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Association of estradiol and HPV/HPV16 infection with the occurrence of cervical squamous cell carcinoma

LING DING, CHUNLIANG LIU, QIN ZHOU, MEIJUAN FENG and JINTAO WANG

Department of Epidemiology, School of Public Health, Shanxi Medical University, Taiyuan, Shanxi 030001, P.R. China

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Abstract. The associations between human papillomavirus (HPV) infection or hormonal exposure and cervical cancer risk are well established. However, to the best of our knowledge, the association between high endogenous estradiol levels in conjunction with HPV/HPV16 infection and the risk of cervical squamous cell carcinoma remains unknown. To investigate this, the current study conducted a matched case-control study in Shanxi Province, China, in which clinical samples were obtained from 74 females with newly diagnosed uterine cervix squamous cell carcinoma and 74 matched healthy females who were selected from 582 healthy females according to age, place of residence, marital status and menopausal status. From all participants, DNA was extracted from cells obtained from a cervical smear and serum was separated from venous blood withdrawn during days 5-8 of the menstrual cycle. HPV/HPV16 DNA and estradiol expression levels in the serum were measured by general polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. Significant differences were identified in the positive HPV and HPV16 DNA expression rates between patients and controls, with odds ratios (95% confidence interval) of 3.74 (1.84-7.59) and 4.04 (1.97-8.28), respectively. Expression levels of estradiol in patients were significantly higher compared with the controls (P<0.001), however, this was only identified when the HPV16 E2 or E6 oncogene status was negative. Considering 40 ng/ml as the cut-off estradiol level, 78.38% of patients exhibited high estradiol levels, which was significantly higher than the percentage of controls (P<0.001). An additive interaction pattern was revealed between estradiol expression levels and HPV/HPV16 infection. The results suggest that among the various types of HPV, HPV16 may be most likely to cause uterine cervix squamous cell carcinoma and an abnormally high level of endogenous estradiol may further increase this risk. Therefore, estradiol therapy may represent a new treatment strategy for cases of cervical cancer associated with HPV infection.

Correspondence to: Professor Jintao Wang, Department of Epidemiology, School of Public Health, Shanxi Medical University, 56 Xin Jian Nan Road, Taiyuan, Shanxi 030001, P.R. China
E-mail: wangjt59@163.com

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Introduction

Cervical cancer is the fourth most common type of cancer in females worldwide (1), with >52,000 new cases diagnosed annually and a morbidity rate that is markedly high in the Shanxi Province of China (2). Cervical cancer has a complicated etiology. Infection with human papillomavirus (HPV), particularly high-risk HPV, has been implicated in cervical squamous cell carcinoma (3,4), however, not all females with HPV infection develop cervical cancer (5,6), which indicates that other factors are responsible for inducing cervical carcinogenesis. The HPV oncogenes E2 and E6 exhibit differential effects during carcinogenesis (7,8). In our previous study (9), it was identified that both the expression levels of HPV16 E6 and E2 were significantly higher in patients with cervical cancer compared with healthy controls and the expression level of E6 was higher compared with E2 in each group. The cervix is the narrow portion of the uterus at the point that joins with the top of the vagina. The majority of cervical cancer cases are squamous cell carcinomas, which occur in the squamous epithelial cells that line the cervix (10,11). This transformation zone, where the majority of cervical cancer cases arise, is the most estrogen-sensitive region of the cervix (12). DNA damage by estrogen metabolites may also contribute to cervical carcinogenesis (12,13). Furthermore, females taking oral contraceptives are at an increased risk of developing cervical cancer (14,15), which suggests an association between hormonal exposure and cancer risk. Parity has also been demonstrated to be a cofactor, along with HPV, in increasing the risk of developing cervical cancer (16). Similarly, lesions have been observed in mice that were perinatally exposed to diethylstilbestrol (17,18).

The role of female hormones in cervical carcinogenesis has traditionally been studied using cervical cell lines and HPV-16 transgenic mice (19,20), however, the role of estradiol in the development of precancerous and cancerous lesions, particularly the association between estradiol and HPV infection, remains unclear (21). Periodic changes in the levels of endogenous hormones are under tight regulation, however, this regulation may be disrupted in certain conditions, including disease and certain environmental exposure. Therefore, further

studies are required to investigate the association and mechanism between endogenous hormones and the development of cervical cancer. Therefore, we conducted a case-control study to examine the association between estradiol level and the risk of cervical cancer, and to determine the potential synergistic effect of estradiol and HPV on the progression of cervical cancer.

