncogenic potential (22-27).

More than 1 year of persistent HPV infection may be an important risk factor for the progression of cervical cancer and its precursors (28,29). HPVs can establish their progeny, spread their viral genes, infect basal cells and further promote

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epithelial-mesenchymal transition (30,31). However, HPV infection itself is not sufficient for the initiation and establishment of malignant cell transformation (32,33). Accordingly, HPV tests have a poor positive predictive value, since HPV infection will progress to cervical cancer in only a few women (34,35). Clinical data have indicated that HPV infection is self-limiting and regresses in several cases, suggesting that other biomolecular mechanisms are involved in the progression of cervical cancer. A number of studies have demonstrated that DNA methylation is involved in the carcinogenic process of cervical cancer (36-40). The hypermethylation of CpG islands in the promoter regions of specific genes, including tumour suppressor genes, leads to the silencing of the gene and inhibits the downstream pathways. By contrast, disruption of epigenetic processes can lead to the activation of oncogenes, and the accumulation of epigenetic changes is an essential step in the development of cervical cancer (41,42).

An increasing number of studies have indicated that DNA methylation is an early event in tumorigenesis and plays a major role in tumour initiation and the progression of cervical cancer (43). Therefore, it is crucial to identify reliable prognostic and predictive DNA methylation-related biomarkers that may help in the early diagnosis and treatment strategies for cervical cancer. In the present study, to elucidate the effect of the combination of HPV genotypes and DNA methylation, these methylation biomarkers in HPV-16- and HPV-18-positive women with cervical carcinoma were analysed using a human Illumina Human Methylation 850 K BeadChip. The purpose of the present study was to identify the hypermethylation of CpG islands of genetic variability from samples tested positive HPV-16 and HPV-18 and to elucidate possible methylation biomarkers of HPV-positive women with a risk of developing cervical cancer.

Materials and methods

Human tissue specimens. A total of six paraffin-embedded specimens, including three cases of HPV 18 (HPV-18 group)- and 3 cases of HPV-16 (HPV-16 group)-positive cervical carcinoma tissues, were collected and diagnosed on a pathological basis according to the FIGO (2009) clinical staging criteria (44). Normal cervical tissues were obtained from three women with hysteromyoma who underwent total hysterectomy (normal group) from July, 2014 through December, 2017 at the Department of Obstetrics and Gynecology of the First People's Hospital of Lanzhou and Gansu Provincial Hospital. All experiments performed in the present study were approved by the Ethics Committee of The First People's Hospital of Lanzhou and Gansu Provincial Hospital (approval no. (2016-02)). Written informed consent was obtained from all the patients, and the time of signing the agreement was the time of sample collection.

HPV genotyping and grouping. DNA extraction was performed using a QIAGEN QIAamp DNA Mini kit (cat. no. 51304; Qiagen GmbH) from formalin-fixed and paraffin-embedded tissue sections according to the manufacturer's instructions. The DNA samples were identified and quantified using a NanoDrop™ 8000 spectrophotometer (Thermo Fisher Scientific, Inc.) and agarose gel electropho-

resis. Genotyping with positive samples was performed by using the HPV Genotyping Detection kit, the Assay Kit for Genotyping Human Papillomavirus (PCR-reverse dot blot) (cat. no. CP.008.022; Guangzhou, LBP Medicine Science & Technology Co., Ltd.) for subtypes HPV-16 and HPV-18. Samples were tested following the manufacturer's instructions (45). The HPV-positive control and -negative controls were set in each experiment. In total, 12 samples were genotyped with the HPV Genotyping Detection kit, and three HPV-16-positive specimens and three HPV-18-positive specimens were randomly selected (Table SI).

DNA methylation chip. An Illumina Human Methylation 850K BeadChip (Illumina, Inc.) was used to detect the whole genome methylation status of HPV-16- and HPV-18-positive tissues. Genomic DNA of the normal group (three cases normal cervical tissues), HPV-16 group (HPV-16-positive specimens with cervical cancer), and HPV-18 group (three cases of HPV-18-positive specimens with cervical cancer) was extracted using the QIAamp DNA Mini kit (cat. no. 51304; Qiagen GmbH) and bisulfite-converted using the EZ DNA Methylation kit (cat. no. D5001; Zymo Research Corp.). The converted DNA was hybridized to an Infinium Human Methylation 850 K BeadChip. The subsequent bioinformatics analysis was performed by Genegy Co. The Illumina 850 K methylation chip analysis data have been uploaded in the GEO public database repository (accession no. GSE169622, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169622>).

Pyrosequencing. Through pyrosequencing, nine candidates of the HPV-16 group screened by the 850K methylation chip were verified. An EZ 96-DNA methylation kit (Zymo Research Corp.) was used for bisulfite conversion in accordance with the manufacturer's standard procedures, with fully methylated and unmethylated samples as test controls. PyroMark Assay Design 2.0 was used for the synthesis of bisulfite-PCR primers, which were synthesized by The Beijing Genomics Institute (BGI). A list of bisulfite PCR primers is presented in Table SII. The bisulfite PCR amplification conditions were as follows: Pre-denaturation at 95°C for 3 min; 40 cycles at 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min; and a final elongation at 72°C for 7 min. The HotStarTaq DNA polymerase (Qiagen Ltd.) was used for disulfide PCR amplification. Compared to the PyroMark Q96 ID (Qiagen Ltd.), Pyro Q CpG software (version 2.0.6, Qiagen GmbH) automatically analysed the methylation status of each site.

