The Identification and Validation of EphA7 Hypermethylation, a Novel Biomarker, in Cervical Cancer

Wenfan Zhang

Tianjin Medical University

Huiling Cao

Tianjin Medical University General Hospital

Jing Zhao

Tianjin Medical University General Hospital

Jinhao Yang

Tianjin Medical University

Zheng Liang

Tianjin Medical University General Hospital

Xiaodong Kang

Tianjin Medical University

Rong Wang (✉ wangrong825@tmu.edu.cn)

Tianjin Medical University <https://orcid.org/0000-0003-3502-5446>

Research

Keywords: EphA7, DNA methylation, CRISPR-dCas9, Cervical cancer

Posted Date: November 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1064970/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Aberrant methylation of EphA7 has been reported in the process of carcinogenesis, but not including cervical cancer. Therefore, an integration study was performed to explore the association between EphA7 hypermethylation and cervical cancer and validate the potential value of EphA7 hypermethylation in the diagnosis of cervical cancer.

Results

Here, we performed an integration study to identify and validate the association between EphA7 methylation and cervical cancer. First, data on EphA7 methylation and expression in cervical cancer were extracted and analyzed via bioinformatics tools. The results showed that EphA7 promoter methylation levels were significantly increased in cervical cancer compared to normal tissues ($P < 0.001$) and negatively correlated with EphA7 expression. Subsequently, these prediction results were confirmed in cell lines; moreover, CRISPR-based methylation perturbation tools (dCas9-Tet1/DNMT3a) were constructed to further demonstrate that DNA methylation participates in the regulation of EphA7 expression directly. Ultimately, the clinical value of EphA7 methylation in cervical cancer was validated in cervical tissues and Thinprep cytologic test (TCT) samples by methylation-specific PCR (MSP) and quantitative methylation-specific PCR (QMSP), respectively. Consistently, the methylation level and the positive rate of EphA7 gradually increased with severity from normal to cancer stages in TCT samples ($P < 0.01$).

Conclusion

Above all, EphA7 presents hypermethylation in the cervical cancer, which is a potential biomarker in the diagnosis of cervical cancer.

Background

Cervical cancer (CC) is the fourth most commonly diagnosed cancer and the fourth leading cause of cancer death in women. Approximately 604,000 new CC cases and 342,000 cases of death were recorded worldwide in 2020(1). Recently, the World Health Organization (WHO) called for global action toward the elimination of CC by 2030(2). This is a challenge for China, with an estimated 109,741 new cases and 59,060 deaths in 2020 according to WHO. Cervical carcinogenesis is a complex process with multiple factors and stages(3). In addition to high-risk human papillomavirus (hr-HPV) infection, epigenetic abnormalities, especially alterations in DNA methylation, are involved in the development of CC(4, 5).

Eph receptors represent the largest family of receptor tyrosine kinases. Including the physiological roles, recent studies revealed deregulation of some of the Eph genes in human malignancies(6). For example,

EphA1 and EphA4 are upregulated in gastric cancer(7), and EphA8 stimulates the proliferation, invasion, and migration of gastric cancer cells(8). EphA2 overexpression in mammary epithelial cells induces tumorigenesis (9).

As a member of the Eph receptor group, EphA7 is associated with carcinogenesis as well(10) but plays a contradictory role in different cancers(11–14). EphA7 is overexpressed in hepatocellular carcinoma(15), glioblastoma multiform(11), gallbladder adenocarcinoma(16) and lung carcinoma(17), which contribute to malignant transformation, aggressive progression, and poor prognosis. In contrast, EphA7 may act as a tumor suppressor since EphA7 downregulation is induced by promoter hypermethylation in prostate cancer patients(18). Similar results have also been observed in gastric carcinoma(19), colorectal cancers(20), and oral squamous cell carcinoma(21). Wang J (19, 20)confirmed that the promoter of EphA7 was hypermethylated because EphA7 expression was restored after demethylation treatment with 5-aza-2-deoxycytidine (5-aza-dc). However, the role of EphA7 in CC remains unclear. Therefore, this study aims to reveal the association of EphA7 methylation and cervical cancer and to validate the potential value of EphA7 methylation in the clinical diagnosis of CC.

Methods

Pool analysis from databases and web tools

Gene expression and DNA methylation data of EphA7 in cervical tissues were extracted from The Cancer Genome Atlas (TCGA, <https://cancergenome.nih.gov/>) and Genotype-Tissue Expression (GTEx, <https://commonfund.nih.gov/GTEx>) databases. The expression data of EphA7 were collected from GEPIA (<http://gepia2.cancer-pku.cn/>)(22) based TCGA and GTEx. The web tools UALCAN (<http://ualcan.path.uab.edu/>)(23) and Wanderer (<http://maplab.imppc.org/wanderer/>) (24) were utilized to compare the level of methylation within adjacent and tumor tissues. The relationships between EphA7 expression and methylation were found by MEXPRESS (<https://mexpress.be/>)(25). JASPAR(<http://jaspar.genereg.net/>) (26) was applied to predict the transcription factors that bind to the promoter of EphA7. LinkedOmics (<http://www.linkedomics.org/login.php>) (27) and the Human Protein Atlas (<https://www.proteinatlas.org/>) were used to explore DNA methylation and the expression and survival of EphA7, respectively, based on the TCGA database.