Materials and methods

Subjects. The present study was conducted with females that were engaged in agriculture and mining in Shanxi Province, located in the north of China. Cervical cancer mortality rates in this population are relatively high (22). Participants were assigned to either the case group or the control group according to their pathologic diagnosis. According to our pilot study, the HPV infective rate among all normal individuals was 28%, with an odds ratio (OR) of 3. The following sample size calculation formula was used to determine that a sample size of 74 for each group was required: $n=\{Z_{1-\alpha/2}^{*}[(2^{*}\rho^{*}(1-\rho)]^{1/2}+Z_{\beta}^{*}[P_1^{*}(1-P_1)+P_0^{*}(1-P_0)]^{1/2}\}^{2}/(P_1-P_0)^2$, $P_1=(OR^{*}P_0)/(1-P_0+OR^{*}P_0)$, $\rho=(P_1+P_0)/2$, where $\alpha = 0.05$ and $\beta = 0.10$ (Z: critical value of standard normal distribution, P: HPV exposure rate). Therefore, a total of 74 patients with cervical cancer that were newly diagnosed by histologic assessment were recruited, and 74 healthy controls were selected at random among 582 healthy females who were diagnosed without cervical intraepithelial neoplasia, invasive cancer or other gynecologic diseases during a routine physical examination between January 2016 and December 2016 at Shanxi Province Tumor Hospital (Taiyuan, China). The controls were matched to patients according to age, place of residence, marital status and menopausal status. Pregnant females, females with ovarian disease or other types of cancer, and those who had been treated with steroid hormones in the prior six months were excluded. Written informed consent was obtained from each participant prior to study initiation. The mean ages of women included in the case and control groups were 47.32 years (range, 25-75 years) and 47.42 years (range, 29-77 years), respectively.

Clinical information was obtained by a questionnaire following ethical institutional approval. All subjects were interviewed according to the questionnaire, which focused on demographic characteristics, lifestyle, personal hygiene behavior and reproductive factors. Cervical tissue specimens were surgically obtained from all females in the case group, and cells were obtained from a cervical smear from females in both the case and control groups. In addition, venous blood was collected during days 5-8 of the menstrual cycle from all participants and the serum was separated for subsequent analysis of estradiol levels. Informed consent forms were signed by all individuals who agreed to participate in the study. The study was approved by the Science Research Ethics Committee of Shanxi Medical University (Taiyuan, China).

General polymerase chain reaction (PCR) analysis. Genomic DNA was extracted from cervical tissues and cells using the standard phenol-chloroform method (23). HPV DNA was detected by PCR with the following consensus primers from the L_1 regions of HPV type 16, 18, 33, 6, 11 and 31: Forward, 5'-CGTAAACGTTTTCCCTATTTTTTT-3' and reverse, 5'-TACCCTAAATACTCTGTATTG-3' (24). High-risk HPV16 DNA was detected using multiple PCR to amplify the E6 and E2 gene sequences with the following primers: HPV16 E2 forward, 5'-AAGGGCGTAACCGAAATCGGT-3' and reverse, 5'-CATATACCTCACGTCGCAG-3'); and HPV16 E6 forward, 5'-CTTGGGCACCGAAGAAACC-3' and reverse, 5'-TTGGTCACGTTGCCATTCAC-3' (25). PCR was performed using 50-μl samples, containing 100 ng template DNA, 25 mM $MgCl_2$, 10 mM each of deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytidine triphosphate and deoxyguanosine triphosphate, 25 pM of each consensus forward and reverse primer, and 1 U of Jump-Start Taq DNA polymerase (cat no. D0089: Bio Basic Inc., Markham, ON, Canada). The following conditions were used: 30 cycles at 95˚C for 30 sec, 55˚C for 60 sec, 72˚C for 60 sec and a final extension at 72˚C for 10 min. 10 μl PCR products and 2 μl ethidium bromide were mixed and then subjected to electrophoresis on 2% agarose gels. A 253-base pair (bp) fragment represented HPV-positive cases, and two bands of 351 and 208 bp, which were produced by the HPV16 E2 and E6 genes, respectively, represented HPV16-positive cases.