Bioinformatics analysis. The 850K chip data analysis was implemented in R language. The whole analysis model is based on the highly integrated R analysis package ChAMP (Version: 2.8.9), which inherits the methods of Minfi, Limma, Sva, and IMA analysis packages. The graph was acquired using self-written R script, the basic function in GGplot2 implementation. Microarray data were normalized using BMIQ (Beta MIXture Quantile dilation). SVD (Singular Value Decomposition) was applied to evaluate the major components of variables in the data set, and then a Bayesian model-based Combat method was used to eliminate the batch effect. Quality control is achieved through a set of functions provided by ChAMP, such as CpG.GUI, champ.QC, and

QC.GUI, and then MVP (Methylation variable position), DMR (Differently methylated regions), Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis are also implemented with ChAMP. Among them, MVP uses the method of Limma R package. Limma firstly establishes multiple linear regression model for all data as a whole, and then applies the regression model to each probe line and calculates the modified T-statistic and P-value in combination with the Bayesian model. The sites with $P \leq 0.05$ after the FDR correction were considered as differential methylation sites.

Cervical cancer data sets. The DNA methylation data from The Cancer Genome Atlas (TCGA)-Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma (CESC) were downloaded from TCGA website (<https://portal.gdc.cancer.gov/>). β -values were extracted to evaluate the DNA methylation level of each probe. The candidate significantly differentially methylated CpG sites of the HPV-18 group were screened using the 850 k methylation chip were verified in (TCGA-CESC).

Statistical analysis. Statistical analysis was performed using SPSS software (Release 13.0, SPSS Inc.) Statistical analysis data comparisons between two groups were analysed using the Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Methylation analysis and general characteristics. The 850K methylation sites in the HPV 16- and HPV 18-positive cervical carcinoma and normal cervical tissues were analysed using the Illumina 850K methylation chip (GSE169622). The data from cervical carcinoma and normal cervical tissue samples were normalized (negative and positive controls were provided by Genergy Co.) and processed. To analyse DNA methylation differences between the cervical cancer group and the normal group, $\Delta \beta$ and P-values were used to construct a volcanic map of CpG sites, in order to reflect the magnitude and statistical significance of differences ($P < 0.01$). As depicted in Fig. 1, the gene methylation profiles of HPV-16 (HPV-16 group) and HPV-18 (HPV-18 group) cervical carcinoma samples differed significantly from those of the control samples (normal group). In total, it was observed that 106,378 sites demonstrated differential expression in HPV 16-positive cervical carcinoma tissues compared to normal cervical tissues, of which 101,152 were hypermethylated and 5,226 were hypomethylated (Fig. 1A-a). In addition, 70,744 sites showed differential expression in HPV 18-positive cervical carcinoma tissues compared to normal cervical tissues, of which 53,168 were hypermethylated and 17,576 were hypomethylated (Fig. 1B-a). In addition, a cluster analysis map was generated to show the methylation status of different groups. As demonstrated in Fig. 1, there were significant differences in methylation patterns and states between cervical cancer and normal samples in the HPV-16 group and HPV-18 group. Thus, there may be biomarkers suitable for cervical cancer screening among these differential methylation sites.

GO functional analysis. The GO functional annotation analysis of the HPV-16 differentially expressed methylation sites revealed that these enriched genes were mainly involved in Biological Process, Cellular Component and Molecular Function (Fig. 2A). The results of cellular component analyses revealed that molecules distributed in the cell periphery, plasma membrane, cell junction and cell membrane components were significantly enriched. Important functions, such as cell migration, transport and synthesis of substances all occur place at these sites. At the molecular level, functional annotation analysis revealed that the highly enriched genes were related to calcium binding, protein binding, cytoskeletal protein binding, metal ion transmembrane transport activity and phosphotransferase activity (Fig. 2A). The GO functional annotation analysis of the HPV-18-differentially expressed methylation sites demonstrated that these genes were mainly enriched in Biological Processes, Cellular Component and Molecular Function (Fig. 2B). The biological processes of the two groups of methylation differential genes were mainly concentrated in the biological development process and anatomical structure development.

KEGG signalling pathway analysis. KEGG pathway functional analysis annotates and classifies the functions of pathways in the KEGG database according to whole genes and differential genes. The signalling pathways were further investigated using the KEGG database. According to the criteria of $P < 0.01$ and $FDR < 0.05$, the top 20 related signalling pathways of different methylation sites were selected (Fig. 3). The most prominent major signalling pathways for the HPV-16-positive samples were focal adhesion, pathways in cancer, glutamatergic synapse and the regulation of actin cytoskeleton, suggesting that these pathways are major regulatory factors of cancer behaviours (Fig. 3A). Additionally, focal adhesion, pathways in cancer, glutamatergic synapse and circadian entrainment signalling pathways were significantly upregulated in the HPV-18-positive samples (Fig. 3B). It was observed that focal adhesion and pathways in cancer are among the top pathways in the comparison of the two groups. This suggests that it may be possible to monitor the degree of cervical lesions by detecting gene differential methylation sites in these pathways.

