RESEARCH ARTICLE

Possible Epithelial Ovarian Cancer Association with HPV18 or HPV33 Infection

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Abstract

The present study was conducted to investigate the prevalence of HPV infection in epithelial ovarian cancer (EOC) in Hunan province. DNA samples were collected from paraffin embedded ovarian tissue from 322 patients with EOC, 99 with ovarian benign tumors and 199 normal persons. The polymerase chain reaction and direct sequencing were used to identify the HPV types in the samples. The relationship between the infection of human papillomavirus (HPV) and the epithelial ovarian carcinoma (EOC) was investigated combined with clinical data. The prevalence of HPV18 and HPV33 in EOC group and benign group was higher than in the normal group. HPV18 and HPV33 may play a role in the development of both EOC and ovarian benign tumor and may participate in the development of EOC with traditional risk factors, family history and abortion, possibly exerting synergistic effects.

Keywords: EOC - HPV - paraffin specimen - risk factor

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Introduction

HPV (human Papilomavirus) is usually a double helical molecule in the cell, and showed strong squamous epithelium-like characteristics. So far, more than 130 gene types of HPV were reported, which directly involved in the benign and malignant hyperplasia of the epithelium of the genital and oral mucosa. HPV16, HPV18, HPV31, HPV33, HPV35, HPV45, HPV51, HPV52, HPV5 and HPV59 have been classified as high-risk type of cervical cancer by The National Institutes of health (Remmerbach et al., 2004).

Studies indicated that a variety of epithelial malignancies such as cervical cancer (Hu et al., 2015), squamous cell carcinoma of the head and neck (HNSCCs) (Linxweiler et al., 2015), Oral and maxillofacial tumor (Dalla et al., 2014), colorectal cancer (Meshkat et al., 2014), esophageal squamous cancer (Michaelsen et al., 2014) and so on, was found to be associated with high-risk HPV infection.

Ovarian cancer is one of the most dangerous malignant tumor (Khanra et al., 2012), and more than 90% ovarian cancer origins from the epithelial ovarian (Chih et al., 2012). Epithelial ovarian cancer has no specific symptoms in the early stag, and lack of appropriate screening index, so that more than 70% patients have advanced at the time they have been diagnosed (Ho et al., 2014).

HPV was confirmed to be associated with the cervical cancer. The same as the female reproductive organ, the association of HPV and EOC was noticed by researchers. In 1992, HPV was detected from both normal ovary tissue and EOC tissue by Chyong-Huey Lai, but no conclusion was drawn that there was a correlation between HPV and ovarian cancer (Chyong-Huey Lai et al., 1992). Years later, Tom *P*. Manolitsas detected HPV16 DNA from both cervical and ovarian tissue from a 52-year-old women who was confirmed III CIN and bilateral ovarian neoplasia. He speculated that the incidence of ovarian cancer may be related to HPV infection. In Italy, the sample size was increased to 71 cases by Giordano G, all of which are EOC. Only 4.22% were detected HPV DNA by PCR. He therefore proposed at least for Italian women, HPV not important enough to be seen as a factor of epithelial ovarian cancer (Giordano et al., 2008). But to Atalay F's point of view, more sensitive technique, larger number of samples, set up control group and other research methodsit is necessary for develop studies (Atalay et al., 2007).

Materials and Methods

Cases selection and specimens collection. This study involved 620 females (median age=50.0; range

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from 13 to 86) including 322 patients with EOC, 99 patients with benign epithelial ovarian tumors and 199 normal, from the Hunan Cancer Hospital (China, hunan province) between June 2011 and June 2013. All of the patients did not receive radiation therapy, chemotherapy, immunotherapy, hormone replacement therapy or any other treatment before surgery, and diagnosed by pathological examination.

After fixed by 10% formalin, the ovarian tissues from cases were embedding the tissue in paraffin, then sectioned into 10 mm slices and placed into individual sterile autoclaved microcentrifuge tubes.

DNA extraction. The tissue samples were deparaffinized with washes in xylene twice, followed by single wash in ethanol at 100%, 95% and 75% respectively. The next steps of extraction were performed according to the manufacturers' instructions of DNA Isolation Kit® from Invitrogen. The extracted DNA pellets were all stored at -20°C until PCR amplification.

DNA quality evaluation. The yield and purity of DNA was tested by ultraviolet spectrophotometer (Eppendorf) according to the value of the absorption in A260 and purity A260/A280. The results were read at 260 nm and 280 nm. The DNA was qualified to be the template of PCR in the condition of its concentration and purity reaching the standard that A260 was no less than 50 and A260/A280 1.7-1.9.