Enzyme-linked immunosorbent assay (ELISA). Estradiol levels in the serum were measured using an Estradiol enzyme immunoassay (EIA) kit (cat no. BC-1111; Biocheck Inc., San Francisco, CA, USA), according to the manufacturer's protocol.

Statistical analysis. SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA) was used to analyze the data. Demographic and clinical variables are presented as means ± standard deviations, with ranges for continuous variables, and frequencies and percentages for categorical variables. Each variable was compared between the case group and the control group. For continuous variables, a two-sample t-test was used, while categorical variables were compared with a chi-square test to identify significant differences in HPV/HPV16 infection and estradiol levels between the two groups, and to obtain maximum-likelihood estimates of the OR and corresponding 95% confidence intervals (CIs). Effect modification between estradiol and HPV/HPV16 positivity in cervical cancer cases was measured according to additive interaction patterns (26), Additive interaction is a type of biological interaction which means the effect of two (or more) factors acting on a disease is not equal to the sum of the independent effects of these factors separately (27). Furthermore, the extent of additive interaction with the relative excess risk of interaction (RERI), the attributable proportion of interaction (API) and synergy index (S) were estimated. If there was no association, RERI and API were equal to 0 and S was equal to 1. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Demographic characteristics of the subjects. There was no significant difference between the ages of the case and control groups (t=0.06; P=0.952). In addition, no significant differences were identified between the groups with respect to education level, frequency of vaginal cleaning, marital status and menopausal status (all $P>0.05$). However, a significantly higher number of women in the case group were identified

Table I. Logistic regression analysis comparing characteristics of individuals in the case group and control group.

Variables	Patients, n (%)	Controls, n (%)	χ^2	P-value	OR (95%CI)
Education level (high school or above)	25 (33.78)	29 (39.19)	2.77	0.096	0.47 (0.19-1.15)
Occupation (farmer)	70 (94.59)	62 (83.78)	3.98	0.046	3.67 (1.02-13.14)
Frequency of vaginal cleaning (≥3 times a week)	21 (28.38)	29 (39.19)	2.14	0.144	0.46 (0.16-1.31)
Marital status (married)	49 (66.22)	45 (60.81)	1.02	0.312	0.77 (0.47-1.28)
Menopausal status (post-menopause)	29 (39.19)	19 (25.68)	3.20	0.074	0.50 (0.23-1.07)
Number of parity (≥3 times)	26 (35.14)	12 (16.22)	6.96	0.008	2.22 (1.23-4.03)

OR, odds ratio; CI, confidence interval.

