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The multiple HPV infections and their possible significance in predicting the risk of the cervical cancer

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ABSTRACT

Infections with human papillomavirus (HPV) are detected frequently. The occurrence of persistent infection with oncogenic HPV type associates with the development of cervical cancer. However, the part of HPV infections consist of the multiple viral types. The influence of HPV co-infections on the risk of the development of cervical cancer is not clear, but the occurrence of more than one viral type seems to increase the risk of advanced lesions of the uterine cervix. Additionally, possible interactions (both positive and negative) between particular HPV types in co-infections may influence the risk of the lesions progression. Although, the issue of HPV co-infections is not well known, the previous scientific reports make that the introduction of the molecular methods, allowing the identification not only single but also multiple HPV infections, in routine diagnostics seem to be important. Here, we present the PCR-RFLP and Real-Time PCR methods as the useful tools in the diagnostics of HPV infections.

Keywords: human papillomavirus, HPV co-infection, cervical cancer, PCR-RFLP, Real-Time PCR

1. INTRODUCTION

Persistent infection with human papillomavirus (HPV), the most common sexually transmitted virus, is a necessary factor that contributes to the development of the cervical cancer (CC) [51] – the third most common cancer among women worldwide. Every year, more than 550 000 new cases are diagnosed (~569 847 in 2018) and about 300 000 women die (~311 365 in 2018) because of the cancer of the uterine cervix [8]. In Poland, the cervical cancer is the seventh most common cancer among women. About 3000 new cases (~3220 in 2018) and about 1500 deaths are recorded annually (~1947 in 2018). Among the Polish women between 15 and 44 years the cervical cancer ranks the fourth place [9].

Majority of HPV infections are spontaneously cleared within two years. About 10% of HPV infections become the persistent infections that can contribute to the development of the cervical cancer [16]. Carcinogenesis within the uterine cervix has been developing for many years and several stages of pre-malignant lesions can be distinguished. The cytology results differentiate the cervical lesions on: ASC (Atypical Squamous Cells) divided into ASC-US (Atypical Squamous Cells of Undetermined Significance) and ASC-H (Atypical Squamous Cells cannot exclude HSIL), LSIL (Low - grade Squamous Intraepithelial Lesions) and HSIL (High – grade Squamous Intraepithelial Lesions). In turn, the histology classification includes: CIN1 (Cervical Intraepithelial Neoplasia grade 1), CIN2 (Cervical Intraepithelial Neoplasia grade 2) and CIN3 (Cervical Intraepithelial Neoplasia grade 3) [27]. Although, the cytology results are not equivalents of the histology terms, LSIL are usually considered to be referred to CIN1 and HSIL as it corresponds to CIN2/CIN3 histology.

The transient infections with HPV virus are very common. The meta-analysis indicates that the global HPV burden among women with normal cytology is about 11% [10]. In turn, in the case of the cervical cancer almost all specimens contain HPV DNA [51]. Although, infections with single HPV type are the most frequently identified [53], the development of the molecular methods that are used for identification and genotyping of HPV infections cause, that co-infections with multiple HPV types are more and more detected.

2. AIM OF THE STUDY

Although the impact of the HPV co-infections on the development of the carcinogenesis process remains unclear, some studies suggest, that the occurrence of the multiple high-risk HPV genotypes [4, 14, 45] and infections with the combined high- and low-risk types contribute to the increased risk of the high-grade intraepithelial lesions/cervical cancer. Additionally, it seems, that the risk of the development of the high-grade lesions increases with the increasing number of HPV types, regardless of the oncogenicity of HPV genotypes [4].

The issue of the meaning of the HPV co-infection is still an open field of research. Determining specific and repeatable methods of the HPV co-infection diagnostics can contribute to the identification of patients with potentially increased risk of the cervical cancer and will provide an opportunity to better control of the progression of the cervical lesions. The paper discusses the current state of knowledge on the occurrence of the HPV co-infections and discusses the molecular methods: PCR-RFLP and Real-Time PCR methods as useful tools in the diagnostics of the HPV infections.