Primers Design. DNA sequence files for HPV33 is obtained from Genbank. Primers (F 5'-ACGCCATGAGAGGACACAAG-3, R 5'-TAGTAATCGGCTGTGGCTGG'-3, with a product of 163 bp) was designed for it, and unique specificity was confirmed by BLAST analysis (http://www.ncbi.nih.gov /genbank/). The primers for PGMY, GP5+/6+, HPV18 were bases on the pertinent literature respectively (Romero-Pastrana F, 2012).

PCR-based HPV detection. Positive and negative controls were used in each assay to appraise whether the DNA was contaminated

The PCR mix. All the PCR amplifications were carried out in a volume of 20 μl containing 10 μl of PCR mix (2×Gold Star Best Master Mix), 10 μM of each primer and 1 μl of DNA template. The final reaction volume was completed to 20 μl using double distilled water.

Amplification with consensus primers. The outer PGMY09/11 primer set was used to amplify approximately 450 bp of the highly conserved L1 region among a broad spectrum of HPV types. Thermal cycling conditions consisted of initial denaturing step at 95°C for 9 min, 40 cycles of 1 min at 95°C, annealing beginning at 60°C and ending at 52°C for 1 min, and extension at 72°C for 1 min. The annealing temperature was lowered 2°C every four cycles until it reached 52°C; this annealing temperature was kept until the end of the cycling process, followed by 7 min of final extension at 72°C.

The negative products for the primary amplification with PGMY09/11 primers were further assessed by secondary amplification with the internal GP5+/6+ primers, which was performed under the following conditions. Initial denaturing for 5 min at 95°C, 40 cycles for 30 sec at 95°C, annealing beginning at 58°C and ending at 48°C for 1 min, and extension at 72°C for 30 sec. The annealing temperature was lowered 2°C every two cycles until it reached 48°C, which was kept until the end of the cycling process, with a final extension at 72°C for 7 min.

Amplification with subtype primers. HPV18: Initial denaturing step at 95°C for 15 min, 10 cycles of 30 sec at 94°C, 90s at 65°C, and 90 sec at 72°C, followed by 30 cycles of 30 sec at 94°C, 90 sec at 63°C, and 90 sec at 72°C, with a final extension at 72°C for 10 min. HPV33: Initial denaturing step at 95°C for 5 min, 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C, with a final extension at 72°C for 10 min.

Sequencing and Identifying. The amplification products obtained from all the PCRs were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide, band sizes were etimated by comparison with a 1500 bp molecular weight marker, and gels were photographed in a Gel imager. Products of GP5+/6+ and subtype amplification were sequenced by Sanggong Biotechnology (Sanggong, China), while the PGMY identified according to nucleic acid molecular hybridization by Human Papillomavirus Genotyping Kit For 23 Types (Yaneng Bio, China).

Statistical analysis. Analyses were conducted with SPSS20.0 for Mac (SPSS Inc, USA). Chi-square, AVOVA and t-tests were used to analyze distribution differences in continuous and categorical variables. A p-value of less than 0.05 for associations was considered significant. Logistic regression analysis were used to estimate odds ratios (OR) for the association between EOC risk and factors in this study

Results

HPV18 positive in 58 cases (7.76%) in group M, 9 cases (9.09%) in group B, 2 cases (1.01%) in group N. Statistical analysis showed that there were differences between the three groups (x^2=12.597, P=0.002). HPV 33 was detected in 39 of 322 (12.11%) EOC samples, 6 of 99 (6.06%) benign samples and 3 of 199 (1.51%) normal samples, also different between the three groups (x^2=19.830, P<0.0001) (Table 1)

Table 1. Human Papillomavirus (HPV) Prevalence of EOC

Result	No. of group M(%) (n=322)	No. of group B(%) (n=99)	No. of group N(%) (n=199)	x^2	P-value
HPV18 positive	25(7.76)	9(9.09)	2(1.01)	12.597	0.002
HPV33 positive	39(12.11)	6(6.06)	3(1.51)	19.83	0

There was no significant difference in the detection rates of HPV18 and HPV33 between the group M and the group B (HPV18: x^2 =0.180, P=0.672, HPV33: x^2 =2.904, P=0.088), however, the detection rates of HPV18 and HPV33 in group M was higher than those in N group (HPV18: x^2 =11.434, P=0.001, HPV33: x^2=9.785, P=0.002). So does the group B (HPV18: x^2=12.159, P=0.001, HPV33: x^2 =4.065, P=0.044).

