

ORIGINAL PAPER

Comparative detection of high-risk HPV (16, 18, 33) in cervical bioptic material of County Hospital of Tg. Mures

Z. PÁVAI¹⁾, T. FÜLE²⁾, E. HORVÁTH³⁾, M. MÁTHÉ²⁾,
ZS. PAP¹⁾, L. DENES¹⁾, J. JUNG³⁾

¹⁾Department of Anatomy,
University of Medicine and Pharmacy, Tg. Mureş

²⁾1st Institute of Pathology and Experimental
Cancer Research "Semmelweis", University of Budapest

³⁾Department of Pathology,
University of Medicine and Pharmacy, Tg. Mureş

Abstract

The purpose of this study was to collect data about the incidence of high-risk HPV (16, 18, 33) types in paraffin embedded cervical bioptic material, including LSIL, HSIL and cervical cancers using immunohistochemistry and nested PCR methods. In our study were included randomly selected 10 LSIL, 18 HSIL and 30 cervical cancer cases. We analyzed the expression of HPV in this specimens with immunohistochemistry used DAKO K1H8 antibody and CHEMICON Mab HPV 16, 16 antibody using LSAB method and Tiramín amplification method, and nested PCR for HPV 16, 18 and 33. In LSIL cases three, in HSIL cases eight and in carcinoma 20 cases were positive for HPV 16 or 18 for immunohistochemistry or PCR. Although this proportion is lower than those reported in the literature, our work signals the existence of the infection in our country and presents a relatively cheap diagnostic method.

Keywords: Human Papilloma Virus, cervix, cancer, immunohistochemistry, nested PCR.

Introduction

Human papillomaviruses (HPVs) are associated with various benign and malignant lesions including genital condyloma and anogenital cancer. Epidemiological data show that about 90% of all cervical cancer patients are HPV positive.

The purpose of this study was to collect data about the incidence of high-risk HPV (16, 18, 33) types in the population of examined women by our hospital and to compare the efficiency of our immunohistochemistry and molecular biology methods.

Material and methods

58 randomly selected cases were examined retrospectively for the presence of HPV infection by the PCR and immunohistochemistry.

In our study was included: 10 low-grade squamous intraepithelial lesions (LSIL – CIN I, cervical intraepithelial neoplasia), 18 high-grade squamous intraepithelial lesions (HSIL – CIN II and III), and 30 cervical cancer cases (*in situ* carcinoma, squamous invasive carcinoma, small-cell carcinoma).

We analyzed the expression of HPV in these specimens with immunohistochemistry used DAKO Monoclonal Mouse Anti-Human Papillomavirus (HPV) antibody (Clone K1H8) and CHEMICON International Mouse Anti-Human Papilloma Virus Type 16 and 18 Early E6 Protein Monoclonal Antibody (Clone C1P5)

using heat induced epitope retrieval method with LSAB and Tiramín amplification method.

Human papillomavirus typing was done by polymerase chain reaction amplification using primers from the E6 gene DNA sequences of HPVs 16, 18 and 33. The nested PCR was made using the method of Nawa A *et al.* [1].

The 5'–3' sequence of primers for E6 gene was: outer F ACCGA AAACG GTTGA ACCGA AAACG GT, outer R AATAA TGCT ATATT CACTA ATT, inner 16F ATGTT TCAGG ACCCA CAGGA, inner 16R CCTCA CGTCG CAGTA ACTGT, Inner 18 F ATGGC GCGCT TTGAG GATCC, inner 18R GCATG CGGTA TACTG TCTCT, bp, inner 33F GCAGT AAGGT ACTGC ACCAC, inner 33R CCTCA GATCG TTGCA AAGGT. These primers yielded 307, 123, 177 and 146 -bp fragments, respectively.

We used 30 micron sections from the paraffin embedded material. Samples were deparafinated and digested overnight with proteinase K (final concentration 400 µg/ml).

The second day the samples was centrifuged at 1300 g, the supernatant transposed in 0.5 ml Eppendorf tube, the enzyme inactivated at 95°C, 5 minutes, and cooled at ice, than stored at -20°C.

The first amplification was carried out in 25 µl of reaction mixture (2 × REDTaq™ ReadyMix™ PCR Reaction Mix (Sigma), 2 pmol of each primer, 7.5 µl H₂O, 1 µl of sample with 40 cycles of amplification.

The second amplification has been made also in 25 µl reaction mixture (2 × REDTaq Ready Mix, inner primer F 10 pmol/µl and R 10 pmol/µl, 5 µl H₂O, 1 µl of product with first amplification, using 40 cycles.