3. BIOLOGICAL CHARACTERISTIC OF HUMAN PAPILOMAVIRUS

3. 1. Genetic diversity of human papillomavirus

Human papillomavirus belongs to *Papillomaviridae* family [6, 12] that contains a very diverse group of viruses that infect various mammals [6]. The actual phylogenetic classification of HPV viruses is based on the analysis of the sequenced genomes for the occurrence of genetic diversity of the most conserved L1 region. L1 encodes one of the proteins that are part of the virus capsid. The similarity between L1 sequences of two viruses lower than 60% lets to assign them to various genera, that are marked with letters of the Greek alphabet (α , β , γ , μ and ν). Various species, that are marked with numbers (e.g. $\alpha 9$), are identified when similarity between L1 sequences is between 60 - 70%. The different types can be distinguished within the species, also marked with numbers (e.g. HPV-16). Types are characterized with the occurrence of less than 90% of similarity between L1 sequences [12]. Currently, more than 220 various HPV types were identified [International Human Papillomavirus (HPV) Reference Centre; http://www.nordicehealth.se/hpvcenter/reference_clones/]. The most attention is paid to viruses from α genus, because of their association with the cervical cancer [24]. 13 species and over 60 types within α genera are distinguished [12, 24] [International Human Papillomavirus (HPV) Reference Centre; http://www.nordicehealth.se/hpvcenter/reference_clones/]. About 50 types of *α -papillomaviruses* infect the genital tract [13].

3. 2. Tissue tropism and oncogenic potential

Human papillomavirus infects the cells of the multi-layered squamous epithelium of skin and mucosa. Each HPV type prefers to infect one kind of tissue, so they are divided into the cutaneous and mucosal types. In the case of the cervical epithelium, the virus usually attacks cells, that are located within the transformation zone – the area where the junction between ectocervix (containing squamous epithelial cells) and endocervix (that contains columnar epithelial cells) occur [24]. HPV viruses differ in their oncogenic potential. The International Agency for Research on Cancer (IARC) divided the HPV types from α genus (for which there were enough epidemiological data) into three groups, based on the oncogenic potential: carcinogenic (1), possibly (2A) / probably (2B) carcinogenic and non-carcinogenic (3) [24].

The HPV types that were assigned to particular groups are listed in Table 1.

Table 1. Division of HPV types belonging to α genus based on the oncogenic potential (based on [24]).

Group 1	Group 2		Group 3
Carcinogenic High risk (HR)	2A Possibly carcinogenic	2B Probably carcinogenic	Non-carcinogenic Low risk (LR)
	Probably high risk (pHR)		
16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59	68	26, 30, 53, 66, 67, 69, 70, 82, 85	2, 6, 7, 11, 13, 27, 28, 29, 32, 40, 44, 57, 61, 62, 72, 74, 77, 81, 83, 84, 86, 87, 89, 90, 91, 106

The most frequently detected HPV types are HPV-16 and HPV-18 that are responsible for about 70% of cervical cancer cases [8, 13, 16].

Although the cervical cancer is the most frequently occurring disease associated with human papillomavirus infection, persistent infections with the high-risk HPV types can contribute to the development of cancers in another anatomical sites of the human body e. g.: vagina, vulva, anus, penis or head and neck [8, 16, 24]. In turn, infections with the low-risk HPV types lead to the development of the benign lesions like genital warts. Only in small percentage of cases, especially in patients that are not immunocompetent, the infections with LR HPV types can contribute to the development of cancers [24].

3. 3. Organization of HPV genome

The high-risk virus type 16 (HPV-16) is connected with most of the cervical cancer cases as well as anogenital and oropharyngeal cancers, causing significant morbidity and mortality in the humans [8]. Because of this, HPV-16 is the best-studied HPV type and the data on the genome organization and virus life cycle presented in this paper are mainly based on type 16.