Identification of different methylation sites in the HPV-16 and HPV-18 groups. Subsequently, the 3,000 methylation variable positions in genes with the most significant difference were further analysed by KEGG according to the selected significantly different signalling pathways, including focal adhesion and pathways in cancer from HPV-16- and HPV-18-positive samples. The results indicated that a total of 10 genes, including CHRM2, GNG4, LAMA4, CHAD, ITGA8, COL11A1, FGF10, IGF1, TEK and COL11A2, were screened from the focal adhesion and PI3K-AKT signalling pathways from the HPV-16 group. Moreover, the Gene network analysis revealed that the most significantly different CpG sites, cg24575234, cg20818778, cg14289461, cg06818777, cg08361126, cg06381931, cg08976810, cg20881548, cg08264401 and cg25459558, can be found in the CHRM2, GNG4, LAMA4, CHAD, ITGA8, COL11A1, FGF10, IGF1, TEK and COL11A2 genes, indicating their possible diagnostic roles in cervical cancer development

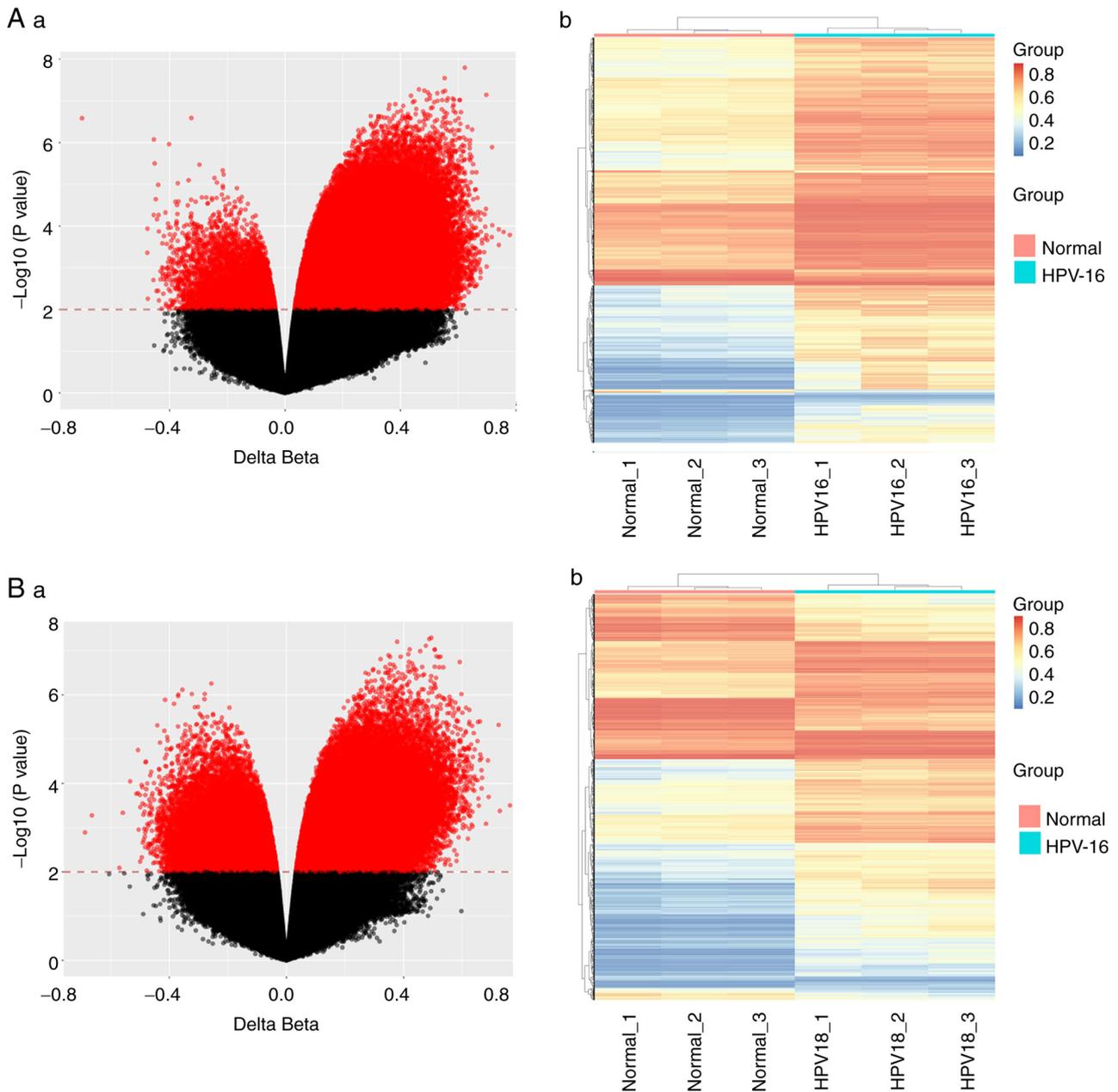


Figure 1. The magnitude and statistical significance of the methylation difference between the cervical cancer group and the normal group (A-a and B-a) were reflected by the volcano map ($P < 0.01$, The $\Delta \beta$ value > 0 corresponds to hypermethylation, and the $\Delta \beta$ value < 0 to hypomethylation). (A-b and B-b) The hierarchical cluster analysis is depicted by the heatmap of differentially methylated CpG company sites between the cervical cancer group and the normal group (DNA methylation levels are color-coded. Blue represents CpG with the lowest methylation level and red represents CpG with the highest methylation level). HPV, human papillomavirus.

