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Simultaneous Detection of the HPV L1 Gene and the Human β-Globin Gene in the Blood Components of Cervical Cancer Patients Living in Yakutia

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Abstract

Background: To create a test for the early detection of cervical cancer (CC), we conducted exploratory studies on detecting HPV genes and genes of the human β-globin locus in plasma (Pl) and red blood cell (RBC) suspension (RBCsus) samples from patients with newly detected CC (NDCC).

Methods and Results: Smears of venous blood containing K3-EDTA from five anonymous patients aged from 45 to 55 years (residents of Yakutia), with NDCC were obtained. Three types of blood component samples were prepared – Pl, RBCsus, and the erythrocyte fraction treated with trypsin (RBCsus-Try). To detect circulating cell-free DNA (cfDNA) in NDCC patients, we studied the presence of genes corresponding to the HPV L1 protein region and genes of the human β-globin locus by real-time PCR (qPCR) using appropriate primers.

The genes of β-globin locus and HPV L1 were detected in Pl of NDCC patients in 20% of cases, and in RBCsus in 60% of cases. The amplified gene products using primers were present in RBCsus-Try in only one patient (20% of cases).In patients with uncertain amplification, electrophoresis showed the absence of amplified products in Pl and their presence in RBCsus.

Conclusion: In NDCC patients, HPV L1 and β-globin genes can be detected in both Pl and RBCsus. In addition, in RBC samples, these genes were detected more often than in plasma samples, and no absolute absence of amplification products was observed in RBC samples. The research needs to be continued.(**International Journal of Biomedicine. 2022;12(1):109-114.**)

Key Words: cervical cancer • human papillomavirus • circulating cell-free DNA • quantitative PCR

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Abbreviations

CC, cervical cancer; **Pl**, plasma; **RBC**, red blood cells; **NDCC**, newly detected CC; **HPV**, human papillomavirus; **ctDNA**, circulating tumor DNA; **cfDNA**, circulating cell-free DNA; **qPCR**, quantitative PCR; **csbDNA**, cell-surface-bound extracellular DNA.

Introduction

Cervical cancer (CC) is common cancer among women around the world. The high burden of CC is among people living in low- and middle-income countries where access to public health services is limited.(1) In Russia, a middle-income country,(2) cytological screening is primarily used for CC early detection. Cytological screening is carried out

free of charge for Russian citizens once every 3 years. At the same time, in Russia, the CC incidence and mortality have not been decreasing and significantly exceed the target level established by the WHO.[3] Therefore, research on creating new tests for CC early detection is highly relevant. For such tests, the researchers suggest, among others, the detection of ctDNA isolated from human bodily fluids since in CC patients, ctDNA has a high genetic similarity to the tumor cell DNA.[4,5] ctDNA can be detected before treatment in patients with early-stage primary cancer; however, ctDNA is generally detected at a lower rate than in advanced cancer.[6-8]

For CC detection, researchers often detect circulating cell-free HPV DNA, which can be considered as CC-ctDNA, since it is believed that cfDNA containing one or more copies of the HPV genome originates from transformed cells.[5,9]

The aim of our study was to create an affordable and easily reproducible method for the early detection of CC using cfDNA as a CC biomarker.

To detect cfDNA in NDCC patients, we studied the presence of genes corresponding to the HPV L1 protein region and genes of the human β-globin locus by real-time PCR (qPCR) using appropriate primers. It is known that CC-ctDNA contains human genes and HPV genes.[5] We detected cfDNA, which can be a CC biomarker in NDCC patients, in samples of plasma (Pl) and red blood cell (RBC) suspension (RBCsus) since these blood fractions do not contain human cell nuclei.

A meta-analysis conducted by Y. Gu et al.[9] showed that detection of HPV cDNA in patients with CC could be used as a noninvasive early dynamic biomarker of tumors, with high specificity and moderate sensitivity.