The first PCR cycle included a denaturation step at 94°C for 1.5 minutes, an annealing step at 42°C for 1.5 minutes, and a chain elongation step at 60°C for 2 minutes using DNA Thermal Cycler (Hybaid).

The second PCR included steps at 94°C for 30 seconds, 54°C for 30 seconds, and 70°C for 2 minutes. The polymerase chain reaction run was completed by extension for 10 minutes at 70°C.

Negative controls for background contamination did not add to the DNA template. The positive control was used for HPV 16, 18 and 33 with proved positive cases.

Polymerase chain reaction products were analyzed

on 2% agarose gel with ethidium bromide staining and their molecular weight was determined by comparison with a 100-bp DNA ladder.

Results

Of 120 consecutive cases of gynecologic pathology from the material of the Pathology Laboratory, between 2001–2002 (18 LSIL, 36 HSIL, and 66 CC) we were able to include 70 for immunohistochemical and PCR processing, because the rest of the paraffin blocks was inaccessible because of a flood affecting the storage area (Table 1).

We excluded 12 cases, because after isolation we could not obtain interpretable genetic material, beta-globin control being negative (we used the GH20/PCO4 of 267 bp, beta globin sequence).

Table 1 – Age distribution of cases according diagnosis and HPV positivity

| Age [years] | <29 | | 30–39 | | 40–49 | | 50–59 | | 60–69 | | >70 | | Total | | Min. | Max. | Mean | | | | |
|--------------|-----|---|-------|----|-------|----|-------|---|-------|---|-----|---|-------|----|------|------|------|----|------|------|------|
| | T | + | T | + | T | + | T | + | T | + | T | + | T | + | | | | | | | |
| LSIL | | | 4 | 1 | 5 | 2 | 1 | | | | | | | 10 | 3 | 30 | 31 | 50 | 49 | 40.2 | 40.0 |
| HSIL | 2 | 1 | 7 | 5 | 5 | 1 | 4 | 1 | | | | | | 18 | 8 | 21 | 21 | 58 | 50 | 39.3 | 37.7 |
| CC | | | 4 | 4 | 12 | 8 | 7 | 4 | 3 | 2 | 4 | 2 | 30 | 20 | 31 | 31 | 82 | 73 | 52.1 | 50.0 | |
| Total | 2 | 1 | 15 | 10 | 22 | 13 | 12 | 5 | 3 | 2 | 4 | 2 | 58 | 31 | | | | | | | |

Of the 58 processed cases, 31 presented HPV, 16 or 18 positivity showing the following distribution. Of the 10 LSIL cases, three had positivity. Of the 18 HSIL cases, eight were positive, while of the 30 carcinomas 20 cases were positive (Table 2).

Table 2 – Type of tumors and HPV positivity in tumors

| | The type of tumor | HPV + HPV - Total | |
|------------|--|-------------------|-------|
| | | HPV + | HPV - |
| Large cell | <i>squamous non-keratinizing cell cc</i> | 9 | 5 |
| | <i>squamous keratinizing cell cc</i> | 10 | 5* |
| Small cell | | 1 | 1 |
| | Total | 20 | 10 |

1* was well differentiated one

The age range of the patients was between 21 and 82 years, and the HPV positive cases ranged from 21 to 73 years. The mean age of the LSIL and HSIL groups is almost identical (40.2 and 39.3 years with standard deviation 7.87 and 9.38; standard error 2.48 and 2.21), and concurs with the mean age of the corresponding HPV positive groups (40 and 37.3 years).

The mean age of patients with carcinoma is considerably higher (52.1 years with standard deviation 13.23 and standard error 2.41), and the age of the HPV positive cases also does not deviate much (50 years). The data corresponds approximately to the descriptions from the literature.

We processed 38 biopsies, eight conisation pieces, and 12 total hysterectomies. The positive cases ratio was the following LSIL-HSIL-Cc: biopsies 8/2–10/2–20/14, conisations 1/1–5/5–2/1, and hysterectomies 1/0–3/1–8/4.

The ratio of patients regarding provenance (rural, small town, large towns) did not show significant differences. In most cases, the presumed clinical diagnosis and histopathologic diagnosis matched each other.

In two cases of hysterectomy for prolaps, the diagnosis was CIN III-HSIL, one being HPV+, and a case labeled hyperkeratotic papilloma turned out to be epidermoid carcinoma. Of the 30 carcinoma cases, nine were large cell keratinizing carcinomas, 10 large cell non-keratinizing carcinomas and a small cell carcinoma. The distribution of the cases corresponds to literature data. Using immunohistochemistry with K1H8 antibody we obtained positivity in the HSIL and the LSIL cases (Figure 1).