Cell lines and clinical sample collection

Cell culture

Human CC cell lines, including CaSki and SiHa, and a human embryonic kidney cell line, were purchased from Zhong Qiao Xin Zhou (Shanghai, China) ,which have tested for mycoplasma by PCR and were authenticated using STR profiling. The above 3 cell lines were cultured in RPMI 1640, MEM and DMEM (HyClone, USA) supplemented with 10% fetal bovine serum (BI, USA) and 1% penicillin-streptomycin (Solarbio, China) and incubated at 37 °C in 5% CO₂ and saturated humidity.

Clinical specimens

Cervical frozen tissues and TCT specimens were obtained from the Department of Gynecology and Obstetrics, Tianjin Medical University General Hospital from January 2016 to June 2019. This study was approved by the Medical Ethics Committee of Tianjin Medical University General Hospital, and all patients gave informed consent. None of the patients received radiotherapy or chemotherapy prior to surgery. According to the results of the pathological diagnosis, patients in the study group were divided into normal, cervical intraepithelial neoplasia II (CIN_{II}), CIN_{III} and cancer groups.

A total of 57 frozen tissue specimens were collected for the test. The frozen tissue samples consisted of 25 normal, 24 CIN_{II/III} and 8 cancer samples and were stored at -80 °C. In addition, 114 TCT specimens were collected, including 28 normal cervical samples, 24 cases of CIN_{II} stage, 45 cases of CIN_{III} stage and 17 cases of CC specimens. Specimens were obtained using a disposable cervical specimen collection brush and stored in TCT preservation solution (BD Surepath, USA) at 4 °C.

Selection of sgRNA sequences and creation of U6-sgRNA PCR cassettes

The sgRNAs were designed to target the interesting CpG island of EphA7 using the public tool CRISPR-ERA (<http://crispr-era.stanford.edu/>) as previously described(28). U6-sgRNA PCR cassettes were created from an sgRNA vector (#84477) by using primers (Table 1) that amplify a U6 promoter fused to the single guide sequence (sgRNA) reverse complement and sgRNA forward complement fused to the remaining sgRNA scaffold cassettes, followed by another round of overlap-extension PCR to amplify the full U6-sgRNA PCR cassette. Fuw-dCas9-Tet1CD (#84475), Fuw-dCas9-Tet1CD_IM (#84479), Fuw-dCas9-DNMT3a (#84476) and Fuw-dCas9-DNMT3a_IM (#84478) plasmids were purchased from Addgene(29) . Three active sgRNAs (act-sgRNAs) for demethylation and 4 repressive sgRNAs (rep-sgRNAs) for increasing methylation were designed and listed in Table S1; Please see Fig. 1 for the gene map and sgRNA sites.

Transient transfection

Plasmids of dCas9-Tet1 or dCas9-DNMT3a with U6-sgRNA cassettes were transfected into cells in 6-well plates using OMNIfect Transfection Reagent (Transomic Technologies, USA) according to the manufacturer's instructions. The ratio of dCas9-Tet1/-DNMT3a to sgRNA individually was 1:1. For the experiments in which sgRNAs were transfected together, the amount of each sgRNA was equal to aliquoted parts of the total amount of sgRNA. Plasmids of dCas9-Tet1CD-IM (Tet1^m) or dCas9-DNMT3a-IM (Dnmt3a^m) were considered negative controls.

RNA extraction and quantitative real-time PCR (qRT-PCR)

The harvested cells were dissolved in TRIzol® reagent (Invitrogen, USA), and total mRNA was then extracted following the manufacturer's protocol. QRT-PCR was performed using SYBR Green PCR Mix (Tiangen, China) and a Stratagene Mx3005P sequence detection system (StrataGene, Agilent, USA). The

amplification parameters were as follows: 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. The $2^{-\Delta\Delta C_t}$ method was performed with the GAPDH gene as an internal control, and the relative quantification procedure was selected. The primer sequences are listed in Table S2.

MSP (Methylation-specific PCR)

Primers were designed via Methy Primer Express v1.0 (Applied Biosystems, USA) and synthesized in Sangon Biotech (Shanghai, China). The primers were shown in Table S2. In total, 1.5 µl bisulfite-treated DNA were amplified in a 30 µl reaction mixture consisting of 1x PCR Buffer with 0.5U AmpliTaq Gold DNA polymerase (Applied Biosystems, USA), 0.2 mM dNTP mix, and 0.3 µM of each primer. The PCR conditions were as follows: 95 °C for 10 min, then 40 cycles of 95°C for 60 s, 55°C for 60 s, 72°C for 1 min and finally an elongation step of 7min at 72°C. PCR products were separated on a 2% agarose gel, prestained with Gelred (Shanghai Life iLab Bio, China) and visualized by UV transillumination. Leukocyte (leu) DNA from healthy women was used as a negative control, and in vitro methylated (iv) leukocyte DNA was used as a positive control.