Human β-globin is expressed by cervical carcinoma cells and plays a cytoprotective role against oxidative damage.[10] In cancer patients, cfDNA carries tumor-related genetic alteration of transformed cells.[11] Therefore, we expected that in CC patients, genes of the human β-globin locus should be detected in blood fractions that do not contain human cell nuclei in significant quantities. In ovarian cancer, for example, genes of the human β-globin locus are detected in plasma in significantly greater numbers than in healthy patients.[12]

Genes corresponding to the HPV L1 protein region and genes of the human β-globin locus are often detected in scientific research and in clinical practice; their primers are available on the market and have an optimal value. In addition, we decided to find out whether the cfDNA detected from RBCsus is the RBC-bound cfDNA. Therefore, we determined the presence of cfDNA in the erythrocyte fraction treated with trypsin (RBCsus-Try).

Materials and Methods

Smears of venous blood containing K3-EDTA from five anonymous patients aged from 45 to 55 years (residents of Yakutia), with NDCC were obtained. The blood was collected in 2018-2019 and in the same years, it was investigated. At the time of blood sampling, the patients were not given any type of cervical cancer treatment.

Three types of blood component samples were prepared – Pl, RBCsus and RBCsus-Try. To obtain blood fractionation, blood samples were centrifuged at 1600 g for 10 minutes. After fractionation, PI samples were obtained.

RBC samples were obtained from 1ml of the erythrocyte fraction then washed three times in a phosphate buffer. RBCsus-Try samples were obtained as follows: The RBC samples described above were split in half; a solution of 0.25% trypsin was added to one half in a ratio of 1:1 and incubated at 37°C for 10 minutes. After incubation, it was centrifuged to obtain sediment. Then the lower part of the sediment was washed three times in a phosphate buffer, and these samples were included in this study as RBCsus-Try.

All samples were stored at a temperature of –20°C before DNA isolation and qPCR. For DNA extraction, 200 µl of previously prepared samples with markings Pl, RBCsus, RBCsus-Try were used. TRIzol™ and chloroform were added to each sample in an amount of 1000 µl and 200 µl, respectively. The concentration of the isolated DNA was determined on a spectrophotometer, following the manufacturer's instructions.[13]

To detect cfDNA, the CFX96 Touch Real-Time PCR Detection System was used. qPCR was performed using the 5x qPCRmix-HS SYBR+LowROX, designed for setting up PCR with SYBR Green I in the presence of a reference dye ROX, according to the manufacturer's amplification protocol.[14]

Genes of the human β-globin locus were detected using PC03/04 primers (5'-ACACAACTGTGTTCACTAGC-3'/5'-CAACTTCATCCACGTTCACC-3').

Primers MY09/11 (5'-CGTCCMARRGGAWACTGATC-3'/5'-GCMCAGGGWCATAAYAATGG-3') were used to detect genes corresponding to the HPV L1 protein DNA region. The length of the amplicons was determined by DNA electrophoresis in 2% agarose gel.

Given the small number of patients and the exploratory nature of the study, the results are presented as a percentage.

The study was conducted in accordance with ethical principles of the WMA Declaration of Helsinki (1964, ed. 2013) and approved by the Ethics Committee of the M.K. Ammosov North-Eastern Federal University (protocol No. 13 of April 4, 2018, decision No. 2). Written informed consent was obtained from each patient.

Results

In all 15 samples (3 samples [Pl, RBCsus, and RBCsus-Try] from each of the five CC patients), DNA was detected and isolated in sufficient amounts (Table 1).

The results of qPCR using primers PC03/04 and MY09/11 (Table 2) showed the joint presence of genes corresponding to the human β-globin region and HPV L1 protein DNA region in Pl from Patient #5 (20% of cases), in RBCsys from Patients #3-5 (60% of cases) and in RBCsus-Try from Patient #5 (20% of cases).

Uncertain amplification results using both primers were detected for DNA isolated from Pl of Patients #1 and #4 (40% of cases), for DNA isolated from RBCsus of Patients

Table 1.

