

# In Vivo Application Of 5-Aminolevulinic Acid In The Treatment Of Papillomavirus Infection In Women With Cervical Lesions After Detection And Genotyping Using PCR Technique

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## Citation

G Ekonjo, Y Saleh, J Kasiak, M Grybo?, E Teterycz, J Korzeniewski, M Siewi?ski, A D?browski, M S?onina. *In Vivo Application Of 5-Aminolevulinic Acid In The Treatment Of Papillomavirus Infection In Women With Cervical Lesions After Detection And Genotyping Using PCR Technique*. The Internet Journal of Gynecology and Obstetrics. 2005 Volume 6 Number 1.

## Abstract

**Objective:** The aim was to study the HPV viral genotyping in patients with abnormal PAP smears and the use of Photodynamic Therapy (PDT) for treatment of the HPV associated cervical lesions and for HPV eradication.

**Methods:** 478 samples were collected consecutively from patients with abnormal Pap smears who visited the Department of Obstetrics and Gynecology and HPV genotyping were performed. Oncogenic HPV types were correlated with ages of the patients. PDT was given to some of patients were analyzed with regards to the outcome.

**Results:** Out of those 478 patients, 227 (47 %) patients were HPV positive. In 123 patients (54%) out of the 227 women, oncogenic HPV type 31/33 was identified. A total of 77 patients (33%) disclosed HPV type 16/18, 27 patients (5%) disclosed HPV type 6/11. HPV 31/33 type incidence was significantly higher in age between 26-35 years. Out of the 227 patients HPV positive, 20 cases with high-risk cervical lesions HPV infection (16/18 genotype) were treated with PDT using 20% 5-ALA in five courses. 16 patients (80%) out of these 20 cases with cervical lesions associated with HPV infection were negative after treatment.

**Conclusion:** HPV 31/33 was the most common HPV type in patients with abnormal PAP smears. Most women with cervical lesions associated with HPV infection can be successfully treated by PDT.

## INTRODUCTION

HPV is ubiquitous worldwide and is markedly heterogeneous, such that more than 80 different genotypes have so far been identified by nucleotide sequence similarity [29]. Infection with certain oncogenic types of human papillomavirus (HPV) is considered a prerequisite for the development of the disease [23]. According to HPV PCR results, individuals with high-risk type-specific persistent infection during follow-up have a higher risk of persistent squamous intraepithelial lesion (SIL) than those with low-risk type-specific persistent infection or non-type-specific infection [12, 20, 23].

More than 80 known HPV types are specific for epithelial cells, including those of the skin, respiratory mucosa, and genital tract, and more than 35 distinctive types infect the genital epithelium. HPV types 16 and 18 are found most frequently in cervical carcinoma specimens and HPV types 31, 33, 35, 39, 45, 51, 52, 56, and 58 may also be associated with cervical cancer or premalignant lesions [4, 21, 24, 26]. Each of the carcinogenic genital HPV types presents a different risk to patients, with the greatest burden of risk attributed to types 16 and 18. In addition, these types are interesting, as there are possible differences in the clinical properties of cervical neoplasia according to HPV type.

DNA microchip systems composed of oligonucleotides [22] or robotically spotted DNAs [25] permit genome scale analysis of gene expression patterns and have been used recently in applications such as mutation detection [9,10] and genome mapping [28]. To date, more than 85 HPV types have been identified, of which at least 35 types have been found in female genital tract infections [7, 8, 32]. HPVs have been categorized into “high risk” (HPVs 16, 18, 45, and 56), “intermediate risk” (HPVs 31, 33, 35, 51, 52, and 58), and “low risk” (HPVs 6, 11, 42, 43, and 44) groups based on their relative risks for the occurrence of a high-grade cervical lesion and an invasive cancer [16].

