

## Human papilloma viruses 16 and 18 in patients under 40 years of age with operable squamous cancer of the uterine cervix

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Numerous investigations support the belief that human papilloma viruses (HPV) play an important role in the etiology of cervical cancer. Our study carried out in 31 patients with operable squamous cervical carcinoma (SCC), who were under 40 years of age, was aimed to investigate the incidence of HPV 16 and 18 infection in this group of patients, their sexual behavior as well as the efficacy of Slovene gynecological service in cervical cancer detection. HPV 16 was found in 67% and HPV 18 in 22% of our patients, which is consistent with the data reported by other authors.

**Key words:** human papilloma viruses cervix neoplasms-etiology

### Introduction

In the majority of western countries the incidence as well as the mortality of SCC have been gradually decreasing since 1950, as a result of their carefully planned and systematically practised strategy for early detection of this disease. On the other hand, the facts that cervical cancer related deaths are the most frequent in women under 40 years of age, and that an increasing trend in SCC incidence has been reported in some areas in that particular age group, should not be ignored.<sup>1,2</sup> A prerequisite for a successful mass screening for pre-cancerous conditions of the uterine cervix and non-invasive cancer is certainly the simple and painless method of cervical smear taking at the time of gynecological checkup. Papanicolaou

test, or shortly "Pap test" (PT) was introduced in 1941 by Papanicolaou and Traut, and it has become a synonym for a complete gynecological examination. Though the value of PT is indisputable, some drawback of this diagnostic method nevertheless need to be pointed out. The most relevant is certainly the fact that cytomorphology is unable to foretell which of the seemingly indistinguishable (identical) pre-cancerous changes will sooner or later turn into intraepithelial or invasive carcinoma.

The changes caused on cells by papilloma viruses were described already in 1933. Due to the lack of suitable cultures (growth media) and some serological tests, it was only after 1970 that a possible correlation between HPV and cervical cancer became apparent on the basis of several experimental, clinical and epidemiological studies. Advances in molecular biology, particularly the discovery of polymerase reaction (PCR) which is the most sensitive method of HPV detection (as compared to others such as in situ hybridization and dot blot hybri-

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dization), resulted in the isolation of over 60 different HPV genotypes. The types 6 and 11 are associated with benign changes in the uterine cervix. Their presence most probably does not represent an increased risk of cervical cancer. HPVs 16, 18, 31 and 33 are, on the other hand, considered to be oncogenic HPV types, as they can be frequently seen in intraepithelial cervical dysplasias (CIN II\*), in severe dysplasias or in noninvasive squamous carcinoma of the uterine cervix (CIN III) and invasive SCC. According to some data, HPV 16 and 18 can be found in 1.5% of healthy women, in 20–40% of patients with squamous intraepithelial changes (CIN I), in 60–80% of patients with changes described as CIN II and III, as well as in 80–90% of patients with SCC.<sup>1,3</sup>

The present report was aimed to explain the role of certain suspected risk factors in the sexual behavior of patients, believed to be associated with the appearance of SCC. We also wanted to establish the number of gynecological checkups performed within two years before diagnosis, as well as the number of cytological smears and cytological findings in the same period, and to determine the percentage of patients with HPV 16 and 18.

### Patients and methods

The study included 31 patients younger than 40 years, who were treated for operable squamous carcinoma of the uterine cervix at the Department of Gynecology of the University Clinical Center, and at the Institute of Oncology in Ljubljana, in the period from February 1990 to February 1992.

The data were collected by means of a questionnaire comprising general as well as specific

\* It seems that the international classification of intraepithelial cervical neoplasms (CIN), which was accepted in 1973, will be replaced by a new classification from Bethesda, where CIN I stands for "low grade" squamous intraepithelial lesion, whereas CIN II and III denote "high grade" squamous intraepithelial lesion; this classification system is based on the degree of probability to develop cancer.

questions related to the patient's life style, her gynecological and reproductive history and sexual behavior (age at first sexual intercourse, the number of partners in the last two years), and the type and duration of contraception used. We also collected the data on patients' imminent gynecological problems just before diagnosis and on the number of cytological smears taken during the last two years before diagnosis.

Each patient had cervical smear for the determination of HPV 16 and 18 taken on gynecological checkup. Tests for the presence of HPV 16 and 18 were done at the institute of Microbiology in Ljubljana. The serum levels of vitamin A and beta-carotene were also determined in each patient in order to establish possible correlation between a decrease in these values and the appearance of SCC metastases.