DNA concentration (ng/ml)

	Pl	RBCsus	RBCsus -Try
Patient 1	High	High	High
Patient 2	High	High	High
Patient 3	High	3773	High
Patient 4	High	High	High
Patient 5	3390	2808	2753

"High" means that the concentration of the isolated DNA is higher than the linear range of the spectrophotometer.

#1 and #2 (40% of cases), and for DNA isolated from the RBCsus-Try of Patients #1, #3 and #4 (60% of cases). No amplification results using both primers were detected for DNA in Pl from Patients #2 and #3 (40% of cases) and in RBCsus-Try from Patient #2 (20% of cases).

The results of qPCR products were validated based on the DNA gel electrophoresis. The agarose gel electrophoresis confirmed the qPCR amplification and no amplification. Regarding CC patients with uncertain amplification for DNA isolated from Pl (Patients #1 and #4), in Patient #1, electrophoresis showed the presence of amplified products using PC03/04 primers, but electrophoresis to confirm amplification using MY09/11 primer products could not be performed because we ran out of biomaterial; in Patient #4, electrophoresis showed the absence of amplified products using both primers.

Patients #1 and #2 with uncertain amplification for DNA isolated from RBCsus, electrophoresis showed the presence of amplified products using both primers. In Patients #1, #3, and #4 with uncertain amplification results for DNA isolated from RBCsus-Try, electrophoresis did not confirm the presence of amplified products using both primers.

Electrophoresis revealed that the size of qPCR products ranges from 50 bp to 200 bp. This size corresponds to the recommended amplicon sizes for obtaining consistent and reliable results by the real-time PCR method.[15]

Thus, in NDCC patients, genes corresponding to the HPV L1 protein DNA region and the human β-globin region were detected in RBCsus more often than in Pl. Also, in RBCsus, there was no undoubted absence of amplified products using primers for these gene products. Considering that the amplified gene products using primers were present in RBCsus-Try in only one patient, we can say that the cfDNA that can be a CC biomarker might be an RBC-bound cfDNA.

Discussion

HPV cDNA has become a major focus, providing a strong basis for early diagnosis and prognosis in cervical cancer.[5,16,17] The E7 proteins encoded by the high-risk type HPVs, such as HPV 16 and HPV 18, bind Rb with a much higher affinity compared to those encoded by the low-risk type HPVs, such as HPV 6 and HPV 11.[18] The ability to target the retinoblastoma (Rb) family of proteins and p53 and to induce telomerase are some of the critical events that contribute to the development of malignancy.[19]

HPV DNA for CC detection is isolated by researchers mainly from plasma and serum samples(6) using primers for HPV16/18 E7.[20-22] It is believed that the DNA fragment corresponding to the HPV16/18 E7 protein region is the optimal candidate for a CC biomarker.[22,23]

The papillomavirus major capsid protein, L1, encoded by late gene 1, can spontaneously self-assemble into a highly immunogenic structure,virus-like particles, that closely mimic the natural surface of native papillomavirus virions. The late phase of the viral life cycle, during which new virions are assembled, occurs only in keratinocytes, which form the outer layer of the skin (epidermis), as well as the surface of other stratified squamous epithelia, including the genitals.[24]

Table 2.

The results of qPCR using primers PC03/04 and MY09/11

	Pl		RBCsus		RBCsus -Try	
	PC03/04	MY09/11	PC03/04	MY09/11	PC03/04	MY09/11
Patient #1	-+ (+)	-+(N/A)	-+ (+)	-+ (+)	-+ (-)	-+ (-)
Patient #2	- (-)	- (-)	-+\+	-+ (+)	- (-)	- (-)
Patient #3	- (-)	- (-)	+ (+)	+ (+)	-+ (-)	-+ (-)
Patient #4	-+ (-)	-+ (-)	+ (+)	+ (+)	-+ (-)	-+ (-)
Patient #5	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)

The "+" indicates the amplification; the "-"indicates no amplification; the "-+" indicates an uncertain amplification. In parentheses - the results of electrophoresis with the RT-PCR products.