Human papillomavirus consists of icosahedral, non-enveloped capsid containing the circular dsDNA. HPV genetic material includes between 7 and 8 kbp depending on the type and variant of the virus. The HPV genome contains nine known open reading frames (ORFs), that in several cases overlap (Fig. 1). HPV ORFs can be divided into two categories:

- early (E), which encodes proteins that are expressed at the beginning and in the middle of the productive virus life cycle (in the lower and middle layers of stratified epithelium),
- late (L), which encodes proteins that are expressed at the final stage of the productive life cycle of the virus (in the upper epithelial layers).

The functions of the early and late open reading frames products are shown in Table 2.

Apart from the above mentioned two regions, the so-called long control region (LCR, named also upstream regulatory region, URR) occurs in the HPV genome. LCR is a non-coding region that contains the origin of the replication (which consists of E1-binding site), four known E2-binding sites and multiple sites that are targets for the cellular transcription factors (Fig. 1) [24].

Table 2. Main functions of proteins that are expressed from early and late ORFs.

Open Reading Frame (ORF)	Main functions of expressed protein
E1	E1 protein is a helicase that binds to the viral origin at the long control region. E1 takes part in the replication, unwinds HPV DNA and interacts with several cellular replication factors [5].

E2	E2 protein binds to several E2-binding sites at the long control region. It takes part in the transcription activation and repression (mainly in E8 ^{E2} form) by interacting with cellular transcription factors. E2 also takes part in the initiation of the replication by enhancing the function of E1 protein. During mitosis, E2 protein takes part in the division of HPV DNA to the daughter's cells by tethering the viral genome to host chromosomes [29].
E4	The main form of E4 protein is E1 ^{E4} that is formed as a result of splicing of the HPV transcripts. E4 takes part in the assembly and releasing of new virions and in the replication of the virus genome [23].
E5	E5 protein is considered to be the third HPV oncogene with a weaker oncogenic capacity than E6 and E7 proteins. E5 is a transmembrane protein that probably leads to the alterations in the cell membrane and influences the activity of several cellular proteins like EGFR, which is associated with the cell proliferation. It seems that E5 protein also takes part in the productive life cycle [22].
E6	E6 protein occurs in various splicing forms and fulfills various functions in the virus life cycle. The most important is influence on immortalization of the cell. E6 is present in the cell mainly as a complex with E6AP protein (E6 associated protein). E6-E6AP complex takes part in the inhibition of the apoptosis by degrading p53 in a way of ubiquitination (in the case of HR HPV types, LR HPV types only inactivates p53). Additionally, the blockade of apoptosis, in both HR and LR HPV types, is reached by degradation of another protein – Bak. E6 also contributes to the immortalization of the cell by the activation of the telomerase. The activation of the telomerase occurs only in the case of HR HPV types [50].
E7	E7 protein mainly takes part in transforming and immortalization of the cell. One of the best studied targets of HR HPV viruses E7 protein are proteins from the Retinoblastoma family. E7 associates with pRb (p105), p107 and p130 and, in the case of pRB, leads to its degradation. The consequence of the degradation/inactivation of the Retinoblastoma protein family is dysregulation of the cell cycle. Additionally, E7 protein promotes chromosomal instability, which is the result of mitotic abnormalities (e. g. anaphase bridges, disturbances in centrosome duplication). E7 is also involved in the cytokine modulation that promotes immortalization and induces the double strand breaks in DNA [42].
E8	E8 ORF is very short (~ 40 nt). The functional product of E8 ORF is a fusion of E8 and a part of E2 ORF and is called E8 ^{E2} . The function of E8 ^{E2} protein is the repression of the replication [25].
L1	L1 protein is a major part of the virus capsid. During infection, L1 protein interacts with the cellular membrane protein, leading to the virus entry into the cell [11].
L2	L2 protein, together with L1 protein, forms the virus capsid. Apart from that, the conformational changes of L2 protein are necessary to the successful establishing of the infection [52].