Two of the LSIL cases were positive for HPV 16 and one for HPV 18. By immunohistochemistry, all three were positive for both anti capsid antibody and anti E6 antibody. In HSIL cases, the anti capsid antibody was detected in only three cases, while in carcinomas it presented no positivity. This corresponds to the fact, that the antibody is manufactured against the capsid protein and during tumor genesis, practically only the E6–E7 integrates into the genome. In the tumor cases, positive immunohistochemical reaction using the antibody against E6–E7 has been obtained only following tiramin amplification (Figures 2 and 3).

When using tiramin amplification a strong background appears, care which makes difficult the interpretation of results. In many cases we noticed cytoplasmic immuno-labeling, which under ordinary conditions would not have been considered positive, but in the same cases we obtained PCR positivity.

In five cases we obtained unspecific PCR lines for HPV 16, 18 or 33, in the same time being negative for immunohistochemistry as well. In these cases we performed a control with general primers for HPV MY11/MY15, and GP5+/GP6+, but because of the lower sensibility of these, we could not obtain interpretable results.

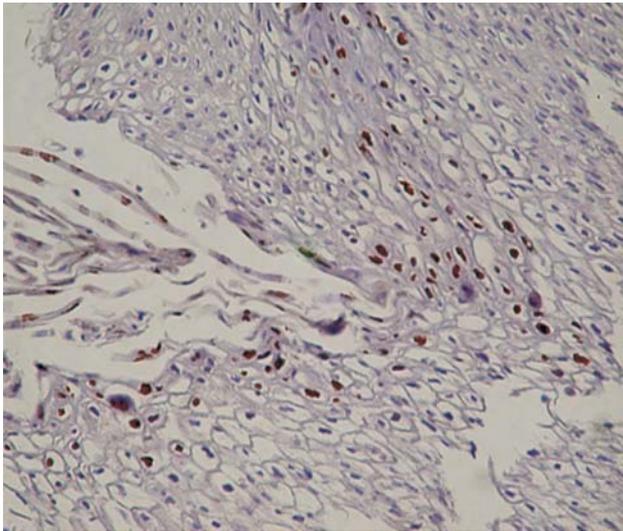


Figure 1 – 21 years, condyloma with CIN II, HSIL (LSAB method, PCR HPV18+, ob. ×40)

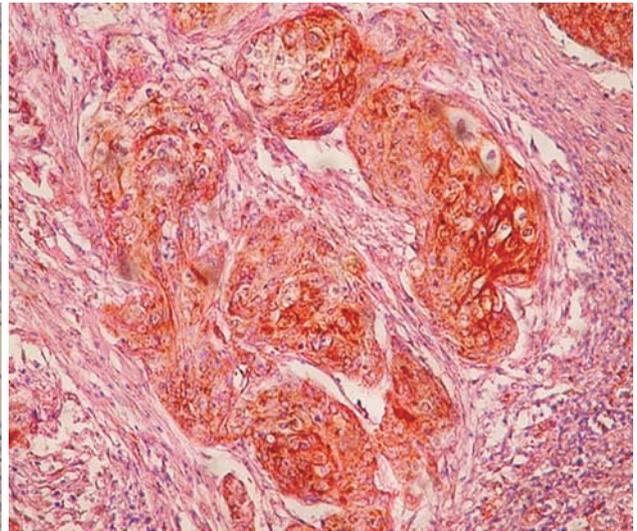


Figure 2 – 42 years infiltrating keratinizing cell cc, strong background (tiramine method, PCR+, ob. ×20)

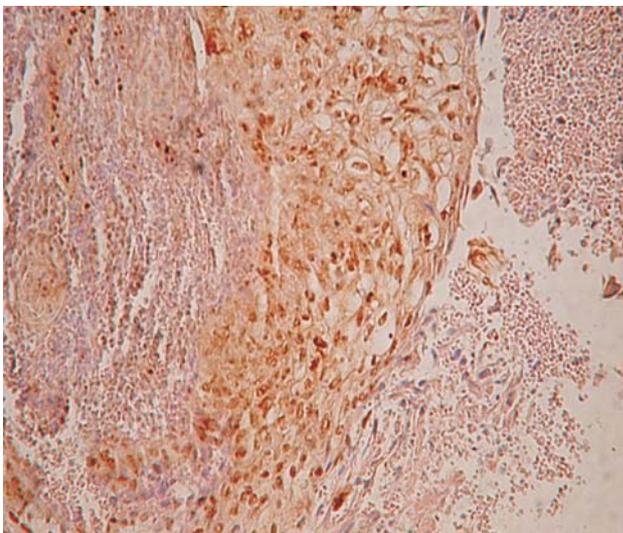


Figure 3 – 54 years non-keratinizing cell cc HPV+ immunoreactivity, strong background (tiramine method, ob. ×20)

