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THE FREQUENCY OF HUMAN PAPILLOMA VIRUS TYPES 16, 18 IN UPPER GENITAL TRACT OF WOMEN AT HIGH RISK OF DEVELOPING OVARIAN CANCER

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***Aim*: To investigate the incidence of human papilloma virus (HPV) types 16, 18 in upper genital tract of women considered at a high risk (HR) of developing epithelial ovarian cancer (EOC). *Methods*: HPV 16 and 18 *E6* ORF specific semiquantitative PCR was used to screen the incidence of HPV in 20 women at HR of developing EOC and 10 women with no ovarian disease (control). *Results*: The HR subset of fallopian tubes and ovarian tissues showed greater positivity for HPV *E6* ORF (40%) as compared to control (10%) tissues. Of all the samples, two (10%) were positive for HPV 16, two (10%) were positive for HPV 18, and four (20%) showed positivity for mixed HPV 16/18 infection. The presence of HPV *E6* ORF was found both in the fallopian tubes and ovarian DNA from 6 (30%) patients. In two cases (10%) we detected HPV ORF only in the fallopian tube derived genomic DNA. *Conclusion*: It has been shown the presence of HPV in the upper genital tract in women at HR of developing EOC in close proximity of HPV susceptible tissue cervix.**

***Key Words*: HPV, high risk of ovarian cancer.**

The devastating and lethal epithelial cancer of the ovaries (EOC) is characterized by an asymptomatic clinical course, the absence of a reliable diagnostic marker(s), clearly defined etiologic, pathologic, and genetic parameters at an early stage [1, 2]. Various lines of evidence point to hormonal, genetic and external environment as significant factors in the development of the disease [2, 3]. In addition to the global and local environmental factors such as occupational exposure, hormones, parity, and age, it is thought that there may be a genetic predisposition for developing EOC [4]. Genetic contribution to cancer risk is indicated by the accumulation of ovarian and breast cancers in either a first-degree relatives or a second-degree relatives in a cohort of women with ovarian or/and breast cancers. The most studied risk factor for ovarian cancer or breast-ovarian cancer syndromes is an inherited germline mutation of highly penetrant DNA repair genes *BRCA1* (locus 17q21) and *BRCA2* (locus 13q12.3). *BRCA1* and *BRCA2* mutations are responsible for about 5–10% of all ovarian cancers. The penetration of heterozygous *BRCA1* and *BRCA2* mutations are thought to have a dose effect dependent upon the mutated allele transcript and genetic alterations of tumor suppressor genes, particularly *TP*53, in addition to the environmental factors mentioned above [5–7].

The genetic and environmental modifiers and their synergistic effect on the risk for EOC not associated with *BRCA* mutations among women with family history of ovarian and breast cancers are subjects of active investigation at clinical and molecular level [6]. In our previous study, an important DNA repair protein in the Fanconi Anemia pathway, Fanconi Anemia complementation protein D2 (FANCD2) has been implicated as an important EOC risk factor in women without *BRCA* mutations [7].

HPV is a known etiological moderator of epithelial malignancies and has been found in 99.7% of women with high-grade cervical intraepithelial neoplasia [8, 9]. Even though ovarian tissues reside in close proximity of the cervix, the role of HPVs in predisposing women for developing EOC has been relatively controversial and the prevalence of HPV in EOC varies markedly by geographical area [10, 11].

Our present understanding of the productive viral infection of HPVs specifies that they are strictly epitheliotropic [12]. They infect squamous epithelium and establish their genomes as low copy number episomes in proliferating basal cells [13]. As infected cells differentiate, the viral DNA is amplified to high copy number, with concomitant expression of late capsid proteins L1 and L3, followed by the encapsidation and release of infectious virus particles [14]. The HPV genome gets integrated with host cell genome, often accompanied by the loss of early viral regulatory genes *E1* and *E2*, and a dysregulated expression of *E6/E7* gene products. The early viral proteins E6 and E7 directly interact with TP53 and PRb and subvert their expression and hence act as potent oncogenes. Additionally, E6 and E7 interact with factors affecting growth and apoptosis and are critical for neoplastic transformation of the host cell [15]. Elevated E6 and E7 protein levels are indicators of growth changes and transform epithelial cells. High risk (HR) HPV E6 and E7 oncoproteins can each independently induce genomic instability in normal cells by generation of mitotic defects and aneuploidy through the induction of centrosome abnormalities [16].

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Abbreviations used: EOC – epithelial ovarian cancer; HPV – human papilloma virus; HR – high risk.