QMSP (Quantitative methylation-specific PCR)

Quantitative methylation specific PCR was performed with a double-quenched (FAM/IBHQ) labelled hybridization probe. The methylated primers for QMSP were the same as those used for MSP. The probes were designed using Cone Manager 9.0 software and synthesized by Sangon Biotech (Table S2). Twenty-five ng bisulfite-converted DNA was utilized for PCR with 0.3 µM primers, 0.2 µM probe and 1x QuantiTect Probe PCR Master Mix (Qiagen, Germany) in 10 µl reaction for 50 cycles in a 7900HT Fast RealTime PCR System (Applied Biosystems). The ACTB gene was used as a methylation independent internal reference gene. The criteria for the interpretation of positive methylation results were as follows: C_t value < 50 (at least 2 of 3 multiple wells) with sufficient methylated DNA (200 pg DNA). The relative level of EphA7 methylation was analyzed as previously described (30).

Pyrosequencing

Pyromark PCR and sequencing primers were designed and generated by the instructions of PyroMark Assay Design 2.0 (Table S2). PCR amplification was carried out and optimized using a PyroMark® PCR Kit (Qiagen, Hilden, Germany) in a total reaction volume of 25 µl. The PCR volume was 25 µl with 0.2 µM primer mix, 1x PyroMark master mix, 1x CoralLoad Concentrate and 1 µl bisulfite-modified DNA. PCR testing was carried out at 95 °C for 10 min, followed by 45 cycles (94°C for 30 s, 55°C for 30 s, and 72 °C for 30 s, with a final extension of 72 °C for 10 min). The obtained PCR products were then subjected to pyrosequencing (PyroMark Q24 system; QIAGEN) on the PyroMark Q24 platform.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22.0 (IBM Corporation, New York, USA) and Graphpad Prism 8.0 (GraphPad Software, USA). Students' t test was used to compare differences in

median methylation or expression levels between two groups conforming to normal distribution, whereas a Mann-Whitney U test was used for data not conforming to normal distribution. The correlation between the expression and methylation level was investigated via Pearson correlation coefficients. The Kaplan–Meier method was used for survival analysis, and a log-rank test was applied for comparisons between groups. A P -value below 0.05 was considered to be significant.

Results

EphA7 is significantly hypermethylated in CC tissues

From the UALCAN website, the promoter of EphA7 was hypermethylated in 15 out of 23 categories of cancer ($P < 0.05$) compared to their adjacent normal tissues (Fig. 2A). Furthermore, the difference in EphA7 hypermethylation in cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) was the most prominent compared with other cancers, and the median beta value was listed in Table S3.

Therefore, we further analyzed the methylation status of the EphA7 gene from the TCGA database using Wanderer, an online tool. As shown in Fig. 2B, there were a total of 21 HumanMethylation 450 probes located in the EphA7 gene and 11 probes in the promoter region exhibiting significant differences ($P < 0.05$) between tumor tissues and adjacent normal specimens. The differences in methylation in global EphA7 were summarized in Table S4.

Subsequently, pyrosequencing was performed to quantify the methylation level of EphA7 at each CpG site along the sequence positions of the promoter. Compared to normal cervical tissues, the average methylation level of EphA7 was 4.09-fold higher in tumors within a total of 11 CpG sites ($P < 0.05$) (Fig. 2C).

Promoter methylation of EphA7 is inversely correlated with gene expression in CC

Global EphA7 abnormally methylated sites were observed via MEXPRESS with all 21 HumanMethylation450 probes. Among them, 19 probes showed significantly negative expression (Pearson correlation coefficients from -0.211 to -0.522), and the correlation was significantly more pronounced at the highlighted region, which focused on the EphA7 promoter (Fig. 3A).

This inverse correlation was further proven by the UCLCAN and GEPIA web tools. As presented in Fig. 3B and 3C, EphA7 was notably hypermethylated and downregulated in cervical tumors compared with normal tissues ($P < 0.05$).

Consistent with the bioinformatic findings, the expression level of EphA7 was significantly decreased in the CaSki and SiHa cell lines compared with the HEK293T cell line ($P < 0.001$) (Fig. 3D). However, EphA7 showed methylation bands in CaSki and SiHa cells, which were not observed in HEK293T cells via MSP (Fig. 3E).

DNA methylation plays a direct role in EphA7 gene regulation

To confirm that DNA methylation affected EphA7 expression directly, a set of CRISPR-based DNA methylation regulation tools (dCas9-Tet1/DNMT3a) was developed to target the EphA7 promoter region.

Since EphA7 was hypermethylated in CC cells (CaSki and SiHa), we combined the 3 sgRNAs targeted for activation with the dCas9-Tet1 plasmid for demethylation to upregulate EphA7 expression. In CaSki cells, the sgRNA1 (6.20-fold) and sgRNA 3 (2.04-fold) groups had significantly increased EphA7 gene expression compared to the control group (Tet1^m) (Fig. 4A). However, it did not improve more in the combination of all three sgRNAs (sgRNA1+2+3) (1.49-fold). Then, we performed pyrosequencing to detect the methylation level in the promoter region of EphA7 in the sgRNA1 group in CaSki cell lines. As expected, compared with the control (Tet1^m) group, the global methylation level was decreased, and the average methylation level was decreased by 19.10% in CaSki (Fig. 4B, C, Table S5).

