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Detection of Human Papillomavirus Prevalence in Ovarian Cancer by Different Test Systems

Natalia Kisseljova[a] Kirill Zhordania[b] Maria Fedorova[a] Alexey Katargin[a] Adelina Valeeva[a] Julia Pajanidi[b] Larisa Pavlova[a] Olga Khvan[c] Svetlana Vinokurova[a]

[a]Laboratory of Molecular Biology of Viruses, NN Blokhin National Medical Research Center of Oncology, Ministry of Health, Russian Federation, Moscow, Russian Federation; [b]Department of Gynecology, NN Blokhin National Medical Research Center of Oncology, Ministry of Health, Russian Federation, Moscow, Russian Federation; [c]Department of Pathology, NN Blokhin National Medical Research Center of Oncology, Ministry of Health, Russian Federation, Moscow, Russian Federation

Keywords
Human papillomavirus · Epithelial ovarian cancer · PCR-based HPV test

Abstract
Background/Aims: High-risk human papillomavirus (HPV) infection is associated with different malignancies, but its role in the pathogenesis of ovarian cancer remains inconclusive. Published studies demonstrated a wide variation (0–50%) in HPV prevalence in ovarian cancer. To evaluate the contribution of detection tests to controversial results in different populations, we determined the presence of HPV DNA in Russian ovarian cancer patients using 10 different PCR-based tests. ***Methods:*** Epithelial ovarian adenocarcinomas were tested with 5 general primer sets commonly used for HPV screening of cervical and ovarian cancer and 5 HPV type-specific primers. ***Results:*** The use of a single PCR primer set resulted in a wide variation (0–29%) and an underestimation of the incidence of HPV-positive cancers. The combination of MY09/MY11 and GP5+/6+ primers in nested PCR revealed HPV DNA in 53% (18/34) of adenocarcinomas. HPV16 was found in 94% of the HPV-positive cases. In 6/6 positive cases, the active status of HPV16 was demonstrated by RT-PCR detection of E6 and E7 oncogene mRNAs. ***Conclusion:*** These findings indicate the need to employ multiple PCR-based tests to detect all HPV-positive patients. The identification of viral DNA and oncogene transcripts in cancerous tissues indicate the possible role of HPV in ovarian carcinogenesis in Russia.

Introduction

Epithelial ovarian cancer (EOC) is one of the most lethal gynecological malignancies. Little is known about the very early molecular and genetic events associated with ovarian carcinogenesis. Due to the lack of symptoms and markers in the early stage of the disease, EOC is commonly diagnosed only at an advanced stage [1]. The etiology of ovarian cancer remains unclear. Epidemiologic research has demonstrated genetic, reproductive, and other

N.K. and K.Z. contributed equally to this work.

karger@karger.com
www.karger.com/int

Natalia Kisseljova
NN Blokhin National Medical Research Center of Oncology of Ministry of Health of Russian Federation, Laboratory of Molecular Biology of Viruses, Kashirskoe shosse 24
Moscow 115478 (Russian Federation)
natalia-kis@yndex.ru

risk factors for ovarian cancer [2]. In 1987, for the first time, the presence of HPV DNA in some cases of EOC was demonstrated and the involvement of human papillomavirus (HPV) infection in the etiology of at least a subset of ovarian cancers was discussed [3]. Since then, many studies on the prevalence of high-risk HPV (HR-HPV) in EOC, with both positive and negative results, have been published [4–7]. Recently, a meta-analysis including nearly 50 observational studies demonstrated a wide variation in HPV prevalence in ovarian cancer tissues (0–66.7%) [8, 9]. The vast majority of published studies are based on assays to identify DNA HPV in ovarian cancer cells. One of many criteria for determining the role of viruses in human carcinogenesis is the regular presence and persistence of the viral DNA in tumor cells and cell lines derived from the same tumor type [10]. The controversial results of the published studies indicate that detecting HPV DNAs in ovarian cancer remains a big challenge. In the case of ovarian adenocarcinoma, HPV DNA detection is complicated by the very low number of HPV copies per cell. Glandular epithelium does not support productive viral infection, and the level of HPV DNA in ovarian adenocarcinoma is at least one order of magnitude lower than in cervical squamous cell carcinoma where HPV DNA is detected in >99% of cases [11]. As a result, the detection of HPV DNA in adenocarcinoma requires a sensitive detection assay.