There was only 5 patient harboring coinfection of HPV18 and HPV 33, 3 in group B,. No other HPV types were found in this study, 3 in group B, 1 in group M and 1in group N. There was no significant difference in multiple infection rates between the three groups (x^2=3.412, P=0.065).

It seemed that that EOC is not the only outcome after HPV18 and HPV33 infection. It is strange that the same as infected persons, part of the development of benign tumors and part of the development of malignant. Thus the Chi-square test and logistic regression analysis were carried out to identify the difference of demographic characteristics between benign tumor and EOC patients. The group M was set as the case group, and group B the control group. In the Chi-square test, Each variable of P<0.10 was involved in the Logistic regression analysis.

Chi-square test suggest that P values of seven variables between the two groups were less than 0.10, and these variables were further analyzed by logistic regression analysis. The details were shown in Table 2.

Compared with non-family history patients, the one who has a family history have statistically significantly increased risk of EOC (OR=2.747, 95% CI=0.868-8.689), although compared with benign tumor. CA125 higher than 35 U/ml indicates malignant tumor. Among people with HPV infection, those who have abortion experience had a higher risk of developing of EOC (1-2: OR=5.667, 95% CI=2.628-12.223. >3: OR=3.327, 95% CI=1.715-6.454). The incidence rate for EOC was higher among persons pregnant than no pregnancy experience individuals (Table 3).

In this study, the interdependency between pregnancy and the abortion, and pregnancy and birth were analyzed by Pearson bivariate correlation analysis. The result suggested the pregnancy outcomes tend to be abortion (r=0.755, P=0.000) rather than birth (r=0.604, P=0.000).

Table 2. Chi-square Test Results of Demographic Characteristics

Varieties	B n (%)	M n (%)	x^2	P-value
Age				
<50	60(61.6)	149(47.8)	6.222	0.013
≥51	39(38.4)	173(52.2)		
Family history				
Yes	4(4.0)	32(9.9)	3.368	0.066
No	95(96.0)	290(90.1)		
age of menarche				
<14	67(67.7)	189(58.7)	2.563	0.109
≥15	32(32.3)	133(41.3)		
Pregnant				
0	7(7.1)	8(2.5)	6.607	0.037
1-2	23(23.2)	103(32.0)		
≥3	69(69.7)	211(65.5)		
Abortion				
0	30(30.3)	155(48.1)	24.116	0
1-2	33(33.3)	119(37.0)		
≥3	36(36.4)	48(14.9)		
Birth				
0	11(11.1)	18(5.6)	4.751	0.093
1-2	65(65.7)	206(64.0)		
≥3	23(23.2)	98(30.4)		
CA125				
<35U/ml	69(69.7)	92(28.6)	54.225	0
≥35U/ml	30(30.3)	230(71.4)		

Discussion

HPV is a small, non enveloped DNA virus infection of squamous epithelium. So far, it has been found that the high risk type HPV play an fatal role in many kinds of epithelial tumors. HPV infection is an important aspect of studying the pathogenesis of epithelial ovarian cancer, and PCR is the most common method. Recently, a possible relation between HPV and EOC has also been noticed

In the past, fresh samples were mostly used for researches, albeit hard to get and sample size is subject to greater limitations. To minimize the impact of sample size on statistical results and to get larger number of samples in the short term, formalin fixed and paraffin embedded (FFPE) ovarian tissues were used in this study. FFPE is suitable for long-term storage but extracting DNA

Table 3. Demographic Characteristics and Risk Factors for EOC

Factor	No. of EOC (n=322)	P-value	OR(95% CI)
Family history			
No(reference)			
Yes	1.01	0.086	2.747(0.868-8.689)
CA125			
<35U/ml(reference)			
≥35U/ml	1.809	0	6.103(3.560-10.461)
times of pregnant			
0(reference)			
1-2	-1.835	0.005	0.160(0.044-0.575)
times of abortion			
0(reference)			
1-2	1.735	0	5.667(2.628-12.223)
≥3	1.202	0	3.327(1.715-6.454)
Constant term	-0.744	0.009	0.475

α_{in}=0.10, α_{out}=0.15, OR, odds ratio; CI, confidence interval

from it remains challenge. Faure Marin induced DNA fragmentation, reduce the number of DNA to be used, meanwhile, fixed process also shorter the length of the template (Dona et al., 2013).