Photodynamic therapy (PDT) is a novel treatment modality that produces local tissue necrosis with laser light after prior administration of a photosensitizing agent [11]. The optimal treatment of preinvasive cervical lesions is still not clear as all surgical techniques cause substantial cervical stroma destruction with the risk of a possible incompetent cervix. Photodynamic therapy can preserve fertility due to selective tissue destruction [3]. 5-aminolevulinic acid (5-ALA) is a precursor in synthesis of endogenous porphyrins used to sensitize tumor tissues in photodynamic therapy (PDT). It is administered topically into tumor which after the certain time, required for porphyrins to accumulate, is irradiated with visible light from the proper source at established wavelength [13, 35]. The study aimed to determine the incidence of human papillomavirus (HPV) DNA by in situ hybridization analysis in women with cervical dysplasia and other genital organs disorders such as condylomata accuminata of genital areas, LSIL and HSIL in cytology analysis, and suspected HPV infection as compared to HPV negative controls. We investigated photodynamic therapy with topical 5-aminolevulinic acid in eradicating cervical lesions associated HPV infection.

## **MATERIALS AND METHODS**

### **PATIENTS**

Between February 2003 and May 2005, 478 samples were collected consecutively from women who visited the Department of Obstetrics and Gynecology, Wrocław Medical University, Poland. Most of them had persistent LSIL and HSIL in their cytology results, some of the patients had koilocytes in their Pap smears reported by cytologists. Out of those 478 patients, 227 (47 %) patients were HPV DNA positive. The rest of the patients with abnormal Pap smear were HPV negative.

### **SPECIMEN COLLECTION FROM PATIENTS**

Scraping the cervical canal with a small cytobrush after Pap smears samples were collected, the brush was put into a 15-ml centrifuge tube containing phosphate-buffered saline. The specimens were collected as part of an informed consent protocol approved by the studies committee of the Wrocław University Hospital.

### **DNA EXTRACTION FROM CERVICAL SCRAPES**

The HPV DNA was isolated by using a standard procedure [17]. DNA was extracted by using a Wizard genomic DNA purification kit proteinase-K digestion (0.5mg/ml, Fluka, Germany), phenol-chloroform-alcohol izoamyl 25:24:1 single mixture extraction (Fluka, Germany) as well, as nucleic acid salts precipitation in glacial ethyl alcohol (Chempur, Piekary Śląskie). The obtained DNA was centrifuged at 12000 rpm (MPW-211, Poland), then dried under vacuum over penta oxide phosphate, dissolved in deionised water and finally the concentration was measured by using UV/VIS spectrometer (RNA/DNA-calculator Gene Quant, Pharmacia LKB, UK).

### **PCR AMPLIFICATION**

The DNA matrix underwent the next dilution to obtain a solution of 25 ng/ml. The amplification of the EI HPV genome region which is characteristic for types 6,11,16,18,31,33 was conducted by using thermocycler personal Mastercycler 5332 (Eppendorf, USA) according to Van den Brule procedure et al. [31] in 50 µl in the following conditions:- initial denaturation at 94°C for 5 min, and 40 cycles consists: denaturation at 94°C for 1 min., hybridization at 48°C for 1min. and elongation at 72° for 1 min., followed last extension at 72°C for 4 min. The reaction mixture consisted of 130-150ng of DNA matrix, 0.2 mM dNTPmix (Finnzymes Oy, Finland), 3.5mM MgCl<sub>2</sub> (Finnzymes Oy, Finland) as well as primers pair 0.5 µM PrimerMixGP (TibMolbiol, Poznań, Poland). HPV types 6, 11, 16,18,31,33, CATCCGTAACATCATCTTCCA, TCTGTGTCTAAATCTGCTACA, GATATGGCAGCACATAATGAC, CTAAATTTGGTAGCATCATATTG, GATCTTCCTTGGGCTTTTGG, CTGTCACTAGTTACTTGTGTGCAT, respectively. The reaction products, 12 µl were distributed in a 1,5% gel agarose (Invitrogen, UK) in a 0.5 TBE buffer system (Qbiogene, USA) for 1h at 70V [17]. The detection was conducted by using ethidine bromide (Sigma-Aldrich Poland) and UV light source (Transiluminator TII Biometra,

Germany) molecular weight of marker 100 - 600 bp. The positive specimens in which the reactions attained 444 bp contained the HPV particle types as follows: 6, 11, 18, 31, and 33. The specimens without 444 bp PCR product were regarded negative.