Our objective was to estimate the prevalence of HPV infections in EOC in Russian patients by means of a broad range of general primer sets and HPV type-specific primer pairs simultaneously, and to understand if the commonly used tests for HPV detection may contribute to the controversial results of epidemiological studies.

Materials and Methods

Clinical Samples

Ovarian adenocarcinomas (17 samples of frozen tissue and 24 samples of formalin-fixed, paraffin-embedded [FFPE] tissue) were obtained from 34 patients attending the N.N. Blokhin National Medical Research Center of Oncology (Tables 2, 3). The EOC cases included 29 serous adenocarcinomas, 2 mucinous adenocarcinomas, 3 endometrioid adenocarcinomas. The diagnosis was verified by 2 experienced gynecopathologists. All adenocarcinomas were stage I–IV according to FIGO staging 2014. The age of the patients ranged from 30 to 74 years. None of the patients had human immunodeficiency virus (HIV) and they were all immunocompetent, except for patient 19 (Table 3) who was allergic to antibiotics. No patients had cervical cancer or cervical intraepithelial neoplasia (CIN), but patient 2 had metachronous cancer of the uterine body and patient 13 had cancer of the Fallopian tubes (Table 2).

RNA and DNA Isolation

DNAs and RNAs were isolated from frozen tumor tissues by homogenization in 4.5 M guanidine isothiocyanate and centrifugation through a cesium chloride cushion, as described previously [12]. DNAs from FFPE tissue sections, after manual microdissection of the tumor cells under microscope control, were isolated using a DNA isolation kit (ReliaPrep FFPE gDNA Miniprep System; Promega, Madison, WI, USA) as recommended by the supplier, and included additional deparaffinization with mineral oil at 80 °C for 1 min.

PCR

The adequacy of all DNA preparations for PCR was determined by amplification of the *HPRT* gene. PCR primers and conditions are presented in Table 1.

HPV Typing

PCR products were analyzed by electrophoresis in agarose gel, extracted from the gel using a GeneJET PCR purification kit (Thermo Scientific, Vilnius, Lithuania), and then underwent Sanger sequencing (in the Genome Center, Moscow, Russian Federation) using ABI prism 3100-Avant genetic analyser (Applied Biosystems, Foster City, CA, USA), or direct sequencing (PyroMark Q24; Qiagen, Hilden, Germany) [13].

Complementary DNA Preparation and Amplification

Total RNA (1 μg) was reverse-transcribed, using the HPV16-specific reverse primer HPV16 R845 and the Moloney murine leukemia virus reverse transcriptase (Super Script III RT; Invitrogen, Carlsbad, CA, USA), for 1 h at 50 °C in a final volume of 20 μL. An additional DNase treatment step (deoxyribonuclease I amplification grade; Invitrogen) was included for all RNA samples, and DNA contamination was controlled by conducting cDNA preparation without reverse transcriptase for each RNA sample. First-strand cDNAs encompassing the viral *E7* and *E6* genes were subsequently amplified by nested PCR: for mRNA E7, the first round with HPV16 E6^ as forward primer and reverse primer HPV16 R845 and the second round with F651 and 816R; and for mRNA E6, the first round with F99 and R816 and the second round with F108 and R544 and Taq DNA polymerase (Clontech, Mountain View, CA, USA).

Statistical Analysis

The Fisher exact test was used to compare the presence of HPV DNA detected by different PCR tests. Differences were considered to be statistically significant when the p value was ≤0.05.