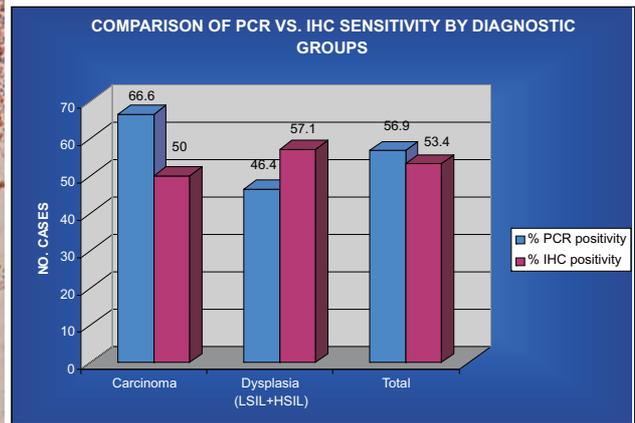


Figure 4 – Comparison of PCR versus immunohistochemistry sensitivity



Figure 5 – Photograph of an agarose gel electrophoresis. HPV16 positivity in cases 1, 6, 8, 9, 11, 15, 19, 20



Figure 6 – Photograph of an agarose gel electrophoresis. HPV18 positivity in case 45 of 68 year old women with infiltrating keratinizing epidermoid cc

As a consequence, these cases were labeled as negative. We cannot exclude the possibility of the presence of another HPV strain, which we cannot detect. There were no significant differences between the sensibility of our immunohistochemistry methods and nested PCR (Figure 4).

Although there were differences, these had no statistical significance ($p > 0.1$). The relatively low number of cases may possibly explain this.

☞ Discussions

Cervical HPV infection is extremely common among young sexually active women. Although cervical screening procedures have reduced the diseases associated with HPV infection, they are expensive and abnormal results cause significant emotional distress.

Therefore, prevention may be an effective strategy for reducing the economic, psychosocial, and disease burden of HPV infection. Multivalent vaccines are now in clinical development. HPV vaccines will be most effective when administered prior to initiation of sexual activity, and vaccination campaigns should aggressively target preadolescent and adolescent populations [2].

The peak prevalence is seen in women 15 to 25 years of age, which may be 25% to 40%, and then declines with increasing age. An increase in HPV DNA positivity in women older than 60 years of age has been identified in some populations [3].

When the virus is transmitted, it enters the epithelial cells via the basal layer and produces two general categories of epithelial change that are relevant to diagnostic classification. The first is viral cytopathic effect in the maturing, terminally differentiated cells, which includes koilocytotic atypia. It is important to note that this process occurs in terminally differentiating cells that are incapable of dividing.

The cellular alterations are mediated via HPV early (E6–E7) gene expression, leading to disruption of normal cell cycle processes and involving disruption of the functions of the p53, Rb, and other cell cycle genes [4].

High-risk HPVs, via the direct effects of viral oncogenes on the stem (immortal) or replicating cell population, initiate progressive alterations frequently accompanied by integration of the virus into the host genome and genomic amplifications at 3q [5].

Although viral DNA remains exclusively episomal in benign papillomas, integration of a partially deleted viral genome is present in most cervical cancers. The E6 and E7 viral oncogenes are preferentially retained and expressed in severe dysplasias as well as in frankly malignant lesions.

Three major categories of cervical squamous cell carcinoma exist, although admixtures and intermediated forms abound: large cell non-keratinizing, large cell keratinizing and small cell.

Other forms may be verrucous, spindle cell, basaloid (squamous cell), lymphoepithelioma like and transitional cell carcinoma.

The better differentiated forms of keratinizing squamous cell carcinoma differ from the others by their apparent lack of relationship with HPV or CIN, these rare tumors tend to be large and locally aggressive [6].

The tumor appears most often in the older age groups but also occurs, with increased relative frequency, in young white females [7].

Lack of detection of HPV in the tumor cells is a poor prognostic sign [8].

The mean age of our cases corresponded to literature data, and in the same time, we did not find any statistical difference between the keratinizing and non-keratinizing carcinomas.

The most common HPV types in invasive cervical cancer were, in order of decreasing prevalence are HPV16, 18, 45, 31, 33, 58, 52, 35, 59, 56, 6, 51, 68, 39, 82, 73, 66 and 70.

