DNA adducts in squamous cell cervical carcinomas associated with HPV infection

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Summary

The aim of the study was qualitative and quantitative evaluation of DNA adducts in squamous cell cervical carcinomas associated with oncogenic HPV infection.

Material: The study material consisted of oncogenic tissue collected during the surgeries of seven women aged 37 to 52 who were undergoing surgical treatment due to squamous cell cervical carcinoma. The control group consisted of 3 tissue fragments from morphologically normal cervix collected from patients undergoing surgery due to uterine myomas.

Methods: DNA from the tissues was isolated using Genomic Prep Plus kit from A&A Biotechnology, Austria. Amplification reactions detecting HPV DNA presence in the tissue fragments were performed using specific starters allowing for amplification of conservative genome fragments within L1, E6 and E7 *Papilloma* viruses. After extraction, the DNA specimens underwent enzymatic digestion to nucleotides and marked on the 5' end using γ32P - post labeling technique. Division and quantitative evaluation of DNA adducts was performed using thin-layer chromatography (TLC) on PEI-cellulose plates. Qualitative radioactivity measurements were performed using Bio-Imaging analyzer in quantitative mode.

Results: In all fragments, including the control, HPV 16 and/or 18 DNA was found. Mean adduct content in cervical carcinoma tissues was 289 adducts per 10^9 nucleotides and was higher than mean adduct content in control tissues (57adducts per 10^9 nucleotides).

Conclusions: The study results suggest that the content of DNA adducts in squamous cell cervical cancer associated with HPV infection may serve as a molecular marker of oncogenesis in this organ.

Key words: Squamous cervical cancer; HPV 16 and/or 18; Adducts.

Introduction

Papilloma viruses, especially the types with high oncogenic potential are considered to be the main factor of neoplastic transformation within the paraepidermal cervical epithelium [1]. However, in a complex, multi-stage and multi-factor process of neoplastic transformations, the role of environmental factors has also been stressed [2]. The result of environmental factor influence on a molecular level are DNA adducts, which represent one of the basic causes of gene mutations [3]. Consequently, evaluation of DNA adductivity constitutes a direct indicator of exposure to exogenous physical and chemical factors and, at the same time, is a highly specific and sensitive biological marker of carcinogenic risk [3]. Thus, the purpose of the study was a qualitative (pattern of distribution) and quantitative evaluation of DNA adducts in squamous cell cervical carcinomas associated with oncogenic HPV (16 and/or 18) infection.

Material and Method

The study material consisted of oncogenic tissue collected during the surgeries of seven women aged 37 to 52 (mean 48.3) who were undergoing surgical treatment due to squamous cell cervical carcinoma at the Ist Department of Gynecology of Lublin Medical Academy. The weight of the collected tissue sections was 0.03-0.4 g.

The control group consisted of three tissue fragments from morphologically normal cervix collected from non-smoking patients aged 40-58 undergoing surgery due to uterine myomas.

In the study group of seven patients with squamous cell cervical carcinoma there were five *macrocellular keratosis* cases, one *macrocellular akeratosis* and one *microcellular akeratosis* case. Depending on the neoplastic cells, grading of the patients was divided into three groups (G1 (n=2), G2 (n=4), G3 (n=1)) according to the WHO classification [4].

According to FIGO clinical staging five women were evaluated as Stage IA, one was classified as IB and one was qualified as IIA.

DNA from the tissues was isolated using a Genomic Prep Plus kit (A&A Biotechnology, Austria) according to the procedure recommended by the manufacturer. Purity of the obtained DNA was controlled spectrophotometrically and electrophoretically on agarose gel. In order to identify viral genes in the DNA isolated from the study and control material, PCR analysis was performed using MY09/MY11 starters with sequences complementary to 33 HPV types, HPV16 L1/L2 starters complemen-

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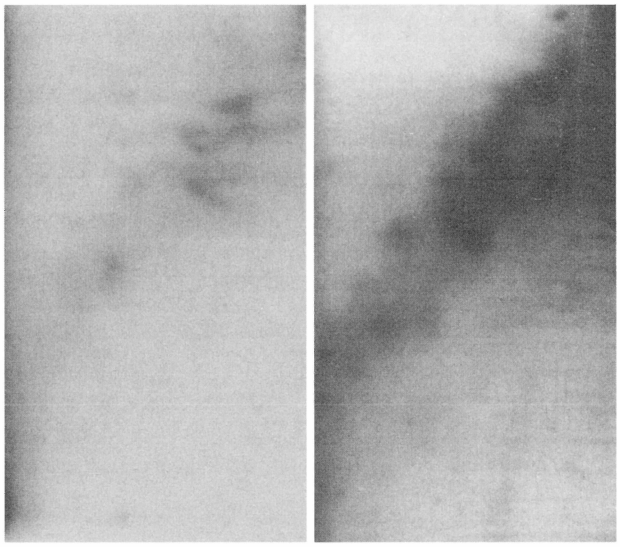
tary to HPV 16 as well as HPV18L1/L2 and HPV 18ME12/50 starters complementary to HPV 18. The degree of purity of the complete human DNA matrix isolated from the study material was evaluated using PC03 and PC04 starters for β-globin as a reference gene. The reaction was performed in the way described by Tuker [5]. In statistical analysis, the percentage of occurrence of the studied feature was given.