In SiHa cells, sgRNA1 (7.54-fold), sgRNA2 (6.80-fold) and sgRNA (all) (5.49-fold) effectively increased EphA7 gene expression (Fig. 4D). In the sgRNA1 group, similar to the results from CaSki, the average methylation level decreased by 17.23% (Fig. 4E, F, Table S3).

Conversely, we selected 4 repression sgRNAs with the dCas9-DNMT3a plasmid to increase methylation to downregulate gene expression in the EphA7 unmethylated HEK293T cell line. As expected, the EphA7 mRNA level decreased by 69.05% (sgRNA1 group), 72.75% (sgRNA2 group), 86.23% (sgRNA3 group), 42.50% (sgRNA4 group), and 83.04% (sgRNA1+2+3+4 group) in each sgRNA group individually and together (Fig. 5A). In the sgRNA2 group, the pyrosequencing results showed that the average CpG sites of the promoter increased nearly 23.67%. (Fig. 5B, C, Table S6).

Validation of the potential clinical value of EphA7 methylation in the diagnosis of CC

Methylation-specific PCR (MSP) was performed in 57 cervical tissues, and the methylation positive rate in tissues was 0% (0/25) in normal tissues, 45.83% (11/24) in CINII/III, and 100% (8/8) in tumor tissues (Fig. 6A). Full-length gel pictures were presented in Supplementary Fig. S1.

Quantification methylation-specific PCR (QMSP) were measured for total 114 TCT specimens. The results showed that the methylation level increase with the progression of disease for cervical ($P<0.05$), the positive detection rates of methylation were 0% (0/28), CIN 4.16% (1/24), and CIN 11.11% (5/45), and cancer 58.82% (10/17) (Fig. 6B), and the methylation level of EphA7 increased with the severity of cervical lesions ($H=27.311$, $P<0.001$).

Furthermore, Kaplan-Meier curves demonstrated that lower methylation was correlated with an increased patient survival period via LinkedOmics (Fig. S2A). The Human Protein Atlas also confirmed that high expression of EphA7 was associated with improved survival (Fig. S2B).

Discussion

With the rapid development of bioinformation knowledge, numerous studies have suggested that the field of precision oncology will greatly benefit from a multiomics analytical approach(31–33). Currently, a branch of nomic-associated datasets and web analysis tools exists that indeed opens a door to explore more novel biomarkers more deeply and widely. However, this novel opportunity also requires creative and skillful solutions to accommodate noisy, unstructured information to offer valuable biological insights. Hypermethylation-mediated silencing of tumor suppressor genes is a critical event in the initiation and progression of various malignancies(34) and is also recognized as a potential biomarker of human cancer. Aberrant methylation of EphA7 has been reported in the process of carcinogenesis. However, until now, there have been no reports about the relationship between EphA7 and CC; therefore, we developed a pipeline with both “dry-lab data and wet-lab experiments” to uncover EphA7 methylation in CC. First, we initially investigated EphA7 methylation via multiweb tools with data from TCGA and GTEx. TCGA is a public database that includes 33 cancer types and matched clinical data. GTEx is a kind of normal tissue expression dataset that contains RNA-seq profiles for hundreds of demographically diverse healthy individuals in multiple human tissues and cell types. From these two datasets, it is feasible to compare the EphA7 methylation and expression levels in CC and normal tissues. However, because the raw data are difficult to interpret from datasets, we explored EphA7 hypermethylation in CC tissues with UALCAN and Wanderer web tools. By MEXPRESS, the methylation level in the promotor region was negatively correlated with the expression, which suggests that EphA7 is a suppressor gene in cervical carcinogenesis. LinkedOmics results demonstrated that lower methylation notably increased the patient survival period. Then, because the bioinformatical results could be verified in cell lines, we further moved to real tissue and clinical TCT samples, and both demonstrated EphA7 hypermethylation in CC.

Additionally, although decades of research have focused on the biological function of DNA methylation, the role of DNA methylation in targeted gene expression remains unclear due to a lack of molecular tools to precisely modify DNA methylation in the genome(35). Recent emergence of the CRISPR system has allowed for precise and stable editing of the epigenome at targeted sgRNA by fusing catalytically dead Cas9 (dCas9) to DNA methylation-modifying enzymes such as DNMTs and TETs (36). DNMTs target cytosines and facilitate methylation of CpG islands, which contributes to the regulation of gene expression(37). DNA methylation is maintained by the methyltransferase DNMT1, while DNMT3a and DNMT3b provide de novo CpG methylation(38). In contrast, TET promotes the process of DNA demethylation. Both DNMTs and TETs activities play important roles in the dynamic changes in DNA methylation(39, 40). X.S. Liu et al.(29) constructed the dCas9-Tet1/DNMT3a vector by fusing Tet1 or DNMT3a with the dCas9 protein for re-editing DNA methylation in mice. Moreover, they determined that the CRISPR vector system has better methylation editing efficiency than the TALE vector(29) and that the off-target effect of dCas9-Tet1 on the editing DNA methylation is minimal(41). Therefore, to establish that DNA methylation plays a direct role in EphA7 expression, both CRISPR-dCas9-Tet1, which targets demethylation, and CRISPR-dCas9-DNMT3a, which targets methylation, were applied in our study. As expected, with the dCas9-Tet1 and sgRNA tools, EphA7 was activated with loss of methylation in the promoters of CaSki (19.10%) and SiHa (17.23%), respectively. Simultaneously, we induced a 23.67% increase in methylation in the first exon of EphA7 via dCas9-DNMT3a, and the expression was indeed