## **THE HPV VIRAL GENOTYPING**

Typing separate HPV genotype was done by using PCR multiplex reaction according to the Van den Brule et al. procedure [30], which amplify the HPV genomes: region E5 for HPV 6, region L1 for HPV 11, 16, 18 and region E1 for HPV 31 as well as HPV 33. In addition in order to confirm the inhibition absence the human characteristic primers-globin was added to reactive mixture. Amplification was performed by using thermocycler personal Mastercycler 5332. 50 µl from the sample when the following conditions was done. Initial denaturation at 95°C for 5 min, followed by 40 cycles consist, second denaturation at 94°C for 1 min and hybridization at 55°C for 2 min and extension at 72°C for 1.5 min and the last extension at 72°C for 4 min. The reaction mixture consisted of 100ng DNA matrix, 0.2 mM dNTPmix (Finnzymes OY, Finland) and seven pairs of primers 0.25 µM (PrMixPCO3/4) (TibMolbiol, PrMixHPV6, PrMixHPV11, PrMixHPV16, PrMixHPV18, PrMixHPV31, PrMixHPV33) (TibMolbiol, Poznań, Poland). The reaction products were then distributed in 2% agarose gel (Invitrogen, UK) in 0.5 TBE buffer system (Qbiogene, USA) for 1.5h at 70v [18]. The detection was conducted by using ethidine bromide (Sigma-Aldrich Poland) and UV light source (Transiluminator TII Biometra firm, German) molecular weight of marker 100 - 600 bp.. The 100 bp PCR product was used in each specimen corresponding human gene globin amplification. The product amplification responsible for HPV regions: 280 bp for HPV 6, 360 bp for HPV 11, 152 bp for HPV 16, 216 bp for HPV 18, 550 bp for HPV 31 and 450 bp for HPV 33.

## **EXPERIMENTAL DESIGN**

20 patients with high-risk cervical lesions HPV infection (16/18 genotype) disclosing more than one oncogenic HPV types underwent photodynamic therapy using 5-Aminolevulinic Acid as a substrate. This concerned patients with cervical, vaginal and vulva HPV infection type 16/18, 31/33, 6/11 with non neoplastic disorders. In all cases, 20 % of 5-Aminolevulinic Acid (Sigma-Aldrich) was used as a substrate. Five hours exposure time was needed, after local application of 5-ALA, this range of time is considered to be enough for metabolism of ALA to PPIX before light

exposure. A red light source (630\_+20 nm) 300W halogen light source (HOP 250) light sources were used. The light source offered a total dose of 120J/cm<sup>2</sup> for 30 min. After the exposure to light source, patients were subjected to avoid exposure to light source for at least 72 hours. Colposcopy follow ups of the patients were conducted every two weeks after light exposure, and four weeks after the previous course and before the following light exposure session, all the patients were exposed five times at an interval of 4 weeks. During follow up colposcopy and PCR-DNA test were used.

## **STATISTICAL ANALYSIS**

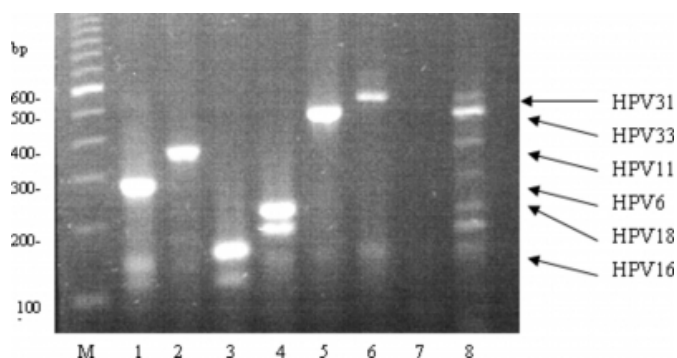
The analyses of the differences between the results obtained in women with (HPV positive) and (HPV negative) were performed with the Wilcoxon rank test, 0.05 level of probability was taken as significant.