We have not found any published studies on CC detection based on the presence of circulating HPV DNA using primers for HPV L1 DNA. Also, we have not found published studies on the detection of HPV DNA and ctDNA in the erythrocyte fraction of CC patients. There is a study that shows that in healthy male blood donors, HPV DNA is found among leukocytes, but not in the erythrocyte fraction.[25]

Protein L1 alone forms the surface of the HPV virion and provides the initial interaction of the HPV capsid with the host cells.[24] In a study by Cao et al.,[26] circulating HPV DNA (in the plasma) was detected by conventional PCR using the L1 G5+/6+ primer (L1 primer) in most patients with HPV(+) oropharyngeal carcinoma. In a study by Cocuzza et al.,[27] HPV DNA detection was carried out in both plasma and cervical samples using type-specific real-time quantitative PCR assays identifying oncogenic HPV 16, 18, 31, 33, 45, 51, and 52. Overall, 34.2% (41/120) of plasma samples were shown to be positive for HPV DNA detection; HPV 45(46.3%), HPV-51(29.6%), and HPV 16(18.5%) were the most frequently identified genotypes. HPV 16 was the most common genotype identified in women found to be HPV DNA positive in both cervical and plasma samples.

We chose primers for HPV L1 and the human β-globin region for early detection of CC, not only in Pl, but also in RBC suspensions because we speculated on the following:

(i) It is known that with the CC progression, changes in an infected cell interrupt the HPV replicative life cycle; therefore, progeny virions cannot be obtained,[28] and L1expression is only poorly supported,[29] but not all infected cells in CC patients undergo cancer transformation, and that means HPV replication may still be present;

(ii) there is a possibility that HPV can bind to the erythrocyte surface; it is known that virus-like particles having the L1 protein derived from bovine papillomavirus type 1 (BPV-1) can bind to surface receptors of erythrocytes;[30] in addition, heparan sulfate, the HPV receptor,[25] has been identified on human erythrocytes.[31]

(iii) With the probable fulfillment of the events described in (i) and (ii), the biomarker of malignancy can be the simultaneous presence of genes corresponding to the HPV L1 protein DNA region, as well as genes of the human β-globin locus, since the human β-globin gene cluster is expressed by cervical carcinoma cells;[10]

(iv) erythrocytes carry extracellular vesicles on their surface,[32] some of which are represented by exosomes[33] including, in our opinion, tumor exosomes, and it is possible that DNA of tumor exosomes contains genes corresponding to the HPV L1 protein DNA region and genes of the human β-globin locus;

(v) erythrocytes are capable of carrying csbDNA[34] and, in our opinion, it is possible that erythrocytes are also capable of carrying csbDNA—RBC-bound ctDNA.

Our study has certain limitations. We do not exclude technical errors, so the study should be repeated. In future research, we should include in the study a larger number of patients, both with CC and cervical neoplasia, as well as healthy patients. We should also get, if possible, consent from patients for more of their medical information.

A review by ASCO and the College of American Pathologists analyzed information on clinical ctDNA assays and found no evidence for clinical utility and little evidence for clinical validity of ctDNA assays in cancer early detection, treatment monitoring, and detection of residual disease. There is also a lack of evidence for the clinical validity and clinical usefulness of ctDNA assays for cancer screening outside of clinical trials.[35] The authors emphasize that "Given the rapid pace of research, re-evaluation of the literature will shortly be required, along with the development of tools and guidance for clinical practice."[35]

We want to emphasize that this study was performed for the detection of CC at any stage. Our study and similar studies by other researchers are relevant, for example, for patients for whom it is important that the procedure for taking their samples for testing is psychologically comfortable.

For example, CC screening tends to induce psychological discomfort, since all patients experience anxiety before a gynecological examination,[36] while the level of anxiety during venipuncture is very low.[37] Patients can be expected to shy away somewhat less from tests that detect CC markers in the blood than from screening for CC.

We hope that the continuation of the study will allow us to create an affordable and easily reproducible method for detecting cfDNA, which can be a CC biomarker.

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Competing Interests

The authors declare that they have no competing interests.

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