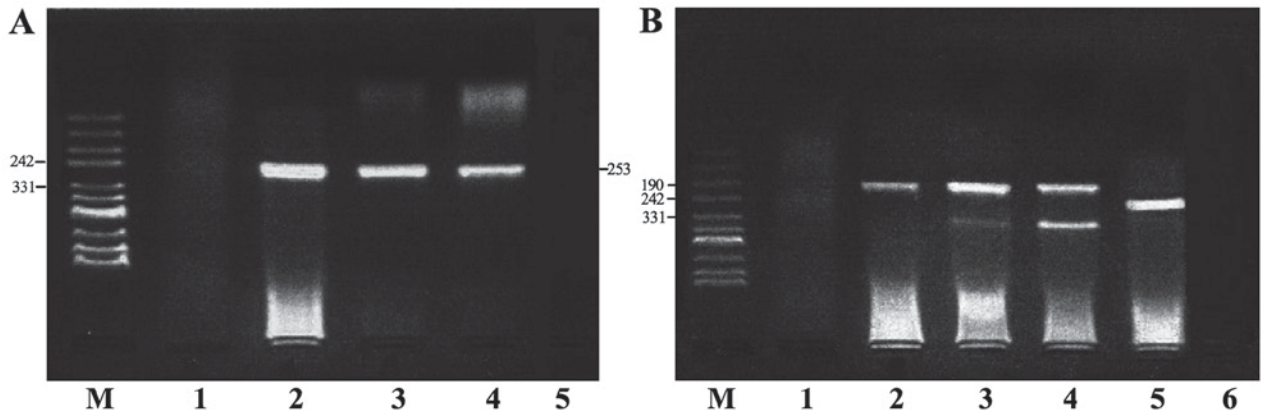

Figure 1. Semi-quantitative PCR. (A) HPV DNA detection by PCR. Lane M contains pUC mix Marker 8. Lane 1 contains a HPV negative control. Lanes 2 and 4 represent positive samples. Lane 3 contains a HPV positive control. Lane 5 contains a blank control. (B) HPV 16 E2/E6 DNA detection by PCR amplification. Lane M contains a pUC mix Marker 8. Lane 1 contains a HPV negative control. Lane 2 represents positive and negative HPV16 E6. Lanes 3 and 4 represent positive samples. Lane 5 contains a HPV positive control. Lane 6 contains a blank control. PCR, polymerase chain reaction; HPV, human papillomavirus.

to have an occupation as a farmer compared with the control group (P<0.05). The full demographic characteristics are summarized in Table I.

HPV and HPV16 infection in the case and control groups. HPV and HPV16 E6/E2 DNA were detected by semi-quantitative PCR (Fig. 1). The HPV16 E2 gene was absent in certain HPV16-positive samples, therefore, HPV16 E6-positive samples were selected to represent HPV16-positive samples. The positivity rates of HPV and HPV16 in the case group (77.03 and 52.70%, respectively) were significantly higher compared with those in the control group (47.30 and 21.62%, respectively), with OR values of 3.74 (95% CI, 1.84-7.59) and 4.04 (95% CI, 1.97-8.28), respectively (Table II). To prevent any deviation caused by differences in tissue and cell specimens, both types of specimens were analyzed consistently for HPV and HPV16 in 24 cervical cancer cases. The results demonstrated that the consistency among the percentage of HPV- and HPV16-positive cases was 91.67% (κ=0.78) and 87.50% (κ=0.75), respectively (Table III).

Estradiol levels in the case and control groups. Estradiol expression levels in the case group (45.98±11.48 pg/ml) were significantly higher compared with the control group (26.96±8.31 pg/ml; Table III). This significant difference between the case and control groups was maintained regardless of the HPV status (Fig. 2A). However, it was identified that estradiol expression level was only significantly higher in the case group compared with the control when the HPV16 E2 or E6 status was negative (Fig. 2B and C).

The lowest concentration of estradiol (40 pg/ml) detected in healthy females during the follicular period was defined as the cut-off value; therefore, estradiol levels >40 pg/ml were considered as abnormally high. Accordingly, estradiol levels in 78.38% (58/74) of the cervical cancer cases were revealed to be abnormally high, whereas the estradiol level was identified as abnormally high in only 27.03% (20/74) of the controls, which indicates a significant difference between the two groups (Table IV).

Association between estradiol level and HPV or HPV16 infection. The risk of cervical cancer in HPV- or HPV16-positive

Table II. HPV and HPV16 infection in the case group and control group.

Infection	Case group (n=74) Positive, n (%)	Control group (n=74) Positive, n (%)	χ^2	P-value	OR (95% CI)
HPV	57 (77.03)	35 (47.30)	13.904	<0.001	3.74 (1.84-7.59)
HPV16 E6	39 (52.70)	16 (21.62)	15.306	<0.001	4.04 (1.97-8.28)
HPV16 E2	31 (41.90)	13 (17.60)	10.479	0.001	3.38 (1.59-7.21)

HPV, human papillomavirus; OR, odds ratio; CI, confidence interval.

Table III. Consistency analysis of tissue and cell specimens for HPV and HPV16.

Variables	HPV, n (%)	HPV16, n (%)
Tissue specimens/ Cell specimens		
+/+	17 (70.83)	10 (41.67)
+/-	1 (4.17)	2 (8.33)
-/+	1 (4.17)	1 (4.17)
-/-	5 (20.83)	11 (45.83)
Total	24 (100.00)	24 (100.00)
Consistency rate (%)	91.67	87.50
κ	0.78	0.75

HPV, human papillomavirus; κ, Kappa.