The patients were followed up for 12 to 36 months after the diagnosis.

### Sample processing

Smear samples were immersed in 2 ml of phosphate buffer saline (PBS) at 7.4 pH, and immediately taken to the laboratory for further processing. After the PBS immersed smears are well shaken on a vortex, the smears alone are removed while PBS with cells is centrifuged at 2000 rpm for 10 min. Using glass slides, two smears from the cell sediment are made for HPV-16 and HPV-18 determination. The slides are then air-dried and fixed in cold acetone for 7–10 min. Thus prepared samples can be stored at  $-20^{\circ}\text{C}$  for a longer period of time till hybridization, or the hybridization procedure can be started immediately.

### Hybridization in situ by means of biotinilated DNA probes

Control slides for negative and positive check were included in each test. The samples were first immersed in 0.3%  $\text{H}_2\text{O}_2$  in methanol for 15–30 min. in order to stop the activity of endogenous peroxidase; 3–5  $\mu\text{l}$  of biotinilated

DNA probe were added to the smears, which were afterwards covered with slips and heated at 100–105 °C on a metal plate for 10 min. with the aim to unbind the double-stranded target DNA.

The procedure is followed by 2-hr incubation in a humid chamber at 37 °C (the incubation can be continued overnight).

Afterwards, the slides are held vertically and rinsed in 2 × SSC with 0.1 % SDS added until the slips fall off. The rinsing is repeated several times, finally with PBS.

Each smear is covered with 10 µl of streptavidin-peroxydase complex and left to incubate for 30 min. at 37 °C before being rinsed with PBS three times.

To obtain staining reaction, a drop of diaminobenzidine with addition of H<sub>2</sub>O<sub>2</sub> substrate is put onto each smear. Thus prepared samples are incubated for 15 min. in dark at a room temperature.

The reaction is stopped by immersing the slides into distilled water. After contrast staining with Mayer's hematoxylin for 1–2 min, the samples were rinsed in running water.

The slides were then dehydrated in growing concentrations of ethanol (to 100 %), and immersed in xylol; after adding a drop of PBS and glycerine (1:10), the slides were covered with cover slips.

Microscopic examination is done by means of a light microscope. We used the reagents produced by Epignost company.

Positive reaction is evident from brown-stained cell nuclei (Figure 1, image 30). There is no brown precipitate in the nuclei seen in negative reaction (Figure 2, image 28).

## Results

The studied patients were younger than 40 years, their mean age being 33 years. The youngest patients was 20 years old.

As to education, 40 % of our patients had primary and vocational school; 55 % of their partners had equal education (the same level of education).

Most patients did not suffer from deficiency of vitamin A in their food either during childhood or adolescence period. Over a half of the patients (58 %) were smokers; they smoked 20 cigarettes daily from 19 years on average.

The data on familial gynecological cancer burden were available for 3 patients only.

Most patients were married (80 %), two were divorced. Their mean age at marriage was 23 years.

Mean age at menarche was 13 years, and at the first sexual intercourse 17 years. In the last two years before diagnosis, all patients claimed having sexual relation with one partner only. Neither the data on urogenital diseases of partners nor on their past relationships SCC patients were available.

Two patients were nulliparous, whereas the rest gave birth twice at an average age of 21 years; 65 % of patients had abortions, the first one at an average age of 21 years. Only one patient had a spontaneous abortion.

65 % of patients were using hormonal contraceptives for 60 months on average, starting at 24 years of age. A half of the patients got IUD protection at an age of 26 years and were using it for 70 months on average. One third of the patients (32 %) were not gynecologically examined within the last two years before the diagnosis, though a majority of them (77 %) claimed that they attended regular annual checkups. In the last two years before the diagnosis, 73 % of the patients underwent one or two, and the rest more than two gynecological examinations.

Before the onset of disease, 45 % of the patients were free of any gynecological complaints while 35 % of them presented with recurrent vaginitis.

The main symptoms before diagnosis were bleeding (55 %), pain (50 %) and vaginitis (23 %). Four patients with SCC were pregnant at the time of diagnosis. 6 % of patients had no gynecological complaints.