DNA adducts were marked using modified γ32P - the post-labeling technique described by Reddy and Randerath in 1986 [6]. After extraction, DNA specimens underwent enzymatic digestion with splenic endonuclease (1 IU) and phosphodiesterase (0.04 IU) in a buffer consisting of 5 µl 200 mM sodium succinate, pH 6 with addition of 20 mM $CaCl_2$ for nucleotides. Digested DNA was treated with 2 µg of P1 nuclease in a buffer containing 1.6 µl of 1mM $ZnCl_2$ and 2 µl of 1M sodium acetate (pH5) at 37°C for 45 minutes. 5' end marking was performed in a bicine buffer solution of pH 9.8 with addition of 70 µCi of $\gamma^{32}P$ ATP and 0.16 µl of T4 polynucleotide kinase. Incubation was carried out at 37°C within 60 minutes. Separation and quantitative evaluation of DNA adducts were achieved using multidirectional thin-layer chromatography (TLC) on PEI-cellulose plates. The first direction (D1) of TLC, removal of non modified nucleotides from the studied specimen, was performed in 2.3 M NaH_2PO_4 buffer of pH 5.7. Marked nucleotides containing adducts were cut out from a PEI-cellulose plate and transferred to a new plate in a solution consisting of 7.7 M of urea and 4.8 M of lithium formate of pH 3.5 (D2). The third (D3) and fourth (D4) direction were performed in solutions containing: D3 – urea 6M, sodium phosphate (NaH_2PO_4) 0.6 M, pH 6.4 and D4 - NaH_2PO_4 1.7 M, pH 6. Quantitative measurements of radioactivity of the marked spots containing adducts were performed using a Bio-Imaging analyzer (BAS 2000, Fuji, Japan) in quantitative mode. The obtained results were recalculated to the number of adducts in 10^9 nucleotides.

Results

In all the sections (7/7-100%), including the control ones (3/3-100%), presence of HPV 16 and/or 18 DNA was observed.

Mean DNA adduct content in the tissues of squamous cell cervical carcinoma was 289 adducts/10^9 nucleotides and it was statistically significantly higher ($p < 0.05$) than the mean number of DNA adducts in the control group tissues (57 adducts/10^9 nucleotides) (Figure 1).



a b

Figure 1. — DNA adducts in control group tissues (a) and DNA adducts in squamous cell cervical carcinoma (b).

Discussion

One of the DNA viruses specifically related to cervical carcinogenesis is human papillomavirus *(HPV)*, which is found in almost 95% of squamous cell cervical carcinomas [1]. In the study results published to date evaluating the occurrence of HPV DNA, it has been reported that the odds ratio (OR) for cervical carcinoma is in a range of 50 to 100. Odds ratios for specific associations (such as HPV-16 with squamous cell carcinoma and HPV-18 with cervical adenocarcinoma) are in the range of 100 to 900 [1]. Thus, the neoplasm occurrence rate in relation to its cause (attributable fraction - AF) is estimated to be over 95%. [1, 2]. In 1995 the IARC *(International Agency for Research on Cancer)* for the first time officially classified HPV type 16 and 18 to the first group of human carcinogens [2]. The results of the IARC study compiled and summarized by Muños *et al.* in 2000 at the HPV 2000 Conference (www.hpv2000.com), indicate that for squamous cell carcinomas, depending on age and study population, OR for cervical cancer associated with HPV was 83.3 (95% CI, 54.9 to 105.3) [7]. The above-mentioned data show that there is a causative relationship between HPV DNA presence and cervical cancer – it is very strong, permanent, specific and universal and it is one of the strongest relationships observed in human neoplasms. Although the evidence on biological probability of the existence of this relationship does not leave any space for doubt, oncogenic *Papilloma* viruses are not the only etiopathogenetic factors in cervical cancer and environmental factors are also known to play an important role. Cellular DNA is constantly influenced by various environmental xenobiotics. Many of these substances are able to form strong covalent bonds with the DNA and consequently to form so-called adducts. Detection of adducts formed as a result of toxic factors influence on human DNA is becoming more and more important for evaluation of increasing environmental risks. Reports from numerous researchers stress the usefulness of utilizing adducts as markers of the influence of genotoxic substances on DNA [3, 8, 9]. Changes in the structure of genetic material caused by the presence of adducts are taken into account as a possible reason for occurrence of gene mutations [3, 8]. This mechanism can lead to irreversible cell damage and, as a result, development of a neoplastic process [10]. Analyzing the available scientific databases (Medline, PubMed) it turns out that DNA adducts are most often studied in cervical cancers in women who smoke cigarettes [11-13]. In their studies, Simons *et al.* [12] demonstrated a higher level of DNA adducts in paraepidermal cervical epithelium in HPV-positive women who smoked tobacco compared to non-

smoking HPV-positive women and that there was a molecular synergism between DNA damaging HPV 16 and tobacco [13]. Melikian *et al.* [11] showed that HPV 16 immortalized cervical cells are more susceptible to DNA damage. These results seem to confirm our initial report revealing higher DNA-adduct content in cervical cancers associated with HPV infection.

Our study results suggest that DNA-adduct content in squamous cell cervical carcinoma associated with HPV infection, also in non-smokers, may serve as a biological marker of oncogenesis in this organ.

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