downregulated. These results offer strong evidence that EphA7 DNA methylation participates in cervical carcinoma through perturbation of gene regulation.

Furthermore, the efficiency of the CRISPR tools in our study is comparable with the results of other studies. Nozomi Hanzawa et al.(42) reduced DNA methylation by 31.5% and 27.6% with different single sgRNAs. The X.S. Liu and J.Wu et al. groups induced gene hypermethylation to approximately 20~30% hypermethylation by dCas9-DNMT3a (29, 43). Although, Nozomi Hanzawa et al.(42) mentioned that co-transfected sgRNAs are known to result in additively DNA demethylation levels (approximately 40%)(42), the efficiency is equal to that of individual sgRNAs when multiple sgRNAs bind in the same region but on the opposite strand, serving as competing sgRNAs (44). Consistent with those results, EphA7 expression was not enhanced more when all the sgRNAs were combined in our studies.

Additionally, DNA methylation may disrupt the binding of transcription factors to regulate the regions of target genes (45), or vice versa, the binding of transcription factors to those regions prevents their methylation(46). In our study, one of the CpG sites of EphA7 presented much lower methylation (Fig. 2C,4E)and almost no difference in the demethylation group and control. Hence, we investigated the possible disrupted transcription factor via *JASPAR*(26). The results show that Yin Yang 1 (YY1) and transcription factor AP-2 alpha (TFAP2A) are closely related to DNA methylation. The YY1/polycomb group (PcG) protein/DNMT complex maintains gene methylation and contributes to gene inactivation(47). DNA methylation could increase the binding of the transcription factor TFAP2A to the target site, leading to suppressed gene expression(48). This could be a reason for the low methylation level in the 8th CpG site(Fig. 2C,4E),but the practical implications require evaluation in a separate study.

Conclusion

Our study is the first to systematically investigate the hypermethylation of EphA7 in CC and employs the CRISPR-dCas9 tools to precisely modify the CpG island in the promoter, which successfully provides direct evidence that EphA7 methylation is involved in its expression. CRISPR provides powerful tools to investigate the functional significance of DNA methylation in a locus-specific manner. EphA7 hypermethylation has the potential to be a biomarker for the diagnosis and screening of CC. In the future, we plan to perform a large cohort evaluation to advance the translation research.

Abbreviations

BLCA: Bladder urothelial carcinoma; BRCA:Breast invasive carcinoma;CC:Cervical cancer; CESC:Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL:Cholangiocarcinoma; CIN:Cervical intraepithelial neoplasia; COAD:Colon adenocarcinoma; CRISPR: Clustered regularly interspaced short palindromic repeats; ESCA:Esophageal carcinoma; GBM:Glioblastoma multiforme; GTEX: Genotype Tissue Expression; HEK293T:Human embryonic kidney 293T;HNSC:Head and Neck squamous cell carcinoma; hr-HPV: high-risk human papillomavirus;KIRC:Kidney renal clear cell carcinoma;KIRP:Kidney renal papillary cell carcinoma; LIHC:Liver hepatocellular carcinoma;LUAD:Lung adenocarcinoma;

LUSC:Lung squamous cell carcinoma; MSP: Methylation-specific PCR; PAAD:Pancreatic adenocarcinoma; PCPG:Pheochromocytoma and Paraganglioma;PRAD:Prostate adenocarcinoma; QMSP: Quantitative methylation-specific PCR; qRT-PCR: Quantitative Real-time;sgRNA:single guide RNA; READ:Rectum adenocarcinoma;SARC:Sarcoma; TCGA: The Cancer Genome Atlas; TCT:Thinprep cytologic test; TFAP2A:Transcription factor AP-2 alpha;TGCT:Testicular Germ Cell Tumors; STAD:Stomach adenocarcinoma; THCA:Thyroid carcinoma;THYM:Thymoma; UCEC:Uterine Corpus Endometrial Carcinoma; WHO: World Health Organization; 5-aza-dc: 5-aza-2-deoxycytidine;YY1:Yin Yang 1.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

RW was team leadership and responsible for whole project administration. WFZ and HLC carried out all the experiments and wrote the Manuscript.JZ and JHY contributed to collect samples and validate results. ZL and XDK analyzed data and provided the advice.All authors have read and approved the manuscript.