We designed this study to investigate the presence of DNA of HPV 16 and 18 in the upper genital tract in women at HR of developing EOC.

MATERIALS AND METHODS

Patients samples. 30 samples from Oregon Ovarian Cancer Tissue Repository which included 10 normal ovaries were obtained from women who underwent oophorectomy for unrelated causes and 20 fallopian tubes and ovaries from HR women undergoing risk reducing oophorectomy after having obtained signed informed consent. The study was approved by the Ethical Committee permission of Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine for studies with human materials.

We define patients considered to be at HR for EOC as women with two or more first degree relatives with ovarian and/or breast cancer, or with a personal history of breast cancer along with first-degree relatives with breast and/or ovarian cancer. Five HR women had mutations in *BRCA1* or *BRCA2* genes. The mean age at the time of oophorectomy for HR patients was 45.7 ± 2.5 with age ranging from 26 to 74 years and prevailing majority of women (12/20; 60%) were aged 26–46 years.

DNA extraction. Formalin-fixed, paraffin-embedded samples were obtained as 20 μm slices, deparaffinized in xylene, rehydrated in absolute and 70% ethanol. Vigorous precautions were taken to avoid sample contamination, including sample preparation of experimental and positive controls in different labs and cleansing the microtome with 75% ethanol before and after cutting each paraffin block. Then samples were processed with QIAamp DNA Mini kit (Qiagen) according manufacturers' instructions. DNA was eluted from columns in a volume of 50 μl.

PCR amplification. The quality of DNA from each tumor specimens was confirmed by PCR using primers for β-globin (224 bp):

β-globin F: CACTCAGTGTGGCAAAGGTGCCC;

β-globin R: GGCACTGACTCTCTCTGCCT.

We detected the presence of HPV genome with type-specific primers for *E6* fragment of high-risk HPV types 16 and 18.

The primers sets were as following:

HPV 16 F: TTAGAATGTGTGTACTGCAAGC;

HPV 16 R: TTGTCCAGATGTCTTTGCTT;

HPV 18 F: CACTTCACTGCAAGACATAG;

HPV 18 R: CTATGTTGTGAAATCGTCGT.

All primers were designed using the Vector NTI software (Invitrogen), homology of designed primers was tested by BLAST algorithm (NCBI nucleic acid database).

All PCR reactions were carried out in a total volume of 50 μl. The reaction mixture contained 1×PCR buffer (Promega, USA), 3 mM $MgCl_2$, 0.2 mM each dNTP, 2.0 μM primers and 1.25u Taq polymerase (Promega, USA) and either 30 or 10 ng of DNA. Samples were amplified on an ABS programmable thermal cycler (Applied Biosystems, USA). The mixture was denatured for 5 min at 92 °C followed by 33 or 37 cycles as follows: denaturation at 92 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 1 min. The final extension was for 10 min at 72 °C. The amplified gene fragments 281bp for HPV 16 and 326bp for HPV 18 were visualized on 2% agarose gels. HeLa and Caski cell lines DNA were used as positive control for HPV 16 and 18 amplification. Water template was used as negative control.

RESULTS

Detection of HPV 16/18 DNA in upper genital tract of patients at HR of developing EOC. The presence of HPV 16 and 18 genomes were detected by PCR of *E6* ORF using HPV type specific primers in fallopian tubes and ovaries samples from a cohort of 20 women at HR of developing EOC and 10 control women. We chose highly specific primers and optimized the PCR conditions such that there was no cross reactivity with other HPV types and secondary amplifications were absent (data not shown). PCR of control material did not suggest the presence of confounding factors or contamination. Moreover, in all cases, the β-globin gene was clearly amplified, indicating an adequate DNA isolation from samples. Negative controls consistently yielded no detectable bands, whereas positive controls showed distinctive bands (Figure).

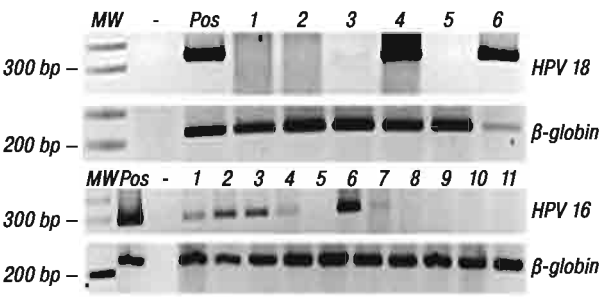

Figure. HPV *E6* specific PCR on genomic DNAs isolated from formalin fixed and paraffinized fallopian tubes and ovarian tissue sections as templates. The products were separated on a 2% agarose gel and stained with ethidium bromide. The marker is marked as MW; lines 1–4, 6, 7 represent the amplification products for HPV 16, lower panel. Lines 5, 8–11 represent HPV 16 negative samples. Lines 4 and 6 represent amplification products for HPV 18, upper panel. Lines 1–3 and 5 represent HPV 18 negative samples. The control for each of these samples was 224bp β-globin and is shown. Negative control (water for PCR) and positive control using genomic DNA either from freshly cultured CasKi or HeLa cells are shown