In squamous cell carcinoma, HPV16 was the predominant type (46–63%) followed by HPV18 (10–14%), 45 (2–8%), 31 (2–7%) and 33 (3–5%) in all regions except Asia, where HPV types 58 (6%) and 52 (4%) were more frequently identified. The overall detection of HPV DNA was similar in different regions (83–89%) [9].

The Hungarian epidemiological data bore closest resemblance to the European ones except some differences. The HPV18 is rather seldom encountered in this country, the occurrence of HPV58 is higher, the incidence of HPV59 is relatively high, HPV33 and HPV66 infections occur in a significantly higher number [10].

E6 open reading frame of HPV 16, 18 or 33 was detected by PCR in 56% of the histological sections of the 50 examined patients with *in situ* cancer. In 92% of the 28 HPV positive patients one HPV type was detected, while in one of the remaining two cases two HPV types (16/33), or all three types could be detected. The three main types of HPV (16, 18, and 33) are probably represented in lower percentages in CIN III in Hungary [11].

In the Polish study the presence of HPV DNA was detected in 53% of cervical cancer patients and in 2% of control group of healthy women. This study showed much lower incidence of HPV in Polish women with cervical cancer than among other populations as reported in world literature [12].

Approximately 90% of squamous precursor lesions that are produced by HPV type 16 exhibit the morphologic features of CIN II-III, and more than 50% of invasive squamous cell carcinomas contain HPV type 16. Nearly 100% of invasive cancers contain HPV [13].

HPV types 31, 33, and 35 may be associated with precursor lesions at the lower end of the neoplastic spectrum (CIN I) [14] and have been associated with “less aggressive” (relative to type 16) invasive carcinomas. HPV 18 has been implicated in “more aggressive” tumors, including small cell undifferentiated carcinomas and adenocarcinomas. In the vast majority of cases, we found only a HPV 16 infection (Figure 5).

We demonstrated HPV 18 infection in one HSIL and three carcinoma cases (Figure 6) and in one case the possibility of HPV 33 infection was raised.

Many different methods with different sensitivity and specificity have been proposed to detect the presence of high-risk human papillomavirus (HR HPV) in cervical samples.

The HC2 is one of the most widely used. Recently, a new standardized PCR-based method, the AMPLICOR HPV test, has been introduced. Both assays recognize the same 13 HR HPV genotypes.

The study amely compared 167 cases shows that the HC2 assay and the AMPLICOR HPV test give comparable results, with both being suitable for routine use [15].

The two methods mentioned above are very expensive to be used routinely in Romania. Immunohistochemistry is considerably lower-priced, and nested PCR is also cheaper.

The K1H8 antibody reacts with a non-conformational, internal, linear epitope of a major capsid protein of HPV [16], the CHEMICON antibody was specific for Virus Type 16 and 18 Early E6 Protein.

Using our LSAB method, we managed to detect the antibody against the capsid in relatively few cases. Tiramin amplification and the antibody against the E6 protein resulted in positive reaction in a much larger proportion, but in this case, the interpretation was difficult because of the background.

Using nested PCR we also did not achieve the proportions mentioned in the literature, but this may be explained by the fact that this is a retrospective study and the DNA was damaged during embedding. In other conditions, the method showed infection in 92% of the cases [17].

Similar conclusions are drawn from the follow-up evaluation of the cancers in the international study that were initially thought to be HPV-negative indicated either that they were false-negative results or that the DNA in the specimens was too degraded for the negative results to be deemed reliable [18].

Until the HPV vaccines become widespread [19], in order to efficiently treat a patient, there is a need for detecting the infection, using the existing funds and methods.

☐ Conclusions

In the indigenous population the HPV is also detectable and the distribution regarding mean age and spread of the infection is similar to Central European data.

Parallel to the severity of the diagnosis increases the infection rate.

In our results, the sensibility of the immunohistochemical reaction using antibodies against the capsid protein was far lower than the tiramin amplification against E6 protein or nested PCR. Although, between tiramin amplification and PCR there were differences, we could not demonstrate that these differences were significant.

Although we did not reach the values stated in the literature, this method may be suitable for a primary patient screening.

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Corresponding author

Zoltan Pávai, Associate Professor, MD, PhD, Department of Anatomy, University of Medicine and Pharmacy, 38 Gheorghe Marinescu Street, 540 000 Târgu Mureş, Romania; Phone +40265–215 551, Fax +40265–210 407, E-mail: zpavai@umftgm.ro

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