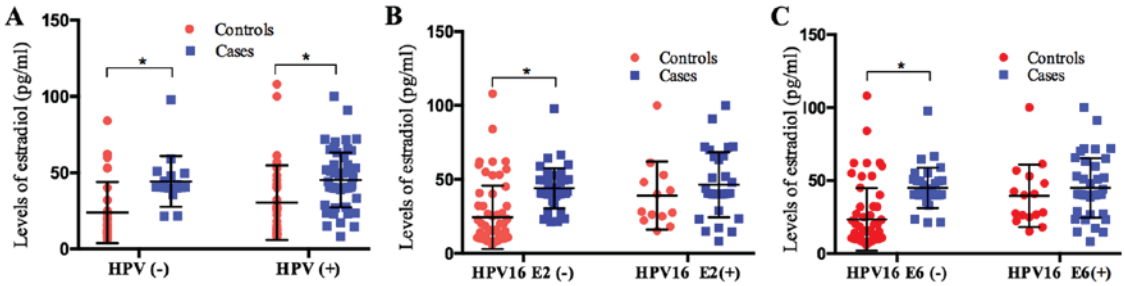

Figure 2. Estradiol expression levels in samples either positive of negative for (A) HPV, (B) HPV16 E2 and (C) HPV16 E6 status. Data are presented as the mean ± standard deviation. *P<0.05.

females with abnormally high levels of estradiol was higher compared with females who were either HPV/HPV16-positive or exhibited abnormally high levels of estradiol. According to a method previously described by Knol and VanderWeele (26), the present study quantitatively analyzed the association between estradiol and HPV/HPV16 infection. and estimated the parameters RERI, API and S (Table V). The results revealed an additive interaction pattern between estradiol levels and HPV/HPV16 infection.

Discussion

HPV infection is relatively common among females (28,29). Persistent HPV infection may cause intraepithelial pre-neoplastic lesions and invasive cervical cancer (30,31). The present study revealed that the positivity rates of HPV and HPV16 in patients with cervical cancer were 77.03 and 52.07%, respectively, which were significantly higher compared with the controls. Among the HPV-positive cases, >68% were HPV16-positive. The results of previous studies, which also investigated HPV infection and cervical cancer in females of Shanxi Province (32,33), in combination with the present results suggest that cervical cancer is closely associated with HPV infection, particularly the high-risk type HPV16, which is considered to be a primary cause of cervical cancer.

To the best of our knowledge, the etiological role of HPV in the development of uterine cervix squamous cell carcinoma remains unclear. Cervical carcinogenesis is a multistep process initiated by HPV, however, infection alone is insufficient to induce malignancy (34). HPV leads to the development of intraepithelial lesions in the presence of other cofactors (35). One such cofactor that has been reported to initiate neoplasia in

Table IV. Association between estradiol level and cervical cancer occurrence.

Group	n	Mean ± SD, pg/ml	Estradiol >40 pg/ml, n (%)	χ^2	P-value	OR (95% CI)
Case	74	45.98±11.48	58 (78.38)	39.14	<0.001	9.79 (4.60-20.82)
Control	74	26.96±8.31	20 (27.03)			

SD, standard deviation; OR, odds ratio; CI, confidence interval.

Table V. Additive interaction between estradiol level and HPV or HPV16 infection.

A, Additive interaction between estradiol level and HPV infection

HPV	Estradiol, pg/ml	Case group, n	Control group, n	OR (95% CI)	RERI	API	S
+	≥40	44	11	41.33 (10.64-160.53)	18.67	0.45	1.86
+	<40	13	24	5.60 (1.06-8.56)			
-	≥40	14	8	18.08 (4.16-78.60)			
-	<40	3	31	1.00			

B, Additive interaction between estradiol level and HPV16 infection

HPV16	Estradiol, pg/ml	Case group, n	Control group, n	OR (95% CI)	RERI	API	S
+	≥40	28	8	32.90 (9.80-110.48)	5.20	-0.16	0.86
+	<40	11	8	12.93 (3.54-47.23)			
-	≥40	30	11	25.64 (8.10-81.13)			
-	<40	5	47	1.00			

HPV, human papillomavirus; OR, odds ratio; CI, confidence interval; RERI, relative excess risk of interaction; API, attributable proportion of interaction; S, synergy index.