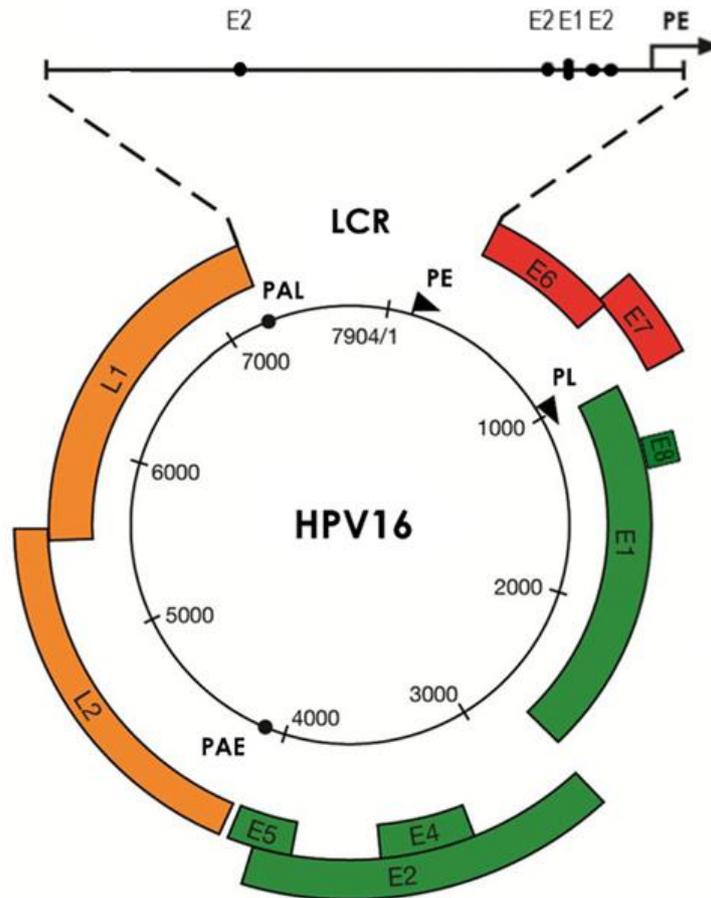


Figure 1. HPV-16 genome with overlapping ORFs is shown. Dots in the LCR region represent E2-binding sites and the oval represents E1-binding site being simultaneously origin of replication. PE – promoter of early ORFs, PL – promoter of late ORFs, PAE – polyadenylation site of early ORFs, PAL – polyadenylation site of late ORFs ([24] with changes).

4. LIFE CYCLE OF HUMAN PAPILLOMAVIRUS TYPE 16

The life cycle of HPV is closely related to the epithelial differentiation. In each layer of the epithelium, different processes of the virus life cycle occur [24]. Human papillomaviruses infect the keratinocytes in the basal layer of epithelium, after the exposure to it as a result of micro-wounds [24]. Basal cells, as the only one within the multi-layered squamous epithelium, undergo a full cell cycle from the G1 to M phase, which is crucial to the establishment of the infection [41].

In the early stage of the cell infection with HPV-16 virus, the virion attaches to the heparan sulfate proteoglycans (HSPGs) located on the extracellular matrix and/or the cell surface by the capsid protein L1. Binding to the receptors on the cell surface triggers the changes in the conformation of both capsid proteins L1 and L2. The cellular enzymes: cyclophilin B (CyPB) facilitates the exposure of the amino terminus L2 protein and the furin convertase cleaves 12 amino acids from the N-terminus of this protein [20].

The virus enters the cell via the pathway of endocytosis. The low pH inside the endosome vesicle and the presence of cyclophilins facilitate disassembly of the capsid, including the release of the vast majority of the L1 protein from the L2 protein, which remains in the complex with the viral genomic DNA. Most of L1 proteins are directed to lysosomes where they are digested. However, a small amount of L1 molecules remain bound to the L2/viral genome complex. A significant portion of the L2 protein penetrates the intracellular membranes and interacts with cytoplasmic factors that facilitate the transport of the L2/DNA complex to the trans-Golgi network (TGN). Inside the cell, the viral genomes utilize its structural reorganization during mitosis to enter the nucleus and the disintegration of the nuclear membrane is a key step to establish an infection [20].