When we analyzed each cohort separately, surprising differences were found. In the control patients cohort consisting of 10 samples, only one ovarian sample (10%) tested positive for HPV 16, rest of the control samples were negative for HPV 18 and 16 *E6* ORF. In the 20 HR persons, a total of 8 (40%) patients tested positive for HPV 16 and 18 genomes (Table). Two patients tested positive for HPV had *BRCA* mutations. Of all the samples, two (10%) were positive for HPV 16, two (10%) were positive for HPV 18, and four (20%) showed positivity for mixed HPV 16/18 infection. The fallopian tubes and ovaries on separate blocks were available for all 20 HR patients. The presence of HPV *E6* ORF was found both in the fallopian

tubes and ovarian DNA from 6 (30%) patients. In two cases (10%) we detected HPV ORF only in the fallopian tube derived genomic DNA.

Table. The frequency of HPV infection types 16 and 18 in the upper genital tract of women at HR of developing EOC

HPV type	HR patients, n (%)	Specimens from HR patients, n (%)	
		Fallopian tubes	Fallopian tubes + ovaries
HPV 16	2 (10.0)	1 (5.0)	1 (5.0)
HPV 18	2 (10.0)	1 (5.0)	1 (5.0)
HPV 16/18	4 (20.0)	0	4 (20.0)
Total	8 (40.0)	2 (10.0)	6 (30.0)

DISCUSSION

The HR HPVs are critical etiologic factors for development of malignancies in the lower female genital tract [8]. The significance of HR HPVs in upper genital tract, including ovarian cancer is controversial. There has been variable frequency reported by various groups regarding the presence or absence of HPV in ovarian tissues and its putative role in cancer development [10, 11]. There have also been recurrent reports where viral sequences have been detected in vessel endothelial cells adjacent to cervical and oral cancers [17, 18]. In our previous study, PCR analysis demonstrated HPV DNA in 17.0% (9 from 53) ovarian serous carcinomas [19]. All HPV 16 and 18 DNA-positive OC tissues showed immunopositivity for E6 oncoprotein, which was adjacent to peritumoral area and was detected in glandular structures of ovarian carcinomas. In our small subset of nine samples that tested positive for diffused HPV E6 staining, we observed an acute downmodulation of p53 expression.

Results from this current study show the presence of HR HPVs in the upper genital tract of women at HR of developing EOC. The specific and sensitive PCR assay developed in our labs could detect HPV DNA derived from a very small number of contributing cells. The possibility of false positive was ruled out with consistent and concordant results among cross-technique and biological replicates (left or right ovarian tissues and corresponding fallopian tubes) and negative controls included in the study. Quite interestingly, we did detect HPV 16, 18 *E6* ORF in the upper genital tissues of HR cohort in a larger proportion compared to previously reported ovarian carcinomas [19]. However, one of the limitations of this study was the small sample size and therefore a higher than previously reported HPV positivity might not reflect a true statistical distribution.

It is known that different subtypes of epithelial ovarian carcinoma are associated with various genetic and environmental risk factors and molecular events during oncogenesis [20, 21]. In *BRCA* carriers cancer initiation may occur in the ovary, fallopian tube, or peritoneum but tumor growth and progression are favored in the ovary [22–24]. These results showing the HPV infection clamber through the genital tract may probably occur by a nonconventional mechanism where endothelial cells possibly acquired whole viral particles by phagocytosis and in turn presented them to ovarian epithelial cells or other differentiating cells of the upper genital tract where additionally to genetic factors appears to be possible environmental modifier of ovarian cancer risk in women at HR of developing EOC. Enhanced opportunistic infectivity in women with an inherent greater genomic instability is highly possible. There are reports in the literature that Fanconi anemia patients have an inherent susceptibility to human HPV-associated anogenital malignancies including ovarian carcinoma [25, 26]. But we also need to unequivocally establish this supposed mechanism of HPV transmission *in vivo* using a three dimensional organotypic culture model and genetic instability of ovarian epithelial cells causing susceptibility to HPV infection. Since our sample size was small, we need to further confirm these results in a larger statistically significant sample set.

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