(Table I). Some of these genes have already exhibited critical roles in several types of cancer, such as thymic, gastric, ovarian, breast, pancreatic and colorectal cancer. For examples, it has been shown that DNA methylation of GNG4 is a common epigenetic alteration in thymic carcinoma (46). LAMA4 and COL11A1 are associated with tumour invasion and metastasis (47,48).

For the HPV-18 group, the 10 most significantly different CpG sites, cg03520644, cg25792518, cg06958829, cg00172849, cg19707040, cg02501779, cg19679123, cg14427009, cg25993718 and cg27423357 was selected. The gene network analysis indicated that they were located in COL11A1, CHAD, CHAD, COL11A1, CTNNA2, CBLN4, SMAD3, PCDH17,

CBLN4 and FLT1 (Table II). In addition, the 10 selected methylation significantly different sites were verified in TCGA-CESC (Table III). All 10 sites exhibited statistically significant differences ($P < 0.05$), and seven sites demonstrated highly significant differences ($P < 0.01$, Table III).

Pyrosequencing verification. In total, 10 candidate significantly differentially methylated CpG sites of the HPV-16 group, including cg24575234, cg20818778, cg14289461, cg06818777, cg08361126, cg06381931, cg08976810, cg20881548, cg08264401 and cg25459558, screened using the 850k methylation chip were verified by pyrosequencing. Among these, cg25459558 was withdrawn from verification

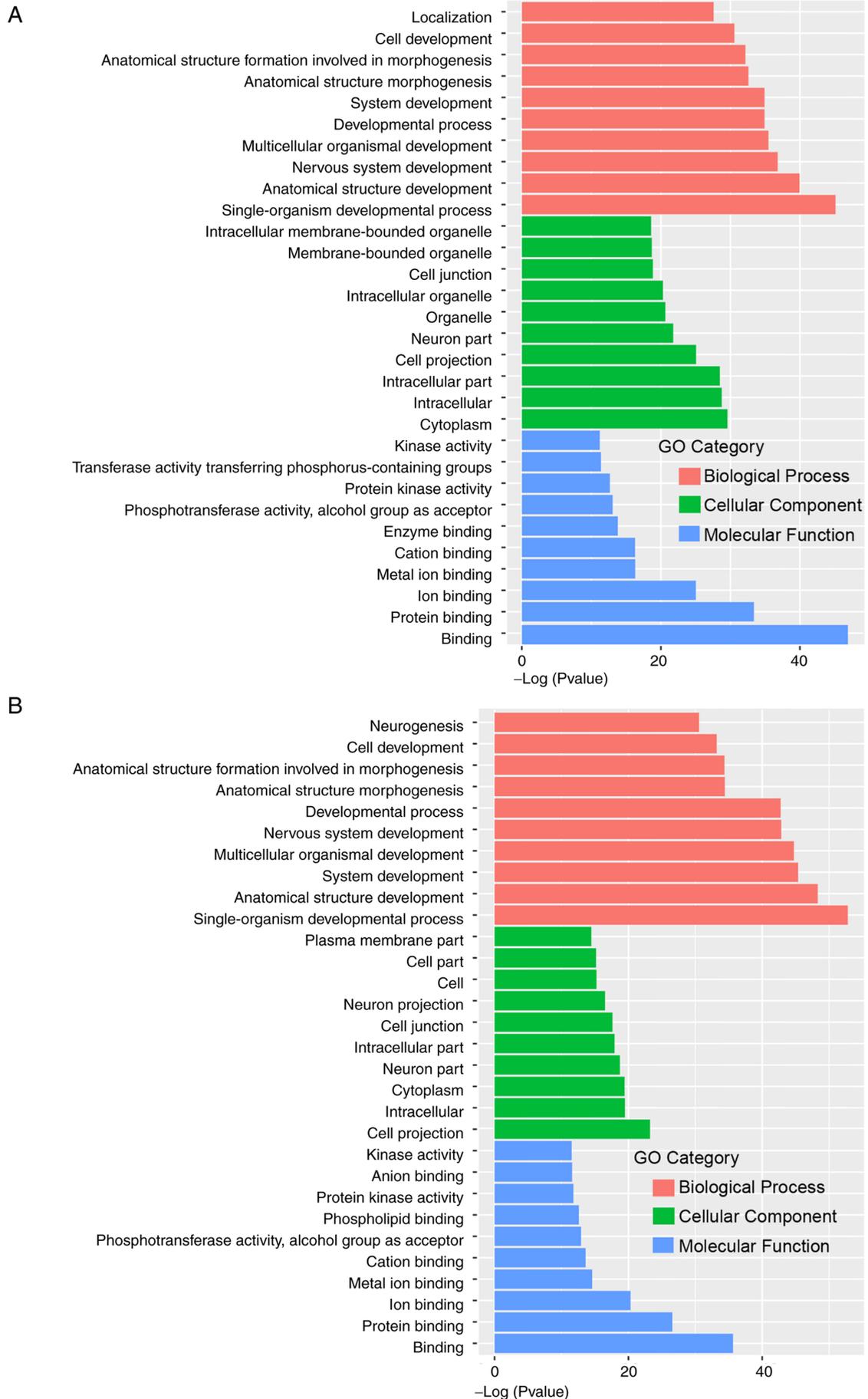


Figure 2. GO functional annotation analysis of the differentially expressed methylation sites from (A) HPV-16- and (B) HPV-18-positive samples. GO, gene ontology; HPV, human papillomavirus.