When the cell enters the process of mitosis (prophase - pro-metaphase), transport vesicles containing HPV16 detach themselves from the trans-Golgi network (TGN), move along the microtubules (MTs) and then accumulate in the vicinity/proximity of the centrosome [20, 21].

In the later stages of mitosis (metaphase - anaphase), HPV16 vesicles move along the spindle of the microtubule and remain adjacent to the condensed chromosomes to the end of the completion of the cell division [20, 21]. At this time, both capsid proteins (L1 and L2) remain in the complex with viral DNA [20].

After the completion of mitosis, when the nuclear envelope is rebuilt (early - late interphase), the capsid proteins separate from each other and the viral DNA is released from the transport vesicles [20, 21]. Then, the released viral genome binds to the promyelocytic leukemia (PML) nuclear bodies (also called ND10 bodies), in the form of DNA - L2 protein complex, to establish the infection [18]. PML nuclear bodies are the groups of proteins that take part in various cellular processes like: transcription, protein degradation, tumor suppression, apoptosis or DNA repair.

When the cell enters the S phase, HPV replicates together with the host genetic material, using the cellular replication factors and their own proteins: E1 and E2. In the basal cells, the episomal viral copy number is maintained at low level [5, 24, 29].

During each subsequent mitosis the HPV episomes are tethered to condensed chromosomes via protein - protein interactions, in which E2 HPV protein takes part, to ensure effective partition of the virus DNA to the daughter cells [29]. The daughter cell, that is formed as a result of division of basal cell, starts to undergo the differentiation process and migrates within the epithelial layers [24].

The biosynthesis of E1 and E2 proteins increase in the cell that are differentiating, facilitating the intensified viral amplification. Accelerated viral amplification aimed at the maintenance of the genome at the beginning and then multiplication of the virus DNA copy numbers [24]. The mode of the replication in the subsequent layers of the epithelium is different from that occurring in the basal layer. It is based on inducing the DNA damage response (DDR) and using the replication factors transcribed in order to repair DNA.

The replication based on DDR makes the HPV replication independent of the S-phase of the cell cycle, allowing the formation of a lot of viral copies in a short period of time [30].

As the viral copy number increases, the amount of E6 and E7 proteins, which are associated with cell proliferation, also increases [24]. As a result of binding and degrading/inactivating of the suppressors of the neoplastic transformation (p53, Retinoblastoma family proteins) (see in Tab. 2), E6 and E7 lead to the deregulations in the cell cycle and, subsequently, to the reactivations of the cell cycle progression in the epithelial layers occurring above the basal layer [24, 42, 50].

When the cell approaches near the epithelial surface, it exits the cell cycle and expression of the late ORFs (L1 and L2) starts. The new virions are assembled and released along with the dead, exfoliating cells, infecting another basal cells [24].

During the HPV infection, the integration of HPV DNA to the host genome may occur. The integration event is frequent and can take place at the early stage of infection as is detected in CINI lesions [39]. Additionally, the percentage of the integration increases with the increasing grade of lesions, regarding to the most prevalence HPV-16 and HPV-18 [26].

The integration is not a part of the HPV life cycle - it ends the possibility to produce new progeny virions, but is a key event in carcinogenesis. The disruption or deletion of the viral E2 ORF, usually occurring during the integration, leads to the abolition of E2-mediated repression of transcription and, in consequence, to the enhanced/deregulated expression of E6 and E7 oncoproteins. In turn, the enhanced activity of E6 and E7 proteins leads to genomic instability, that contributes to the progressing oncogenesis [40].

The viral genome is the most often integrated near or into the common fragile sites – sites that are susceptible on the DNA breaks. Additionally, quite a few studies reported the occurrence of the integration events near or into human oncogenes, enhancing the expression of these genes, and therefore contributing to carcinogenesis [40].

5. HPV CO-INFECTIONS

5. 1. Prevalence of HPV multiple infections

Nowadays, quite a few mentions about the occurrence of HPV co-infections can be found in the literature, but one of the first cases were detected in the epidemiological studies conducted in 1980s and 1990s. The incidence of the HPV multiple infections, from that period of time, in CC cases was about 8% of HPV positive samples [34].