Results

Comparison of Different PCR Tests for HR-HPV Detection in Frozen Tissues

Taking into account the controversial HPV detection results in EOC, we compared different common HR-HPV screening tests by analyzing the set of 17 frozen tissues of EOC (Table 2). Four PCR tests applied separately produced negative results. The PCR with CPI/CPII primers revealed some HPV-positive samples (2/17, 11.8%). Sequencing of these PCR products demonstrated the presence of HPV54

Table 1. Primers and conditions of PCR for HPV detection

Primers	Region of HPV genome	Reference or sequence	Product size, bp	T aneal. (C) and cycles
MY09/MY 11	L1	[11]	450	55°, 40 c
GP5+/GP6+	L1	[11]	145	40°, 40 c
SPF	L1	[12]	65	45°, 40 c
pU-1M/2R	E6/E7	[13]	228–268	55°, 30 c
CPI/CPII G	E1	[11]	188	54°, 40 c
HPV16–1*	E6 F 99 E6 R 227	CTGCAATGTTTCAGGACCCACA CCTCACGTCGCAGTAACTGTTG	128	64°, 45 c
HPV1–2	E6 F 108 E6 R 544	TTCAGGACCCACAGGAGCGA TCTACGTGTTCTTGATGATCTG	436	58°, 45 c
HPV16–3	E7 F 651 E7 R 816	CAGCTCAGAGGAGGAGGATG GCCCATTAACAGGTCTTCCA	165	60°, 45 c
HPV16–4	E7 F 701 E7 R 845	CGGACAGAGCCCATTACAAT GAACAGATGGGGCACACAAT	145	56°, 45 c
HPV16–5	E7 F 728 E1 R1103	CCTTTTGTTGCAAGTGTGACTCTACG CACAGGAAGCAAAACACCATA	375	57°, 45 c
HPV16 E6^	E6 F	ACTGCGACGTGAGGTGTATTAAC	–	
Splice variant (214–418) HPRT	F R	CTGGATTACATCAAAGCACTG GGATTATACTGCCTGACCAAG	230	60°, 28 c

* The positions of all HPV16-specific primers are given according to sequence [GenBank: K02718.1].

and HPV16 DNA in tumor No. 14 and tumor No. 9, respectively. Sequencing of several PCR products of expected size produced by the MY09/MY11 and pUM-pU primers demonstrated 95–100% homology with different DNA regions of *Homo sapiens*, and so these results were classified as false-positive. Nonspecific DNA amplifications in the same size range as the HPV *L1* product recognized by the MY09/11 primers have been described previously [14].

The GP5+/GP6+ primers are complementary to the L1 region located inside the sequence recognized by the MY09/MY11 primer and may be used in nested PCR [15]. The use of nested PCR MY09/MY11– GP5+/GP6+ (MY/GP) might increase sensitivity and specificity of HPV DNA detection. The combination of MY/GP primers in nested PCR enabled the detection of HR-HPV in 10/17 samples (58.8%).

Next, we tried to select effective type-specific primers to evaluate HPV16 prevalence in our sample set (Table 2). Five primer pairs designed for *E6*, *E7*, and the 5′ end of the *E1* gene revealed HPV16 in squamous cell carcinomas of the uterine cervix with 100% efficiency (data not shown). None of these was able to reveal all HPV16-positive samples of ovarian adenocarcinoma, and one had a false-positive result (HPV16 E7 701–845). The presence of HPV16 DNA was confirmed in only 4/10 samples which were positive in nested PCR with MY/GP. These results indicate the low level of HPV DNA copies in ovarian adenocarcinoma compared to in squamous cell carcinoma of the cervix.

The rate of HR-HPV positivity in the same group of EOCs varied from 0 to 59%, depending on which PCR test is applied. There was no discrepancy in the types of HPV revealed by the different primer sets. The MY/GP combination in nested PCR significantly increased the positive results and should thus be used for samples with a low copy number of HPV DNA.

Comparison of Detection of HR-HPV DNA in Frozen and FFPE Tissues

As FFPE tissues are the main source for studies with PCR application and are often used in HPV detection in EOC,

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Table 2. Detection of HPV by different PCR tests in frozen tissues of EOC