We optimized the extraction method, and DNA content and purity of each sample were determined. β-actin amplification for randomly selected samples to ensure there is sufficient DNA in the sample to support the amplification. The length of the primer was controlled within 500 bp in order to cope with the possible existence of a short template so as to avoid missing detection. Selected amplified products randomly for sequence, the results show that the product are the target segment.

More than 80% of women who have had sex have been infected with HPV, most cases are asymptomatic. The HPV can be cleared by the body's immune system in about 6-12 months. Only a small amount infection development to sustained infection and cause intraepithelial neoplasia after period of latency (Tommasino, 2014).

One of the pathological and physiological hypothesis of HPV in ovarian cancer is it ascending from cervix to infect the fallopian tubes and ovaries.

It can be speculated that men were infected with HPV virus, the DNA HPV carried by sperm, reaching the surface of the ovary with the activity of the sperm, subsequent sunk into the epithelium and lead to inclusion cysts. The infection itself do not enough to cause the tumor, some key cell factor of host cells are also required to regulate its gene transcription. High risk type HPV carcinogenic activity reflected in their ability to integrate into the host genome. Through its own long control area which regulates E6 and E7 (Shirish et al., 2010), activated squamous cell undifferentiated basal cells (Paris C et al., 2015), promoting the degradation of p53 (tumor suppressor protein) and pRb (retinal mother cell tumor protein), result in uncontrolled cell proliferation, epithelial cell apoptosis, and apoptosis. Further more, the HPV recruitment and binding with host genome chromatin structure, regulate the gene expression of CCCTC binding factor (CTCF), to balance and control the expression of E6 and E7 (Paris et al., 2015). So E6 and E7 are recognized as the most important molecules leading to the occurrence of tumors. In vitro experiment, The ability to induce transformation of the E6 / E7 of HPV18 is 5 times of HPV16 (Barbosa M S et al., 1989). In a study, the infection rate of HPV16 was higher than that of HPV18 but the risk assessment of HPV18 ratio is higher than the HPV16 (G V M Berlin 2009). All of the above shows that as the earliest discovered high risk subtype, HPV18 has a stronger induced cell malignant transformation.

HPV33 is another type of high risk HPV. Because of its total physical examination rate is far lower than HPV16 and HPV18. The previous study paid not enough attention to HPV33. But in recent years, by the research of the relationship between high risk HPV and multiple malignant tumors, the research on the relationship between them is more and more thorough and detailed. HPV33 and other high-risk types have been brought into the category of large sample research more frequently.

HPV18 was proved to be associate of multiple malignant tumors includes the EOC and its pathogenic mechanism has been explored to some extent. However, the link of HPV33 and EOC deserves higher attention, which means lager sample and case control study of high sensitivity, for it might be an underestimated risk factor of EOC.

Unnoticed in previous research, the prevalence of HPV18 and HPV33 was also different in benign and normal ovarian tissues (1HPV18:x^2=12.159, P=0.001; HPV33:x^2= 4.065, P=0.044). Two assumptions can be made, one of which is the HPV18 and HPV33 not only lead to EOC but benign ovarian lesions, such as ovarian cystadenoma. Or the infection of type HPV33 or HPV18 causes canceration of benign tumor. Experimental literature has not yet been found to support the above assumptions, So we compared the difference of the case data between group B and group M.

In the Logistic regression analysis, abortion experience (1-2: P=0.047, OR=5.667, >3: P=0.047, OR=3.327) and the family history (P=0.086, OR=2.747) Increased risk of ovarian cancer, the value of CA125 higher than the normal value indicats that the ovarian tissue was malignant. The risk of ovarian cancer was lower in those who had who had been pregnant (R=0.160).

Large number of literatures indicated the incidence of EOC is familial aggregation. According to Olaitan A's research, people of the first degree relatives have ovarian cancer suffer higher risk of it (Olaitan A et al., 2014). Although relevant reports are less, it is found this correlation is not limited to the same tumor. For instance, people with a family history of breast cancer, ovarian cancer and prostate cancer, face a higher risk of colorectal cancer (Jang J H et al., 2009).

The familial aggregation of the tumor often associated with one or more gene mutations. The most common known high risk genes are BRCA and HER-2. BRCA-1 germline mutations increase the risk of lung cancer, gastric cancer, breast cancer, etc (Lauren et al., 2013), and carrying of a BRCA1 defect gene is the most common genetic related factors of EOC (Marchetti et al., 2014). As one of the cell growth factor receptor, HER-2 highly expresses in the ovarian cancer, breast cancer, gastric cancer and etc (Satpathy et al., 2014; Rakhshani et al., 2014; Qin et al., 2014).