## **RESULTS**

Table 1 and Figure 1 shows that the oncogenic HPV was detected in 227 women (47%). The six most prevalent HPV types were HPV 6/11 27 (5%), HPV 16/18 77 (16%), and HPV 31/33 123 (26%). Figure 2A showed positive histological staining of cervical lesion associated HPV infection using HE staining in comparison to the cervixes of healthy women (Fig 2B). Oncogenic HPV types were correlated with ages of the women. Table 2 showed that the HPV infection was significantly increased in ages between 26-35 years in comparison to women at the age above 46 years ( $P \leq 0.0001$ ). Positive HPV samples in ages 26-35 were 203 from 478 (43%), while at the age above 46 years only 62 samples were positive (13%). The results from Figure 3 showed that on follow-up after four months and one year after treatment, HPV was eradicated by PDT technique in 80% (16 patients out of 20, were HPV negative). No systemic side effects and no local necrosis, sloughing or scarring occurred due to PDT. Statistically significant differences concerning HPV eradication in comparison to the number of patients before treatment was ( $P \leq 0.0001$ ).

**Figure 1**

Figure 1: A representative PCR and reverse blot assay was performed genotypes in



women with cervical dysplasia from eight patients. The numbers at the bottom figure represent the patients identified in Table 1. Shown on the left side of the figure is a template identifying the probe specific for each HPV type in the assay [M]. 1) HPV type 6; 2) HPV type 11; 3) HPV type 16; 4) HPV type 18; 5) HPV type 33; 6) HPV type 31; 7) HPV negative; 8) Mixture of amplified positive products : HPV 6, 11, 16, 18, 31, and 33.

**Figure 2**

Table 1: HPV Types Distribution of 478 women with Abnormal Pap Smears

HPV types	Total (%)
Negative	251 (53 %)
6/11	27 (5%)
16/18	77 (16%)
31/33	123 (26%)

**Figure 3**

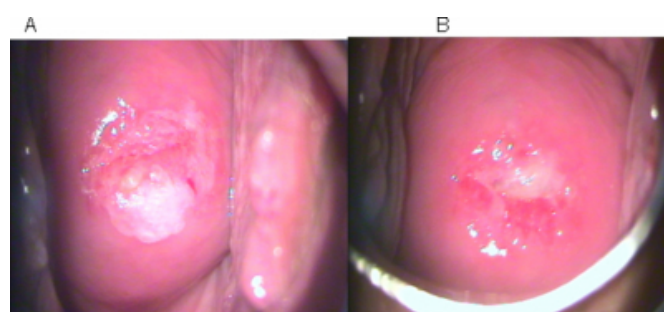
Table 2: The PCR HPV Types Distribution by Age in 478 with Abnormal Pap Smears.

Age / Yr	HPV negative (%)	HPV 6/11 (%)	HPV 16/18 (%)	HPV 31/33 (%)
25 or less (98)	52 (11%)	9(2%)	21 (4 %)	16 (3 %)
26-35 (203)	113 (24%)	10(2 %)	35 (7 %)**	45 (10 %)**
36-45 (115)	57 (12%)	6 (1 %)	11 (2 %)	41 (9 %)**
46 or more (62)	29 (6%)	2(0.4 %)	10 (2 %)	21 (4 %)

\*\*The  $P \leq 0.05$  level of probability was taken as significant.

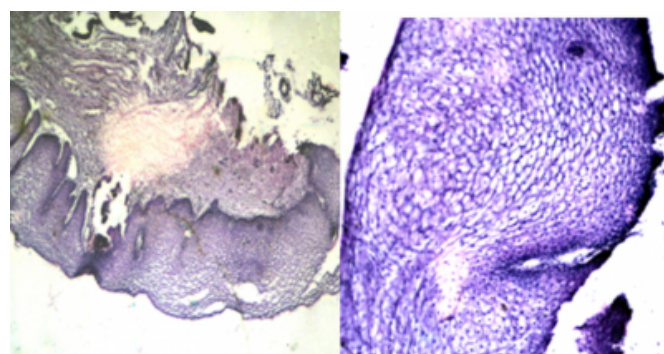
**Figure 4**

Figure 2: A) Colposcopic view observed in a cervical lesion with HPV infections in women before photodynamic therapy. B) Photodynamic therapy improved the cytological and histological feature in cervix and eradicated cervical HPV after the patients were treated with five courses of 5-ALA as a 20% preparation.



**Figure 5**

Figure 3: Histological staining of cervical lesion. A) Cervical lesion associated with HPV infections. B) Healthy cervix without HPV infections. HE was staining 100 xs.