No.	Tumor type, histology	My9/11	GP5/6+	(1) My9/11 (2) GP5/6+[a]	pU1M –Pu	CPI/CPII	SPF	HPV16 E7 (651–816)[b]	HPV16 E7 (701–845)	HPV16 E7/E1 (728–1103)	HPV16 E6 (99–227)	HPV16 E6 (108–544)	mRNA
1	AC ser	–	–	HPV16	–	–	–	–	HS	–	–	HPV16	+
2	AC end	–	–	HPV16	–	–	–	–	HS	–	–	HPV16	n.t.
3	AC ser	HS	–	HPV16	–	–	–	–	–	–	–	–	n.t.
4	AC ser	–	–	HPV16	–	–	–	–	–	–	–	–	+
5	AC ser	–	–	HPV16	–	–	+/–	–	–	–	–	–	+
6	AC ser	–	–	–	–	–	–	–	–	–	–	n.t.	n.t.
7	AC ser	–	–	–	–	–	+/–	–	–	–	–	–	–
8	AC ser	–	–	–	HS	–	+/–	–	+	–	–	n.t.	–
9	AC muc	–	–	HPV16	–	HPV16	+/–	HPV16	HS	HPV16	–	HPV16	+
10	AC ser	–	–	–	–	–	–	–	–	–	–	–	n.t.
11	AC ser	–	–	–	–	–	+/–	–	–	–	–	n.t.	n.t.
12	AC end	–	–	HPV16	–	–	+/–	–	–	–	–	HPV16	n.t.
13	AC ser	HS	–	HPV16	HS	–	–	+	HS	–	–	–	+
14	AC ser	–	–	HPV54	–	HPV54	–	–	HS	–	–	–	–
15	AC ser	–	–	–	–	–	–	–	HS	–	–	–	n.t.
16	AC ser	HS	–	–	HS	–	+/–	–	+	–	–	–	n.t.
17	AC ser	–	–	HPV16	–	–	+/–	–	–	HPV16	–	HPV16	+
N		0	0	10/17	0	2/17	?	2/17	0	2/17	0	4/14	6/6
HPV-positive				58.8%		11.8%		11.8%		11.8%		28.6%	

Ser, serous; end, endometrioid; muc, mucinous; HS, PCR product sequence had 95–100% homology with DNA of *Homo sapiens*; –, there was no PCR product; +, PCR product was presented but not sequenced; +/–, PCR product was hardly visible which meant HPV typing was impossible; n.t., not tested.

[a] HPV type was detected by pyrosequencing (in all other cases, it was done by Sanger sequencing).

[b] Primer positions (in bp).

Table 3. Detection of HPV DNA in FFPE tissues of EOC by PCR and sequencing

No.	Case	Tumor type, histology	(1) My9/11 (2) GP5/6+	CPI/CPIIG	GP5/6+
1	1[a]	AC ser	HPV16	–	–
2	5[a]	AC ser	HPV16	HPV16	–
3	6[b]	AC ser	–	–	–
4	7[b]	AC ser	–	–	–
5	8[b]	AC ser	–	–	–
6	11[b]	AC ser	–	–	–
7	16[b]	AC ser	–	–	–
8	18	AC ser	–	–	–
9	19	AC ser	HPV16	–	–
10	20	AC ser	–	–	–
11	21	AC ser	HPV16	–	–
12	22	AC ser	–	–	–
13	23	AC ser	–	–	–
14	24	AC ser	HPV16	HPV16	–
15	25	AC ser	HPV16	–	–
16	26	AC end	HPV16	HPV16	–
17	27	AC ser	–	–	–
18	28	AC muc	–	–	–
19	29	AC ser	–	–	–
20	30	AC ser	HPV16	HPV16	–
21	31	AC ser	–	–	–
22	32	AC ser	HPV16	HPV16	–
23	33	AC ser	HPV16	HPV16	–
24	34	AC ser	–	–	–
HPV-positive cases			10/24 41.7%	6/24 25.0%	0/24 0%

Ser, serous; end, endometrioid; muc, mucinous; –, there was no PCR product.

[a] HPV16-positive cases in Table 2.

[b] HPV16-negative cases in Table 2.