More recent studies show that multiple HPV infections are detected in about 20% to above 50% of HPV - positive samples [14, 17, 19, 43, 45, 49, 53]. The frequency of the HPV co-infections detection vary depending on the number of the analyzed samples, method used, degree of advancement of the cervical pre-malignant lesions. Additionally, the influence on the percentage of the observed multiple HPV infections has the age of women and the occurrence of additive diseases/infections especially those which impair the immune system (e.g. infections with HIV). The HPV co-infections are the most common identified in young women [17, 43], but some studies recorded also the frequent occurrence in women above 50 years old [53].

In the literature, a few reports, about the occurrence of the multiple HPV infections in Poland, are also available. The HPV co-infections were detected in 39% [48] and above 50% of the HPV-positive samples [38] among women with abnormal cytology result. In turn, the multiple HPV infections constitute about 30% of the HPV-positive cases in a pooled group, consisting of women with and without cytological abnormalities [3].

Considering the issue of the HPV co-infections, the prevalence, in each grade of the cervical lesions, should be discussed. The most reliable data can be obtained from meta-analyzes. Bzhalava et al. analyzed 423 studies and showed that, among the cytology results, multiple HPV infections are the most common in LSIL and HSIL (28% of the HPV-positive cases) and, in the case of ASC-US, the percentage is a little bit smaller (21% of the HPV-positive cases). In turn, overall presence of the HPV co-infections, among the

histology diagnosis, is higher than among the cytology results. In CIN1, the prevalence of the multiple HPV infections is equal to 32% of the HPV-positive cases, than rises, in CIN2 diagnosis, to 39% and, subsequently, decreases to 27% in CIN3. In the CC cases, the multiple HPV infections occur in 12% of the HPV-positive cases and, for comparison, the co-infections are detected in about 4% of women with the lack of cervical abnormalities [13]. These data confirm that infections of the multiple HPV types are common among all cervical lesions.

The number of HPV types detected in multiple infections usually vary from two to four [14, 45, 53], but the most frequent HPV co-infections consist of two various genotypes [4, 14, 19, 53]. Considering oncogenic potential, the most common co-infections consist of only the high-risk HPV types [17] and the most frequent identified is HPV-16 [4,53], probably due to the highest prevalence in the world [13].

An important issue, in reference to the HPV co-infections, is the response to the used treatment, in the case of the cervical cancer cases, and influence on the survival, compared to the infections with single HPV type. Available studies indicate that the occurrence of the HPV co-infections is associated with the reduced or even lack of response on radiotherapy [2, 33]. The occurrence of the multiple HPV genotypes is shown to be also associated with the decreased survivability [2, 37], although in the cited study of Nogueira Dias Genta et. al. the percentage of the co-infections was low. However, in the lights of the present reports, the detection of the multiple HPV infections at the low grade of the cervical neoplasia may be useful to apply the treatment earlier and perhaps extends the life expectancy.

5. 2. Potential interactions between HPV types occurring in co-infections

The studies on cell lines provide evidences that the multiple HPV types can co-exist in one cell [7, 31], what raises the question about the interaction between particular types of the virus. Determining, whether between the various HPV types some interactions occur, both positive and negative, is very important from clinical point of view. Although, there is not unequivocal data about interactions between various genotypes, the reports suggest that both antagonistic (competitive) and non-antagonistic (synergistic) interference can be presented [14, 19, 28, 45-47]. Potentially interactions between two HPV types may occur on various stages of infection. Firstly, on the stage of viral entry, when the competition in binding to the cell surface can occur. Secondly, the viral competition, about the cellular factors necessary to the viral life cycle on the levels of replication and transcription, may be observed. Additionally, in the case, when one HPV type is present in the episomal form and the second is integrated, E2 protein transcribed from the circularized genome may repress the transcription of E6 and E7 protein from the internalized genome, leading to the decrease of the oncogenicity of the integrated HPV type.