cervical cancer is prolonged exposure to sex hormones (14,15). Therefore, the potential association between uterine cervix squamous cell carcinoma and sex hormones has been extensively studied (36,37). A meta-analysis has demonstrated that controlled ovarian hyperstimulation (COH) for *in vitro* fertilization (IVF) could elevate the levels of hormones, however an increased risk of cervical cancer was not identified, which indicates a protective role of IVF (38). However, females undergoing IVF are considered to have stable sexual relationships and a high socioeconomic status, and may be treated for cervical lesions prior to IVF. In addition, HPV infection has been revealed to be significantly lower in females undergoing IVF compared with controls (39). All these factors may reduce the risk of hormone levels on cervical cancer development. By contrast, long-term use of oral contraceptives has been demonstrated to increase the risk of cervical cancer (40). Elevated hormone levels were revealed to induce the proliferation and apoptosis of cervical adenocarcinoma HeLa cells *in vitro* (19). The biological effects of COH or oral contraceptives in the human body may be influenced by numerous factors, such as drug type, dosage, time of taking medicin and so on (40). A number of studies investigating endogenous steroid hormones have demonstrated an association between endogenous hormone levels and cervical cancer based on the general population (41-43). Estradiol is the most important steroid hormone in the normal female endocrine system (44); the present study identified that endogenous estradiol levels in patients with cervical cancer were higher compared with controls, which suggests that high endogenous estradiol levels are associated with an elevated risk of cervical cancer. Estrogen receptor-α binds to the forkhead box P3 promoter and modulates regulatory T-cell function in human cervical cancer (45). Therefore, sex hormones may have an influence on the immune system. In summary, this evidence suggests estrogen exposure may be associated with hyperplasia and squamous differentiation of reserve cells.

Notably, the present study obtained different conclusions according to the expression of the HPV16 oncogene. A significant difference in HPV positivity was identified between patients and controls, however, estradiol levels in patients were significantly higher compared with controls only when HPV16 E2 or E6 status was negative. HPV16 E6 protein is required for cell transformation, while the HPV E2 protein is required for

viral replication and gene expression (46). Estrogen has been demonstrated to upregulate the transcription of HPV E6 oncogenes *in vitro* (47). A number of studies involving HPV-positive transgenic mice that expressed HPV16 oncogenes in their basal keratinocytes have demonstrated that chronic exposure to estradiol is required for the induction of cervical tumors (47-49). Furthermore, based on biological interaction analysis, the present study revealed an additive interaction between endogenous estradiol level and HPV/HPV16 occurrence. In addition, it was identified that the risk of cervical cancer in females with both a high endogenous estradiol level and HPV infection was 18.67-fold greater compared with the risk associated with other factors, accounting for 45% of the risk, and was 1.86-fold greater compared with the sum of risks associated with endogenous estradiol level and HPV infection. A similar interaction was also observed between the endogenous estradiol level and HPV16 infection, with an RERI of -5.20, API of -0.16 and S of 0.86. These results indicate that the additive effect of HPV/HPV16 infection and high estradiol level is greater compared with their individual effects on cancer risk.

The present study possessed a number of limitations. Firstly, analysis of HPV positivity and high endogenous estradiol levels at a single point in a female's lifetime are difficult to interpret as these could represent either a recent change of a long-term status. Secondly, a case-control study can only suggest associations and cannot determine causal associations. In addition, given the complicated association between endogenous estradiol level and HPV infection in cervical cancer, there are numerous other influencing factors that should be investigated.

Despite these limitations, the cervix epithelium is a hormone-dependent epithelium, and the present study provides a strong indication that abnormally high endogenous estradiol levels may be associated with increased risk of cervical cancer and estradiol may serve as a cofactor along with HPV infection in the development of cervical cancer. Further studies, including a prospective cohort study should be performed to provide etiological evidence that may clarify the mechanism of the association between endogenous hormone level and HPV infection in cervical carcinogenesis. In addition, further studies are required to evaluate whether an increased risk of cervical cancer is associated with other factors. Furthermore, the results of the present study suggest that estradiol may represent a new treatment strategy in cases of cervical cancer due to HPV infection, however, this requires further investigation.

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Availability of data and materials

The datasets used or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LD performed data analyses, drafted the manuscript, finalized and submitted the manuscript. LD, and MF contributed to perform the DNA extraction, PCR experiments and ELISA experiments. CL and QZ contributed to the data collection and quality control. JW and LD conceived the idea, designed and led the project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Science Research Ethics Committee of Shanxi Medical University (Taiyuan, China) and all participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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