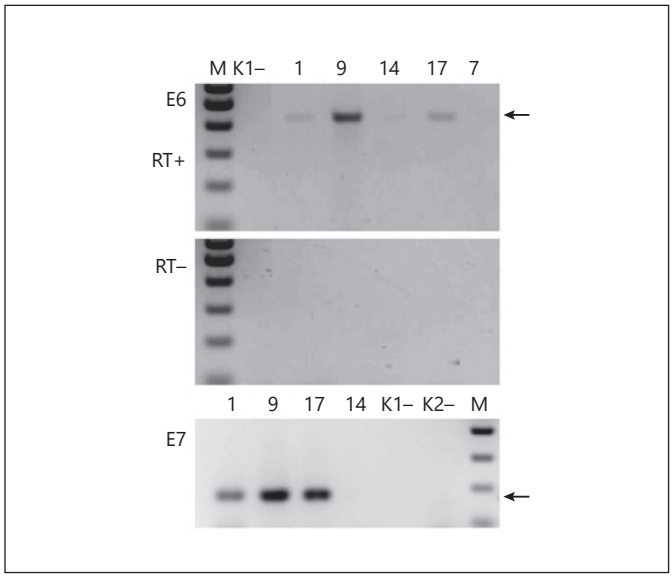

Fig. 1. Analysis of mRNA of E6 and E7 oncogenes in HPV16-positive EOC by RT-PCR. Agarose gel electrophoresis of second-round PCR products are presented. Complementary DNAs were prepared using E7-specific primer R845. RT (–), cDNA preparation without reverse transcriptase, a control of DNA contamination; K1 (–), H_2O was added in PCR instead of cDNA; K2, the primer pair for the first round E7-specific PCR did not amplify HPV16 DNA, a control of DNA contamination; M, molecular marker, 100-bp ladder (SibEnzyme, Novosibirsk, Russia). Numbers under panels indicate samples of EOC (as in Table 2). Arrows indicate PCR products 436 and 165 bp for E6 and E7, respectively.

the efficacy of 2 PCR tests (nested MY/GP and CPI/CpIIG PCR) were was investigated using archival tissues (Table 3). Samples 1–7 were analyzed as frozen tissues and 2 of these were HPV16-positive (Table 2). MY/GP nested PCR confirmed the results of the frozen-tissue analyses and revealed HPV16 DNA in 2/7 samples. In total, HPV16 DNA was detected in 10/24 samples (41.7%). Although this is less than that in the frozen tissues (58.8%), likely due to the common fragmentation of DNA isolated from FFPE tissues, the difference was not significant ($p = 0.35$, Fisher's exact test). The estimated pooled HPV prevalence in both types of tissues was 52.9% (18/34) (Tables 2, 3). The CPI/CPIIG test was found to be less sensitive than MY/GP in both frozen and FFPE tissues. The pooled HPV prevalence estimated by the CPI/CPIIG test accounted for 20.6% (7/34), i.e., significantly different from that revealed by MY/GP (18/34, $p = 0.01$, Fisher's exact test). With regard to GP5+/6+ PCR, the selection of tumor cells by microdissecting FFPE tissues did not have greater sensitivity than the frozen tissues, where a mixture of tumor and normal cells was analyzed. None of the 10 HPV-positive FFPE samples in nested MY/GP PCR was revealed by this test (Table 3).

HPV16 was the predominant genotype in our samples (17/18 HPV-positive samples, 94%). No other HR-HPV (except 1 case of HPV54) or low-risk HPV genotypes were found.

In conclusion, all general and type-specific primer pairs except in the MY/GP test were found to be unsatisfactory for the amplification of HPV DNA from ovarian adenocarcinoma because they tended to underestimate the HPV prevalence.

HR-HPV Expression in EOC

In terms of carcinogenesis, not only the mere presence of HPV DNA but also the expression of the HPV oncogene in tumor tissues is important [10]. To demonstrate

the active status of HPV16 in EOC cells, we determined the transcripts of E6 and E7 oncogenes for samples where RNAs were available (Table 2; Fig. 1).

Six out of 6 HPV16-positive samples were positive for HPV16 oncogene expression, but there was no expression in the HPV16-negative and HPV54-positive samples when using HPV16 type-specific primers. Levels of oncogene expression were low because the presence of E6 and E7 mRNA in tumors was revealed by nested PCR. Our results showed that HPV is indeed present and active in a subgroup of ovarian carcinomas.