Table I. The top 10 methylation difference sites of HPV-16 group screened using the 850 k methylation chip.

Sequence	Probe ID	Normal group average methylation level	HPV-16 group average methylation level	Difference between two groups	P-value	FDR	Gene
1	cg24575234	0.0700±0.0234	0.6615±0.1325	0.592	0.000318	0.00888 ^b	CHRM2
2	cg20818778	0.1310±0.0074	0.6913±0.1583	0.560	0.000888	0.01466 ^a	GNG4
3	cg14289461	0.1687±0.0852	0.7237±0.1104	0.555	0.000511	0.011113 ^a	LAMA4
4	cg06818777	0.0654±0.0250	0.6105±0.1142	0.545	0.000241	0.007846 ^b	CHAD
5	cg08361126	0.0901±0.0376	0.6207±0.1280	0.531	0.000514	0.011149 ^a	ITGA8
6	cg06381931	0.1690±0.0502	0.6946±0.0608	0.526	4.34E-05	0.004157 ^b	COL11A1
7	cg08976810	0.0369±0.0098	0.5484±0.1995	0.511	0.003815	0.033664 ^a	FGF10
8	cg20881548	0.2406±0.0908	0.7451±0.0314	0.505	0.000138	0.006232 ^b	IGF1
9	cg08264401	0.2143±0.0175	0.7006±0.0315	0.486	1.74E-06	0.002313 ^b	TEK
10	cg25459558	0.0932±0.0189	0.5783±0.1569	0.485	0.00171	0.02099 ^a	COL11A2

P-values were obtained using a t-test for comparisons between normal control and HPV-16-positive groups. Variance is uneven; following logarithmic conversion, the data meets the parameter test conditions. ^aP≤0.05 and ^bP≤0.01, compared with the normal group. HPV, human papillomavirus.

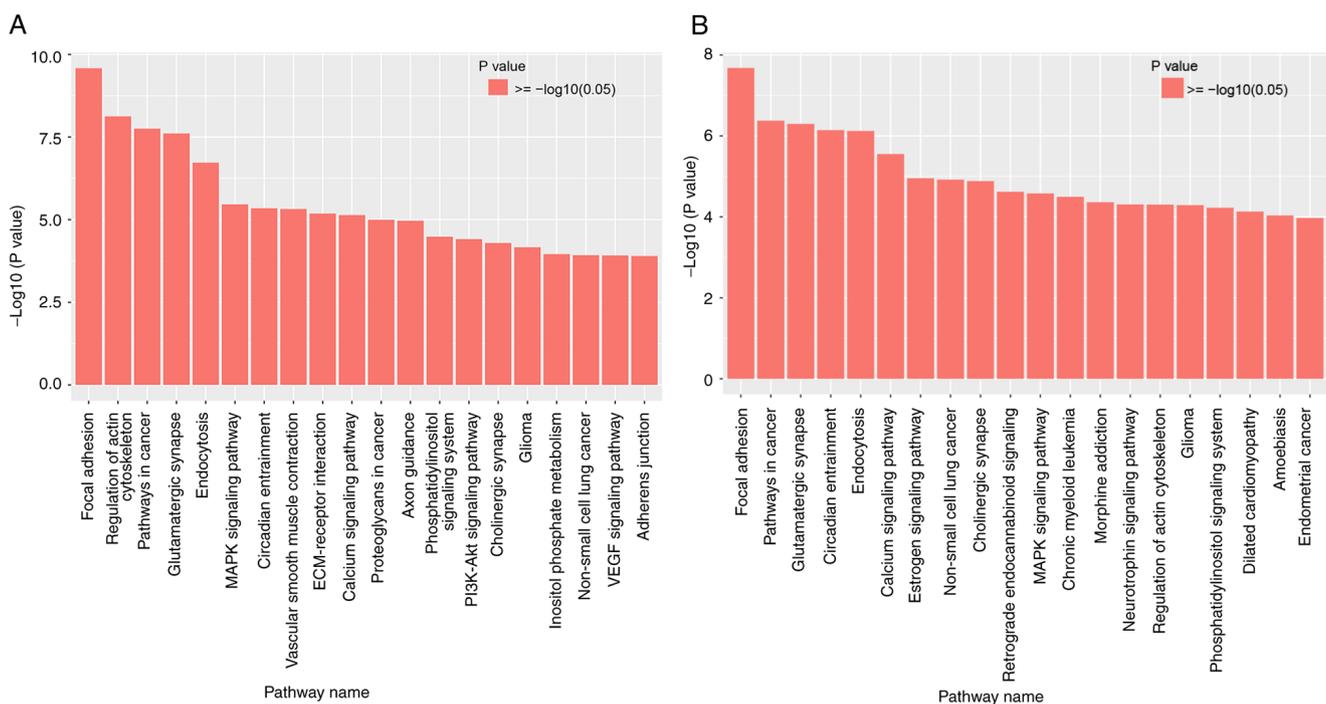


Figure 3. Methylation difference sites were associated with the top 20 signalling pathways in tested (A) HPV-16- and (B) HPV-18-positive samples ($P < 0.01$ and $FDR < 0.05$). HPV, human papillomavirus.