## DISCUSSION

Genital HPV infection is the most common sexually transmitted virus and may be the most common sexually

transmitted disease [15]. Approximately 60-66% of sexual partners of persons with genital HPV-induced disease develops detectable HPV-related lesion [2]. A lot of epidemiological and molecular biological evidence has been accumulated and it is now certain that specific sexually transmitted HPV types are the main cause of most cervical disease proven by using polymerase chain reaction PCR [19]. In situ hybridization analysis showed an HPV 16 prevalence of 17 and 23% in the premalignant lesions from Greenland and Denmark [27].

Our results showed the oncogenic HPV were detected in 227 patients (47%). The six most prevalent HPV types were: 27 patients (5%) with HPV type 6/11, 77 patients (16%) with HPV type 16/18, and 123 patients (26%) with HPV type 31/33. When the oncogenic HPV types from our studies were analyzed statistically, HPV 31/33 was the most dominating infection. Previous epidemiological studies had shown that the onset of sexual activity at an early age and multiple sex partners are risk factors for the development of cervical cancer [6]. These results suggested that women with HPV 31/33 DNA might have a high risk of developing cancer. Previous studies had shown that the frequency of the HPV DNA in cytological normal cervical smears using the PCR method ranges from 5.0% to 52.7% depending on the country [5]. Recently many reports have suggested a strong association of HPV with cervical cancers and pre-invasive cancers. Therefore it is possible that cervical dysplasia could be induced in women after infection with HPV.

To help investigating this problem, we analyzed HPV DNA in cytological normal cervical smears from asymptomatic Polish women by PCR and prospectively followed them up. In the past, dot blot and Southern blot methods carried out the detection of HPV DNA. These techniques have some disadvantages in clinical application in respect of sensitivity or specificity. PCR is a specific and sensitive method of detecting HPV DNA in cervical smears. In particular the use of PCR in routinely processed clinical materials facilitates the analysis of large numbers of samples with a high sensitivity. However adequate attention is needed in order to avoiding false-positives with PCR. To avoid contamination, we used autoclaved disposable pipette tips, microcentrifuge tubes, deionized water and buffer solutions, and disposable gloves. The DNA samples were added after all the other components had been prepared. We included negative and positive controls with each set of amplifications. In this manner, we confirmed that there were no false-positive

results in the PCR.

The difference between our results reported in this study and other reports could be a result of population size and the HPV types distribution geographically. Reports have revealed that younger women (under 35 years of age) show 3- to 5-fold higher rates of HPV infection than women over 35 years of age [8].

In the present paper we show that using a 20% 5-ALA topically applied in photodynamic therapy may be an effective solution in eradicating cervical lesions associated HPV infection. The results of our in vivo experimental treatment confirmed by PCR technique following a routine colposcopy follow up after four months and one year after treatment revealed that HPV was eradicated in 16 cases out of 20 patients without systemic side effects and no local necrosis, sloughing or scarring occurred during PDT. HPV testing requires careful consideration as part of routine follow-up protocol following treatment of cervical intraepithelial dysplasia [1]. Success was defined as the absence of CIN on Pap smear or colposcopic examination at 12-months treatment patients with cervical intraepithelial dysplasia (CIN) using 5-aminolevulinic acid (ALA) as a topically applied photosensitizer for photodynamic therapy (PDT) [14]. Wierrani et al. [34] observed that ectocervical dysplasia and associated HPV infections can be treated by PDT. It has been suggested that HPV detection by DNA analysis might be used as a tool to identify women at high risk of developing cervical dysplasia in Poland. PDT is effective not only in improving the cytological and histological measures when treating cervical lesions but also for eradicating cervical HPV. Further studies are needed with a big number of patients enrolled and with a longer period of observation.

## **ACKNOWLEDGMENT**

The authors is indebted with sincerest gratitude and deepest appreciation to Dr. Tadeusz Dobosz, Prof. of Forensic Genetic, Department of Forensic Medicine, Molecular Technical Unit, Wroclaw Medical University, for his guidance, constructive comments, helpful discussion and his heartily encouragement during the development of the research.

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