The kind of interactions seems to be related to genetic similarity/diversity of two HPV types. The recent study provides evidence that in the case of co-infection with HPV types belonging to various species, the risk of the development of the cervical cancer is decreased. In turn, the multiple infections with HPV types from the same species may contribute to the higher risk of the development of the cervical cancer [45]. However, another report presents the opposite results, showing that the co-infections with HPV types from the same species protect from the development of the high-grade lesions, even if the analyzed HPV types are the high-risk ones [47].

The best studied pattern of the co-infections is HPV-16 ($\alpha 9$ species) and HPV-18 ($\alpha 7$ species). Several studies evaluated the tendency for the interactions between HPV-16 and HPV-18 genotypes and the results suggest the occurrence of antagonistic effect [7, 28, 32, 45, 46]. The recent study on HaCaT cell line shows, that the competition between HPV-16 and HPV-18 occurs during the entry of virus to the cell during the concurrent infection. HPV-16 reduces the binding of HPV-18 particles to the cell through L2 protein, but the exact mechanism is not yet known [7]. Subsequently, if both viral types successfully enter the cell and then the nucleus, the interaction on the level of replication can occur. There are evidences, that E1 proteins of HPV-16 and HPV-18 bind to each other and these interaction inhibits the replication of both viral types [32]. The next possible stage of viral life cycle, on which interactions can be present, is transcription. The occurrence of the competition on the level of transcription between HPV-16 and HPV-18 confirms the decrease of the amount of E1^{E4} transcripts from HPV-18 [7].

Although, the molecular mechanisms of the interactions between the most prevalent and oncogenic HPV-16 and HPV-18 types is, by now, the most widely described, several studies analyzed the occurrence of the interactions between other HPV types. The possible interactions between HPV-16 ($\alpha 9$ species) and HPV-6/-11 ($\alpha 10$ species) were analyzed in serological studies, which indicate the occurrence of antagonistic effect [28, 46]. In turn, synergistic interaction is suggested between HPV-16 ($\alpha 9$ species) and HPV-68 ($\alpha 7$ species) because of the observed significantly increased risk of the cervical cancer/high-grade lesion in the case of the occurrence of such a pattern of co-infection [14]. In the literature, the data about multiple infections with HPV-16 and HPV-33 (both types from $\alpha 9$ species) are also available, but the results are varied. Luostarinen et. al., suggest the tendency to antagonistic effect [46], in contrast to the results obtained by Silins et. al., which imply the occurrence of synergistic interactions between HPV-16 and HPV-33 [28].

Despite the occurrence of the discrepancies in the issue of interaction between some HPV types, the detection and genotyping of the viral types in multiple infections seem to be an important course in prevention of the development of the high-grade lesions of uterine cervix. However, further studies are needed to the unambiguous designation, which HPV types occurring in the multiple infections contribute to the increased risk and which to the decreased risk of the development of the cervical cancer.

5. 3. Molecular methods in diagnostics of multiple HPV infections

Nowadays, the Pap smear is the main screening test determining the occurrence of the pathological lesions within the uterine cervix. The cytological evaluation is based on Bethesda system differentiating the lesions of the squamous epithelial cells (from ASC to HSIL) and glandular cells [27]. However, conventional cytology is not as effective tool in the prediction of the risk of the development of the high-grade lesions and cervical cancer as HPV tests. The HPV tests are considered to be the future of the screening methods [16] and rely on the molecular detection of the present HPV nucleic acids and genotyping [27].

There are a lot of methods, which let to identify infections with human papillomavirus [1]. However, due to the prevalence of the multiple HPV infections, not every method may be useful in the routine diagnostics. The perfect molecular method for detecting and genotyping of HPV infection should allow the identification of as large number of HPV types as possible and be very sensitive and specific. Moreover, the reagents used should be inexpensive,

the time waiting for the results – relatively short and, most importantly, such a method should allow the effective detection and genotyping of the HPV co-infections.