due to the failure of primer design. The statistical comparison of the mean value revealed that the other nine sites exhibited significant differences ($P < 0.05$), of which six sites exhibited highly significant differences ($P < 0.01$, Table IV). The statistical comparison of means revealed that the most significantly different CpG sites between the normal and HPV-16 samples cg24575234, cg14289461, cg06818777, cg08361126, cg06381931, cg08976810, cg20881548 and cg08264401, detected in the CHRM2, LAMA4, CHAD, ITGA8, COL11A1, FGF10, IGF1 and TEK. Unexpectedly, the increasing mean

value of the CpG site, cg20818778, for the gene GNG4 was not statistically significant (detailed statistical analysis data are contained in Table IV).

The mechanism of cervical cancer development remains unclear, while individual differences are significant. Further investigations of the methylation characteristics of a single gene and a single locus as a biomarker for cancer screening will have a high missed diagnosis rate. Multipoint joint detection is suggested for the future improvement of cancer detection rate.

Table II. The top 10 methylation difference sites of HPV-18 group screened using the 850 k methylation chip.

Sequence	Probe ID	G5 normal group average methylation level	G3 cervical cancer HPV-18- positive group average methylation level	Difference between two groups	P-value	FDR	Gene
1	cg03520644	0.1225±0.0342	0.3966±0.0241	0.2741	5.98E-05	0.009034 ^a	COL11A1
2	cg25792518	0.3841±0.0564	0.7650±0.0315	0.3809	8.44E-05	0.009827 ^a	CHAD
3	cg06958829	0.1182±0.0121	0.6611±0.0797	0.5430	4.17E-05	0.008166 ^a	CHAD
4	cg00172849	0.1219±0.0440	0.3997±0.0141	0.2778	8.70E-05	0.009885 ^a	COL11A1
5	cg19707040	0.1011±0.0136	0.4713±0.0504	0.3702	3.63E-05	0.007918 ^a	CTNNA2
6	cg02501779	0.1815±0.0145	0.7214±0.0734	0.5399	3.00E-05	0.007761 ^a	CBLN4
7	cg19679123	0.4993±0.0466	0.8142±0.0246	0.3149	8.42E-05	0.009826 ^a	SMAD3
8	cg14427009	0.2460±0.0267	0.7325±0.0381	0.4865	5.43E-06	0.006396 ^a	PCDH17
9	cg25993718	0.0971±0.0343	0.5181±0.0515	0.4211	4.20E-05	0.00819 ^a	CBLN4
10	cg27423357	0.3920±0.0364	0.7782±0.0226	0.3862	1.20E-05	0.006597 ^a	FLT1

P-values are obtained by using t test for comparisons between normal control and HPV-18-positive groups. Variance is uneven; following logarithmic conversion, the data meets the parameter test conditions. ^aP≤0.01, compared with the normal group. HPV, human papillomavirus.

Table III. The top 10 methylation difference sites of HPV-18 group screened using the 850 k methylation chip were verified in TCGA.

Sequence	Probe ID	Normal group	Cervical cancer group HPV-18-positive	t-test	P-value	Gene
1	cg03520644	0.616±0.0312	0.7381±0.0467	-20.847	<0.001 ^c	COL11A1
2	cg25792518	0.2926±0.1046	0.8885±0.0626	-8.464	0.001 ^b	CHAD
3	cg06958829	0.1529±0.0501	0.7040±0.0587	-12.366	<0.001 ^c	CHAD
4	cg00172849	0.1886±0.0464	0.6807±0.0938	-8.146	0.001 ^b	COL11A1
5	cg19707040	0.0350±0.084	0.5199±0.0944	-8.523	0.001 ^b	CTNNA2
6	cg02501779	0.1541±0.0400	0.6263±0.1349	-5.814	0.004 ^b	CBLN4
7	cg19679123	0.4073±0.1601	0.8318±0.0875	-4.030	0.016 ^a	SMAD3
8	cg14427009	0.2136±0.0373	0.6362±0.0527	-11.327	<0.001 ^c	PCDH17
9	cg25993718	0.1236±0.0384	0.5085±0.1573	-4.118	0.015 ^a	CBLN4
10	cg27423357	0.4072±0.0873	0.7177±0.1584	-2.975	0.041 ^a	FLT1

P-values are obtained by using t test for comparisons between normal control and HPV-18-positive groups. Variance is uneven; following logarithmic conversion, the data meets the parameter test conditions. ^aP≤0.05, ^bP≤0.01 and ^cP<0.001, compared with the normal group. HPV, human papillomavirus.

Discussion

Cervical cancer formation is affected by several risk factors, including HPV infection. It has been reported that HPV16 and HPV18 contribute to >70% of all cervical cancer cases worldwide and are thus entitled as a ‘high-risk’ HPV genotype (49-51). In some studies, HPV-16/18 genotyping is used as a molecular marker reflecting the underlying carcinogenic process. However, HPV infection is self-limiting and regresses in some clinical cases (30,52,53). It is unclear whether HPV infection acts as a key determinant of the progression to cervical cancer. Consequently, HPV positivity is not a specific diagnostic indicator for cervical cancer or diseases.