Funding

This study is supported by National Natural Science Foundation of China (81601836) and Special fund for technological innovation guidance of Tianjin Science and technology program,China(21YDTPJC00720).

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Tianjin Medical University General Hospital.

Consent for publication

Not applicable

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author details

¹ Department of Laboratory Medicine, Tianjin Medical University, Tianjin, China. ²Department of Gynecology and Obstetrics, Tianjin Medical University General Hospital, Tianjin, China.

³Department of Otorhinolaryngology, Tianjin Medical University General Hospital, Tianjin, China. ⁴Department of Medical Technology, Tianjin Medical University, Tianjin, China

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021.
2. Simelela PN. WHO global strategy to eliminate cervical cancer as a public health problem: An opportunity to make it a disease of the past. *Int J Gynaecol Obstet*. 2021;152(1):1-3.
3. Yao J, Li Z, Yang Z, Xue H, Chang H, Zhang X, et al. Long noncoding RNA TOB1-AS1, an epigenetically silenced gene, functioned as a novel tumor suppressor by sponging miR-27b in cervical cancer. *Am J Cancer Res*. 2018;8(8):1483-98.
4. El Aliani A, El-Abid H, El Mallali Y, Attaleb M, Ennaji MM, El Mzibri M. Association between Gene Promoter Methylation and Cervical Cancer Development: Global Distribution and A Meta-analysis. *Cancer Epidemiol Biomarkers Prev*. 2021;30(3):450-9.
5. Ohm JE, McGarvey KM, Yu X, Cheng L, Schuebel KE, Cope L, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat Genet*. 2007;39(2):237-42.
6. Surawska H, Ma PC, Salgia R. The role of ephrins and Eph receptors in cancer. *Cytokine Growth Factor Rev*. 2004;15(6):419-33.
7. Inokuchi M, Nakagawa M, Baogok N, Takagi Y, Tanioka T, Gokita K, et al. Prognostic Significance of High EphA1-4 Expression Levels in Locally Advanced Gastric Cancer. *Anticancer Res*. 2018;38(3):1685-93.
8. Wang YJ, Zhou N, Li P, Wu H, Wang QQ, Gao XD, et al. EphA8 acts as an oncogene and contributes to poor prognosis in gastric cancer via regulation of ADAM10. *J Cell Physiol*. 2019;234(11):20408-19.
9. Zelinski DP, Zantek ND, Stewart JC, Irizarry AR, Kinch MS. EphA2 overexpression causes tumorigenesis of mammary epithelial cells. *Cancer Res*. 2001;61(5):2301-6.
10. Wu X, Yan L, Liu Y, Xian W, Wang L, Ding X. MicroRNA-448 suppresses osteosarcoma cell proliferation and invasion through targeting EPHA7. *PLoS One*. 2017;12(6):e0175553.
11. Wang LF, Fokas E, Juricko J, You A, Rose F, Pagenstecher A, et al. Increased expression of EphA7 correlates with adverse outcome in primary and recurrent glioblastoma multiforme patients. *BMC Cancer*. 2008;8:79.

12. Giaginis C, Tsoukalas N, Bournakis E, Alexandrou P, Kavantzias N, Patsouris E, et al. Ephrin (Eph) receptor A1, A4, A5 and A7 expression in human non-small cell lung carcinoma: associations with clinicopathological parameters, tumor proliferative capacity and patients' survival. *BMC Clin Pathol*. 2014;14(1):8.
13. Eberle FC, Rodriguez-Canales J, Wei L, Hanson JC, Killian JK, Sun HW, et al. Methylation profiling of mediastinal gray zone lymphoma reveals a distinctive signature with elements shared by classical Hodgkin's lymphoma and primary mediastinal large B-cell lymphoma. *Haematologica*. 2011;96(4):558-66.
14. Guan M, Xu C, Zhang F, Ye C. Aberrant methylation of EphA7 in human prostate cancer and its relation to clinicopathologic features. *Int J Cancer*. 2009;124(1):88-94.
15. Zhang SJ, Zhang G, Zhao YF, Wu Y, Li J, Chai YX. [Expression of EphA7 protein in primary hepatocellular carcinoma and its clinical significance]. *Zhonghua Wai Ke Za Zhi*. 2010;48(1):53-6.
16. Liu DC, Yang ZL. MTDH and EphA7 are markers for metastasis and poor prognosis of gallbladder adenocarcinoma. *Diagn Cytopathol*. 2013;41(3):199-205.
17. Li R, Sun Y, Jiang A, Wu Y, Li C, Jin M, et al. Knockdown of ephrin receptor A7 suppresses the proliferation and metastasis of A549 human lung cancer cells. *Mol Med Rep*. 2016;13(4):3190-6.
18. Li S, Wu Z, Ma P, Xu Y, Chen Y, Wang H, et al. Ligand-dependent EphA7 signaling inhibits prostate tumor growth and progression. *Cell Death Dis*. 2017;8(10):e3122.
19. Wang J, Li G, Ma H, Bao Y, Wang X, Zhou H, et al. Differential expression of EphA7 receptor tyrosine kinase in gastric carcinoma. *Hum Pathol*. 2007;38(11):1649-56.
20. Wang J, Kataoka H, Suzuki M, Sato N, Nakamura R, Tao H, et al. Downregulation of EphA7 by hypermethylation in colorectal cancer. *Oncogene*. 2005;24(36):5637-47.
21. Li YF, Hsiao YH, Lai YH, Chen YC, Chen YJ, Chou JL, et al. DNA methylation profiles and biomarkers of oral squamous cell carcinoma. *Epigenetics-U.S.* 2015;10(3):229-36.
22. Tang Z, Kang B, Li C, Chen T, Zhang Z. GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. *Nucleic Acids Res*. 2019;47(W1):W556-W60.
23. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi B, et al. UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. *Neoplasia*. 2017;19(8):649-58.
24. Diez-Villanueva A, Mallona I, Peinado MA. Wanderer, an interactive viewer to explore DNA methylation and gene expression data in human cancer. *Epigenetics Chromatin*. 2015;8:22.
25. Koch A, De Meyer T, Jeschke J, Van Criekinge W. MEXPRESS: visualizing expression, DNA methylation and clinical TCGA data. *Bmc Genomics*. 2015;16.
26. Fornes O, Castro-Mondragon JA, Khan A, van der Lee R, Zhang X, Richmond PA, et al. JASPAR 2020: update of the open-access database of transcription factor binding profiles. *Nucleic Acids Res*. 2020;48(D1):D87-D92.