The HPV tests can be based on hybridization or amplification of the nucleic acids [1]. One of the most useful methods for the detection and identification of HPV for the diagnostics purposes seems to be the polymerase chain reaction (PCR) connected with the restriction fragment length polymorphism (RFLP). PCR-RFLP method is sensitive, specific, simple, relatively cheap and fast and allows the detection of both single and multiple infections [1]. PCR-RFLP allows the distinction of the large number of HPV types by using the properly selected restriction enzymes. The PCR-RFLP methods based on various number of restriction enzymes have been described. One study presents the PCR-RFLP method based on the amplification of ~450 bp fragment of the most conserved L1 ORF and digestion of the PCR product with only one enzyme – *HpyCH4V*, allowing the identification of 39 different HPV types and 2 subtypes (according to current taxonomic affiliation). Although, this method seems to be suitable to the routine diagnostics, it possess some limitation - it cannot differentiate between several HPV types e.g. HPV-11 (LR type) and HPV-30 (pHR type) or HPV-18 (HR type) and HPV-68 (pHR type) [44]. To improve this method, the digestion with *NlaIII* can be performed in the case of undistinguishable HPV types. The RFLP method based on the digestion of both *HpyCH4V* and *NlaIII* is able to distinguish not only the single, but also multiple HPV infections [15].

However, another method also based on the amplification of ~450 bp fragment of the most conserved L1 ORF and, subsequently, the digestion of the PCR product with four enzymes: *PstI*, *HaeIII*, *DdeI* and *RsaI*, has been described. This method discriminates 49 mucosal HPV types and two subtypes, including all the high- and probably high-risk types (12 and 10 HPV types, respectively), 19 low-risk HPV types and 8 HPV types of the undetermined risk. This method enables genotyping of not only single, but also multiple infections [36] and, to our knowledge, allows the identification of the largest number of the mucosal HPV types, so far.

Apart from the detection and typing of HPV, the designation of the physical status (episomal/integrated) of virus in a tissue seems to be also important, because the integration of the HPV genome to the host genetic material is considered to be a key event in the carcinogenesis of the uterine cervix [40]. The Real-Time PCR method allows the detection of the episomal, integrated and mixed forms of particular HPV types by using the specific primers to E2 and E6 ORFs and the intercalating dye (SYBR Green) [35] or probes [39]. The integration of the HPV genome usually leads the disruption of the continuity of the E2 ORF sequence. So, in the case of the occurrence of only integrated form of the virus, the only product targeted within E6 ORF should occur. The starters for HPV-16 are the most widely used in the studies [35, 39] but the Real-Time PCR method with properly designed primers should also let discriminate the physical status of other HPV types. An interesting issue is the occurrence of the integration in the case of multiple HPV infections. The application of the Real-Time PCR, in such cases, should let the indication if some associations between the physical statuses of particular HPV types occur. Additionally, the statement, whether one of the HPV types or even both are present in the integrated form, could allow the selection of patients with the potentially increased risk of the development of the high-grade lesions and cervical cancer.

Among the current available methods for detecting, genotyping and designating the physical status of HPV, PCR-RFLP and Real-Time PCR seem to be the useful tools for the future introduction in the routine diagnostics.

6. CONCLUSIONS

The common prevalence of the multiple HPV infections and the potentially increased risk on the development of the cervical lesions in HPV co-infections cases, lead to a question, if current screening methods towards the differentiation of the cervical lesions are sufficient for the prevention of cervical cancer. In the light of the current available studies, the introduction of the sensitive and specific molecular methods detecting, genotyping and designating the physical status of HPV types occurring not only as a single, but also as multiple infections in the routine diagnostics, can contribute to the decrease of the number of the cases of the HPV-mediated high-grade lesions and cervical cancer. Additionally, it could contribute to the increased survivability, because of the selection of women infected with more than one HPV types and, maybe, apply the treatment earlier in the future. The PCR-RFLP and Real-Time PCR methods seem to be the useful tools in the future diagnostics because these methods allow the analysis of not only the single HPV types, but also HPV co-infections.

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