Cytological smear (PAP) was not taken within two years before diagnosis in 35 % of patients, while 26 % had one and 32 % two smears taken. In almost a half of the patients with cytologic smear taken, this was done within the

year before diagnosis; 35 % of the smears were classified as negative.

The disease was diagnosed by biopsy in 71 % of the 31 patients, whereas the remaining ones underwent conization. On admission, 90 % of patients had Stage I, 81 % of these stage Ib of the disease.

81 % of patients were treated by surgery, and two received preoperative Ra applications. One fifth of the patients were also given postoperative radiotherapy.

#### *Treatment-related complications*

Minor urological complications were noted in 4 patients; two of these were treated by surgery and irradiation.

Three patients presented with recurrence (2 local, 1 distant), all of them within 6 months after primary therapy. Two patients died within the first year after diagnosis.

Normal vitamin A values were established in 84 %, and normal  $\beta$ -caroten in 44 % of patients.

The presence of HPV-16 was confirmed in 67 %, and of HPV-18 in 22 % of patients. Further, HPV-16 was found in 2 of three patients with uncontrollable disease, whereas HPV-18 presence was not established in any of those patients.

### **Discussion**

According to the data of the Cancer Registry of Slovenia, in 1986 cervical cancer was still the sixth most frequent cancer in Slovene female population. From 1977 on, the crude incidence (15/100,000) has remained basically unchanged. A moderate increase (5.3 %) can be observed only among women of 60–64 year age group. Also the relevant mortality rates (5.6/100,000) in the past few decades have been virtually the same.

In 1960 a systematic screening for cervical cancer (CC) was started throughout Slovenia. However, according to the data from 1966, such organized screening for precancerous changes and intraepithelial cervical cancer was

available in some of the Slovene communes only. One of the criteria for the evaluation of the effectiveness of cervical cancer detection is the rate of established intraepithelial vs. invasive cancers; for Slovenia, this rate is 1.2. The small number of patients with intraepithelial CC and the high number of those with invasive CC, as well as consequentially high mortality due to this type of cancer call for a systematic screening for CC, supported by additional investigations, particularly in the littoral communes of Izola, Koper and Piran, and in Maribor. We presume that the poor results of screening for precancerous and early forms of CC in these regions are attributable to the so-far unexplained risk factors such as HPV, as well as to the specific life style believed to be associated with the etiology of CC.

Numerous studies have long been supporting the belief that the risk of SCC is in correlation with woman's sexual behavior (e.g. the first sexual relation before 18 yrs of age, numerous sexual partners), as well as with education level, marriage before the age of 18 yrs, a higher number of births and abortions, smoking, less frequent use of mechanical contraceptives, and oral contraceptive use.<sup>4,5</sup> However, considering the results of multivariate analyses, any conclusion based solely on the presumed association of CC morbidity and sexual behavior is not convincing.<sup>4,6</sup> Our data are consistent with the reported as to the age at first sexual relation, education, smoking, number of abortions and the use of contraceptives. The fact that one third of patients fail to undergo a gynecological examination within the last two years before diagnosis points at the deficient organization of our gynecological service and monitoring of female population at risk, particularly since two thirds of cytological smears are confirmed as pathologic.<sup>7</sup>

According to the results of numerous studies, the two most relevant risk factors associated with the etiology of SCC are 1) the degree of dysplasia of the squamous epithelium of the uterine cervix, and 2) the presence of HPV infection.

Apart from their relevance for diagnosis,

HPVs – particularly HPV-18 – are also considered increasingly important for their prognostic value. It seems that HPV-18 is more virulent than other oncogenic HPVs, and that the fast-growing CC are presumably associated with the presence of HPV-18.<sup>8-9</sup>

It has been found that in adenocarcinoma HPV-18 is present in 30–60% whereas HPV-16 is found in only 20%. In SCC this proportion is just the opposite.<sup>11-15</sup>

Endocervical smear taking, which should be an integral part of every routine gynecological examination, together with testing for HPV-16/18 infection, might – at least to some extent – help to reduce the incidence of SCC. According to our data, the presence of HPV-16 was established much more frequently than the presence of HPV-18.

Contrary to our expectations, HPV-18 could not be found in patients with recurrent disease, which might be attributable to the lesser reliability of the methods used. Perhaps, this is also why the results are somewhat disappointing.

A new study using more advanced methods might help to resolve several interesting questions on possible association between HPV infection and gynecological neoplasms.

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