27. Vasaikar SV, Straub P, Wang J, Zhang B. LinkedOmics: analyzing multi-omics data within and across 32 cancer types. *Nucleic Acids Res.* 2018;46(D1):D956-D63.
28. Liu H, Wei Z, Dominguez A, Li Y, Wang X, Qi LS. CRISPR-ERA: a comprehensive design tool for CRISPR-mediated gene editing, repression and activation. *Bioinformatics.* 2015;31(22):3676-8.
29. Liu XS, Wu H, Ji X, Stelzer Y, Wu X, Czauderna S, et al. Editing DNA Methylation in the Mammalian Genome. *Cell.* 2016;167(1):233-47 e17.
30. Wang R, van Leeuwen RW, Boers A, Klip HG, de Meyer T, Steenbergen RD, et al. Genome-wide methylome analysis using MethylCap-seq uncovers 4 hypermethylated markers with high sensitivity for both adeno- and squamous-cell cervical carcinoma. *Oncotarget.* 2016;7(49):80735-50.
31. Gallo Cantafio ME, Grillone K, Caracciolo D, Scionti F, Arbitrio M, Barbieri V, et al. From Single Level Analysis to Multi-Omics Integrative Approaches: A Powerful Strategy towards the Precision Oncology. *High Throughput.* 2018;7(4).
32. Turanli B, Karagoz K, Gulfidan G, Sinha R, Mardinoglu A, Arga KY. A Network-Based Cancer Drug Discovery: From Integrated Multi-Omics Approaches to Precision Medicine. *Curr Pharm Des.* 2018;24(32):3778-90.
33. Hasin Y, Seldin M, Lusis A. Multi-omics approaches to disease. *Genome Biol.* 2017;18(1):83.
34. Joosten SC, Smits KM, Aarts MJ, Melotte V, Koch A, Tjan-Heijnen VC, et al. Epigenetics in renal cell cancer: mechanisms and clinical applications. *Nat Rev Urol.* 2018;15(7):430-51.
35. Nguyen TV, Lister R. Genomic Targeting of TET Activity for Targeted Demethylation Using CRISPR/Cas9. *Methods Mol Biol.* 2021;2272:181-94.
36. Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, et al. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol.* 2015;33(5):510-7.
37. Deutschmeyer VE, Richter AM. The ZAR1 protein in cancer; from epigenetic silencing to functional characterisation and epigenetic therapy of tumour suppressors. *Biochim Biophys Acta Rev Cancer.* 2020;1874(2):188417.
38. Greenberg MVC, Bourc'his D. The diverse roles of DNA methylation in mammalian development and disease. *Nat Rev Mol Cell Biol.* 2019;20(10):590-607.
39. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell.* 1999;99(3):247-57.
40. Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, et al. Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. *Cancer Res.* 2005;65(14):6305-11.
41. Liu XS, Wu H, Krzisch M, Wu X, Graef J, Muffat J, et al. Rescue of Fragile X Syndrome Neurons by DNA Methylation Editing of the FMR1 Gene. *Cell.* 2018;172(5):979-92 e6.
42. Hanzawa N, Hashimoto K, Yuan X, Kawahori K, Tsujimoto K, Hamaguchi M, et al. Targeted DNA demethylation of the Fgf21 promoter by CRISPR/dCas9-mediated epigenome editing. *Sci Rep.*

2020;10(1):5181.