DNA methylation is one of the mechanisms that has been closely related to the occurrence and development of cervical cancer. The aberrant DNA methylation of human host cell genes or HPV genomic DNA has been closely associated with the dysfunction of various tumour suppressor genes during persistent high-risk HPV (HR-HPV) infection and cervical carcinogenesis (54-56). Studies have indicated that the tumour suppressor genes, p53 and p73, demonstrate a higher degree of methylation in cervical cancer samples than in normal samples (57,58). The aberrant DNA methylation of CpG islands is comparatively rare in normal cells, suggesting that the differentially methylated CpG sites between cervical cancer and normal samples have the potential to become reliable biomarkers of cervical cancer. In addition, DNA hypermethylation has been

Table IV. Verification of different top 9 methylation CpG sites in the HPV-16 group using pyrosequencing.

Sequence	Probe ID	Normal group	Cervical cancer group		t-test	P-value	Gene
			HPV-16				
1	cg24575234	8.68±1.46	33.69±5.71		9.495	<0.001 ^c	CHRM2
2	cg20818778	14.92±0.603	28.17±1.33 ^c		1.916	0.092 ^a	GNG4
3	cg14289461	10.62±3.91	48.76±16.40		5.059	0.001 ^b	LAMA4
4	cg06818777	7.86±2.17	44.13±2.54 ^c		2.775	0.024 ^a	CHAD
5	cg08361126	8.46±1.17	24.53±11.36 ^c		2.833	0.025 ^a	ITGA8
6	cg06381931	26.16±4.54	59.35±10.52		6.474	<0.001 ^c	COL11A1
7	cg08976810	9.39±1.68	52.08±12.63		7.492	<0.001 ^c	FGF10
8	cg20881548	36.35±4.50	60.61±10.68		4.684	0.002 ^b	IGF1
9	cg08264401	23.79±2.77	44.38±12.60		3.568	0.007 ^b	TEK

P-values are obtained by using t test for comparisons between normal control and HPV-16-positive groups. Variance is uneven; following logarithmic conversion, the data meets the parameter test conditions. ^aP≤0.05, ^bP≤0.01 and ^cP<0.001, compared with the normal group. HPV, human papillomavirus.

associated with long-term HR-HPV infection and is therefore considered a marker of cervical intraepithelial neoplasia lesion severity and invasive cervical cancer risk (59). However, the high heterogeneity of those previously published data renders it difficult to determine the appropriate methylation markers for cervical cancer screening (60). Additionally, the expression levels of E6 oncoprotein have a different effect on the carcinogenic potential of HPV. For example, the enhanced expression of the HPV-16 E6 oncogenes may trigger a neoplastic transformation of squamous epithelial cells at the uterine cervix (61). Thus, the HPV promoter methylation profile could be an easy and measurable biomarker for the examination of the high-risk HPV potential carcinogenicity.

In the present study, the genome-wide methylation level was evaluated by comparing HPV-16-positive or HPV-18-positive cervical cancer cases with normal cervical tissues. The results of the present study indicated that 106,378 and 70,744 sites demonstrated differential expression in HPV-16 and HPV-18 cervical cancer tissues as compared with normal cervical tissues, respectively, indicating that the distribution of methylation sites in cervical tissues varies greatly. It has been reported that hypermethylation at CpG islands (CGIs) of genes acting as tumour suppressors is a common mechanism involved in cancer occurrence (62–64). Other studies have also detected an apparently positive association between the hypomethylation of proto-oncogenes and the progression of cervical cancer. In the present study, 101,152 with higher methylation levels and 5,226 with lower methylation levels CGIs in HPV-16-positive cancer tissues than in normal cervical tissues were identified. By contrast, 53,168 CGIs with increased methylation levels and 17,576 CGIs with decreased methylation levels were identified in HPV-18-positive cancer tissues compared with normal cervical tissues. Genome-wide methylation level evaluation can retrieve additional differential methylation sites that have not been previously discovered.

Moreover, the differentially expressed methylation genes were analysed through GO functional annotation. It has been revealed that a number of methylated genes are closely associated with HPV-positive cervical cancer cases, including

SOX1, PAX1, JAM3, EPB41L3, CADM1 and MAL (65,66). For example, expression of SOX1 was shown to be associated with early embryogenesis, central nervous system development, and neural stem cell maintenance. Hypermethylated PAX1 has been detected in cervical carcinoma (67). The aforementioned methylated human gene biomarkers used in combination may be clinically useful for the triage of women with HR-HPV infections. The functional annotation data have previously demonstrated that the highly enriched genes were mainly involved in calcium binding, protein binding, cytoskeletal protein binding, metal ion transmembrane transport activity and phosphotransferase activity (68–71). The results of cellular component analyses revealed that molecules distributed in the cell periphery, plasma membrane, cell junction, and cell membrane components were significantly enriched. Further cluster analysis demonstrated that the differentially methylated genes covered a variety of different functional communities, indicating that there are many types of genes involved in the regulation of the occurrence and progression of cervical cancer (72–74).