Discussion

The comparison of HPV detection in the same sampling of EOC by different sets of general and type-specific primers demonstrated that the use of a single PCR primer set resulted in a wide variation (0–29%) and an underestimation of the incidence of HPV-positive cancers. In our hands, the combination of MY/GP in nested PCR was the most sensitive test, which revealed HR-HPV DNA in 53% of adenocarcinomas. It is possible to determine HPV DNA by a single consensus or type-specific test, but the frequency of occurrence of HPV in these cases is several times lower than in MY/GP in nested PCR (Table 2, primers CPI/CPII or HPV16 E6 108–544). Our result coincides with the data on optimal detection of HPV in cervical carcinomas [16]; the authors demonstrated that multiple consensus and type-specific primers should be used to detect all HPV-positive patients. For the most part, PCR-based studies on HPV prevalence in ovarian cancer have been performed with 1–3 sets of primers. In many of these studies, FFPE tissues have been used and this tends to decrease the sensitivity of PCR if the amplified products are >200 bp in size [4, 5, 7, 9]. Taking into account the technical aspects of HPV detection mentioned above, one can suppose that HPV prevalence in ovarian cancer is underestimated in a portion of studies. The published studies differed according to geographical areas, surveyed populations, DNA detection techniques, and type of tissues (i.e., FFPE or frozen) [8, 9]. It is impossible to exclude variations of HPV prevalence in different geographical regions, but our results demonstrated that the methods of HPV detection contribute substantially to controversial results of epidemiological studies.

In our study, the most frequently detected HPV genotype was HPV16. This result is consistent with the notion data that HPV16 is the most prevalent type in human low genital-tract neoplasias in general, particularly in Russia, and in many EOC [4, 17–21].

The carcinogenic potential of high-risk HPV and mechanisms of its realization is well described. Permanent expression of the viral oncoproteins, E6 and E7, is required for maintaining the malignant growth of cervical cancer cells [22]. We demonstrated not only the mere presence of HPV16 DNA but also E6 and E7 oncogenes transcription in 6 out 6 HPV16-positive tumors. It seems unlikely that the molecular mechanisms of transformation are different in the different types of epithelial cells infected with HPV. Unfortunately, there are only a few published studies that demonstrate the expression of viral oncogenes [6, 23]. The carcinogenic role of HPV infection in ovarian epithelium could be clarified by the detection of viral oncogene expression in clinical samples.

In our study, the identification of high-risk HPV DNA in 53% of EOC and viral oncogene transcripts in cancerous tissues may indicate a possible role of HR-HPV in ovarian carcinogenesis in Russia. The findings provide us with baseline information for future studies regarding the pathogenesis of ovarian cancer. It is known that mucinous and endometrioid endocervical adenocarcinomas metastasize to the ovaries and can simulate primary ovarian carcinoma; most are HR-HPV-associated [24–26]. We cannot exclude the fact that 5 of our samples with mucinous and endometrioid differentiation represented metastases spreading from the endometrium or cervix. In contrast, primary cervical serous adenocarcinomas are considered to be metastases from primary ovarian, tubal, or peritoneal serous adenocarcinomas and are an extremely rare malignancy. [27, 28]; it must be stressed that the diagnosis of primary serous carcinoma of the cervix should be made only after their spread from the ovary, Fallopian tubes, or endometrium has been excluded. It is unlikely that all 29 serous ovarian adenocarcinomas in our sampling metastasized from this rare type of cervical adenocarcinoma. Serous carcinomas account for a major proportion of ovarian cancer (75%) [29]. It is important to understand the role of HR-HPV in their pathogenesis, because currently used vaccines protect against HR-HPV infection and may therefore reduce the risk of at least a subset of ovarian and uterine body cancers.

Statement of Ethics

All tissues were collected under the approval of the Ethics Board of the N.N. Blokhin Research Center in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients.

Disclosure Statement

The authors declare there were no conflicts of interest.

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Author Contributions

N.K. carried out the RT-PCR analysis, interpreted the data, and was a major contributor to writing the manuscript; K.Z. was responsible for ideas, formulation of research goals, and writing the manuscript; M.F., A.K., and A.V. carried out the experimental work; J.P. and L.P. provided data on patients and laboratory samples; O.K. and J.P. performed the pathomorphological analysis; S.V. was responsible for overseeing and leading the research activity and experimental design. All authors read and approved the final manuscript.

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