In this study, family history of tumor show suspicious results both in single factor analysis (x^2 =3.368, P=0.066) and multiple factor analysis(Wald =2.957, P=0.086), which the P value greater than 0.05 and less than 0.10. We speculate that the main reason for this result is the variety of tumor types of the family history. The correlation between the family history of breast cancer, ovarian cancer and the incidence of EOC were proved, but the family history such as leukemia, skin cancer has not been reported associated with EOC. Whether the family history of these diseases associate with an increased risk of EOC need separate detailed study. If not, the results of this study can be considered to be affected by them.

Tsilidis et al. (2011) research suggested that every term pregnancy can reduce the risk of ovarian cancer by 8% . Pregnancy protect ovary based on two main respects. For one thing, the ovarian surface is exposed to progesterone, testosterone or progesterone analogue

during pregnancy, helps prevent ovarian cancer (Cardiff, 2013)(28). Circulating levels of pituitary gonadotropin which can increase the risk of malignancy can be inhibited by pregnancy (Whittemore et al., 1992). On the other hand, no ovulation during pregnancy, then gonadotropin secretion decreased and progesterone levels increased, the retrograde transport from the uterus, fallopian tube to the ovary were disrupted (Tsilidis K K et al., 2011). This disruption may, to a certain extent, block the upward infection of pathogens, reduce the chance of pathogens to invade the reproductive tract of women, so as to avoid the ovarian from chronic inflammation caused by the infection, helps to protect the epithelial tissue of the ovary.

Through the investigation of a total of 274442 women during 1992-2010, Braem MG reported that multiple abortions is associated with an increased risk of epithelial ovarian cancer. And because there are a variety of reasons for incomplete pregnancy, including abortion (natural loss), induced abortion (drug caused or voluntary termination of unwanted pregnancy), etc., the association with the risk of ovarian cancer may vary (Braem et al., 2012). In China, non medical selective termination of pregnancy is one of the most common methods of birth control. In 2008, a report from WHO claimed that in 1990 to 2008 years, about 47000 women died of unsafe abortion, mainly because of infection (Sawaya et al., 1996). Pathogens cause upper genital infection by ascending migration of the cervix, lead to acute pelvic inflammatory disease and other long-term sequelae (Soper, 2010). Vascular endothelial growth factor (VEGF) is one of the cell factors that are closely related to inflammation (Watanabe et al., 2013), which plays an important role in ovarian biology, to maintain the formation and growth of the follicle and corpus luteum, regulate the angiogenesis of ovarian. Its defects appears in the blood vessel formation period may cause a variety of diseases, including malignant tumors (Geva et al., 2000).

But with the increase in the number of abortions, malignant tumor risk is relatively lower. Women who were less than 3 of the number of abortions were less than those who were less than 1-2. We suspect that this phenomenon is not a single factor, but with the synergistic effect of the protective factors. Therefore, we used bivariate correlation analysis, to understand the correlation between pregnancy and abortion of research objects. The results showed that the Pearson correlation coefficient between pregnancy and abortion was 0.755 in this study (P<0.001). Although there is no direct evidence to prove that all the reasons for abortion are all active termination of pregnancy, we still can speculate that under the combined effect of pregnancy and abortion, with the increase in the number of abortions, malignancy risk relatively decreased.

As a widely used tumor marker and biomarker (Liao et al., 2014), CA125 level increases in Various diseases including EOC (Bast et al., 1998). In this study the value of CA125 in 71.4% EOC patients higher than 35U/ml, significantly different from group B, but still within normal limits in 92 (28.6%) EOC patients. Despite the lack of specificity of CA125 and the effect of screening on early EOC is not particularly desirable, but it is still the most commonly used biomarkers of EOC detect (Park et al., 2014).

In summary, it is the first time to explore relationship of HPV infection and ovarian tumor in Hunan province in the south of China using case control study, as yet, which has much more cases involved than other studies including our previous study. In summary, the results of our survey have indicated that HPV18 and HPV33 may associated with EOC, suggesting that they play an important role in ovarian tumor. But the infection of HPV18 and HPV33 do not necessarily lead to EOC but also benign tumor. Family history and abortion as traditional risk factors while pregnant as protection factor were confirmed in our study. They are involved in the complex process of epithelial ovarian lesions.

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