43. Wu J, He K, Zhang Y, Song J, Shi Z, Chen W, et al. Inactivation of SMARCA2 by promoter hypermethylation drives lung cancer development. *Gene*. 2019;687:193-9.
44. Xiong T, Meister GE, Workman RE, Kato NC, Spellberg MJ, Turker F, et al. Targeted DNA methylation in human cells using engineered dCas9-methyltransferases. *Sci Rep*. 2017;7(1):6732.
45. Medvedeva YA, Khamis AM, Kulakovskiy IV, Ba-Alawi W, Bhuyan MS, Kawaji H, et al. Effects of cytosine methylation on transcription factor binding sites. *Bmc Genomics*. 2014;15:119.
46. Stadler MB, Murr R, Burger L, Ivanek R, Lienert F, Scholer A, et al. DNA-binding factors shape the mouse methylome at distal regulatory regions. *Nature*. 2011;480(7378):490-5.
47. Chu YY, Ko CY, Wang SM, Lin PI, Wang HY, Lin WC, et al. Bortezomib-induced miRNAs direct epigenetic silencing of locus genes and trigger apoptosis in leukemia. *Cell Death Dis*. 2017;8(11):e3167.
48. Zhu Z, Meng W, Liu P, Zhu X, Liu Y, Zou H. DNA hypomethylation of a transcription factor binding site within the promoter of a gout risk gene NRBP1 upregulates its expression by inhibition of TFAP2A binding. *Clin Epigenetics*. 2017;9:99.

Tables

Table 1 is not available with this version

Figures

Figure 1

The site of sgRNAs with CRISPR-dCas9 systems targeted in EphA7 promoter and exon 1th. Three active sgRNAs (act-sgRNAs) for demethylation, and four repressive sgRNAs (rep-sgRNAs) for increasing methylation were designed.

Figure 2

The promoter of EphA7 is significantly hypermethylated in CC tissues. (A) EphA7 showed hypermethylation in 15 out of 23 categories of cancer tissues compared with their adjacent normal tissues ($P < 0.05$), and the median of beta value in CESC was the most prominent. (B) The levels of mean EphA7 methylation in total of 21 probes sites. Among them, 11 probes in the promoter region exhibiting significant differences between tumor and adjacent normal specimens ($P < 0.05$). * represents adjusted P

value< 0.05. Plot and P value were produced via Wanderer.(C) Compared with the normal cervical tissues, the average methylation level of EphA7 was 4.09-fold higher in tumors within a total of 11 CpG sites (P<0.05) by performing pyrosequencing. leu: Leukocyte DNA from healthy women was used as a negative control of methylation. iv: In vitro methylated leukocyte DNA was used as a positive control.

Promoter methylation of EphA7 is inversely correlated with gene expression in CC.(A) Among of 21 HumanMethylation 450 probes, 19 probes showed significantly negative expression (Pearson correlation coefficients from -0.211 to -0.522).Statistical significance was indicated in the right side(* $P<0.05$,*** $P<0.001$) and the left highlight region focused on promoter of EphA7. (B) EphA7 promoter methylation level was higher in the cancer tissues compared with the cervical adjacent normal tissues using UALCAN. (C) The expression of EphA7 was downregulated in cevical cancer tissues via GEPIA based on TCGA and GTEx. (D) The expression level of EphA7 was significantly decreased in the CaSki and SiHa cell lines compared with the HEK293T cell line ($P<0.001$). (E) The methylation bands of EphA7 were observed in CaSki and SiHa cells, which were not observed in HEK293T cells via MSP

M:methylation, U: unmethylation.

Figure 4

Targeted DNA demethylation of the EphA7 promoter using the dCas9-Tet1 system in CC cell lines. The expression of EphA7 was upregulated with various levels in CaSki(A) and SiHa(D) by combined dCas9-Tet1 with sgRNAs compared to control(Tet1m, Tet1m was Tet1 with 3 SNPs mutations). Pyrosequencing evidence that the methylation of EphA7 in the promoter decreased in the sgRNA1 group compared to Tet1m in CaSki(B) and SiHa(E).The average methylation level in the promoter region of EphA7 in the sgRNA1 group respectively decrease 19.10%,17.23% in CaSki(C) and SiHa(F) compared to control.

Figure 5

Increase DNA methylation of EphA7 by the dCas9-DNMT3a system. (A) The EphA7 mRNA level was downregulated than DNMT3a^m (DNMT3a^m was DNMT3a with 3 silent mutations) after co-transfected 4 repression sgRNAs with the dCas9-DNMT3a in HEK293T. (B) Pyrosequencing was performed to quantify the methylation of sgRNA2 group compared to control. (C) The results of pyrosequencing showed that the average CpG sites of the promoter increased nearly 23.67%.

Figure 6

Validate the potential value of EphA7 methylation in the diagnosis of CC. (A) The methylation positive rate in normal tissues were 0%(0/25), 45.83% CIN II/III(11/24), and tumor tissues were 100%(8/8) via MSP. Methylation positive results: the methylated bands were more heavy than the unmethylated bands. Methylation negative results: the methylated bands were lighter than the unmethylated bands. Full-length

gel pictures are presented in Fig. S1. (B) The results of QMSP showed that the methylation level of EphA7 increased with the severity of cervical lesions ($H=27.311$, $P<0.001$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Figs1.pdf](#)
- [FigS2.pdf](#)
- [FigS3.pdf](#)
- [SupplementalInformation.docx](#)