Previous data indicate that a variety of cellular pathways can be affected by the methylation status of specific genes. KEGG pathway analysis in the present study revealed that differentially methylated genes were mainly involved in focal adhesion, regulation of actin cytoskeleton, and pathways in cancer. Among these pathways, the most significant pathways were focal adhesion and PI3K-AKT signalling pathways, which are a collection of receptors and ligands on the plasma membrane associated with intracellular and extracellular signalling pathways that regulate cell growth and cell migration. Based on the KEGG pathway analysis results, a total of nine genes, including CHRM2, GNG4, LAMA4, CHAD, ITGA8, COL11A1, FGF10, IGF1 and TEK, associated with nine significantly different CpG sites, cg24575234, cg20818778, cg14289461, cg06818777, cg08361126, cg06381931, cg08976810, cg20881548 and cg08264401, were screened from the focal adhesion and PI3K-AKT signalling pathways of the HPV-16-positive group. However, the pyrosequencing data of the present study indicated that the increasing

mean value of the CpG site, cg20818778, for the gene GNG4 was not statistically significant. Thus, the most significantly different CpG sites are cg24575234, cg14289461, cg06818777, cg08361126, cg06381931, cg08976810, cg20881548 and cg08264401, detected in the CHRM2, LAMA4, CHAD, ITGA8, COL11A1, FGF10, IGF1 and TEK, indicating their possible diagnostic roles in cervical carcinoma development.

Additionally, the 10 most significantly different CpG sites of the HPV-18-positive group, cg03520644, cg25792518, cg06958829, cg00172849, cg19707040, cg02501779, cg19679123, cg14427009, cg25993718 and cg27423357 in COL11A1, CHAD, CHAD, COL11A1, CTNNA2, CBLN4, SMAD3, PCDH17, CBLN4 and FLT1 were selected, indicating their future applications as candidate molecular markers of cervical cancer. Furthermore, the 10 selected methylation significantly different sites were verified in TCGA-CESC. All 10 sites exhibited significant differences ($P < 0.05$), and seven sites demonstrated highly significant differences ($P < 0.01$, Table III).

Some of the genes screened in the present study have already demonstrated potential functions in other diseases. Several studies have revealed that CHRM2 is associated with the pathophysiology of schizophrenia (75). GNG4 has been associated with immune infiltration in the tumour microenvironment, which promotes tumour cell migration and proliferation (76-78). LAMA4 can regulate the proliferation and migration of gastric cancer cells (47) and may be a potential gastric cancer prognostic biomarker and therapeutic target (79). ITGB8 silencing inhibits the invasion and migration of lung cancer cells (80). Increased expression of COL11A1 has been detected in several in various types of cancer, such as ovarian, breast, pancreatic and colorectal cancer, and increased levels of COL11A1 are often associated with poor survival, chemoresistance and recurrence (48). Fgf10 induces migration and invasion of pancreatic cancer cells (81). CHAD, however, has been reported to be involved in confronting hepatocellular carcinoma migration and proliferation and predicting good survival (82). In addition, PCDH17 methylation has been reported as a potential prognostic biomarker in some cancer patient markers, including postoperative renal cell carcinoma (83). Validating whether these markers can be used as novel tools for cervical cancer screening and investigating their role in normal cervical and cervical cancer development will be the focus of our future research.

In summary, the host cell gene methylation test may be a promising method for cervical cancer screening. The Illumina Human Methylation 850K BeadChip methylation chip was used for the methylation site detection in HPV 16- and HPV 18-positive cervical carcinoma and normal cervical tissues. The current findings suggested that the methylation modification sites in cervical carcinoma cells may be abnormal. Hypermethylation and hypomethylation sites occur more frequently and are mainly enriched in functional categories, including focal adhesion, regulation of actin cytoskeleton, tumour growth, and pathways in cancer. The eight most significantly different CpG sites, cg24575234, cg14289461, cg06818777, cg08361126, cg06381931, cg08976810, cg20881548 and cg08264401, were screened and verified from HPV-16-positive samples and were associated with the CHRM2, LAMA4, CHAD, ITGA8, COL11A1, FGF10, IGF1 and TEK genes. The 10

most significantly different CpG sites of the HPV-18-positive group, cg03520644, cg25792518, cg06958829, cg00172849, cg19707040, cg02501779, cg19679123, cg14427009, cg25993718 and cg27423357, which are located in COL11A1, CHAD, CHAD, COL11A1, CTNNA2, CBLN4, SMAD3, PCDH17, CBLN4 and FLT1, were selected and verified in TCGA-CESC. It is important to explore and develop DNA methylation assays of improved sensitivity and specificity in order to ameliorate the early detection of cervical cancer (84-86). The findings of the present study may provide fundamental data for the use of methylation biomarkers for cervical cancer diagnosis; however, further research is required.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The Illumina 850K methylation chip analysis data have been uploaded in the GEO public database repository (accession number: GSE169622, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169622>).

Authors' contributions

JQ and TC conceived and designed this study. YM and CW were responsible for performing the experiments and writing the first draft of the manuscript. JQ was responsible for revising the first draft of the manuscript. MS and ML collected and analysed the data. LL and YM interpreted the data of DNA methylation. JQ and TC confirm the authenticity of all the raw data. All the authors critically reviewed the original manuscript, edited and approved the final version. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. The study was approved by the Medical Ethics Examination of Lanzhou First People's Hospital, China (2016-02).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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