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ORIGINAL ARTICLE

Recurrence of cervical intraepithelial neoplasias with negative cone margins: risk factors

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Summary

Purpose: *The percent of young patients treated for cervical intraepithelial neoplasias (CIN) has been increasing, thus it is very important to define patients in high risk for relapse. The aim of this study was to establish any possible association of persistent human papillomavirus (HPV) infection, age, smoking, parity, use of oral contraceptives, and Chlamydia infection, with relapse of CIN.*

Methods: *Between March 2006 and March 2009 a prospective clinical study was performed at the Clinic of Obstetrics and Gynecology in Nis, with the study group comprising the first 35 patients with disease relapse after conization and the control group consisting of 30 patients with more than one year after treatment without relapse. HPV typization was done at the Laboratory for Molecular Biology and Cytogenetics of the Clinical Centre Nis using polymerase chain reaction (PCR).*

Results: *A statistically significant higher percentage of recurrences with lower pathologic stage (CIN I) was found in younger women (below 29 years) ($p<0.01$). Women in the control group were more commonly non smokers (56.66 vs. 40%) but without statistical significance ($p>0.05$). The distribution of smoked cigarettes in the study and control subjects showed no statistically significant difference ($p>0.05$). Patients with recurrences were more commonly HPV-positive compared to controls (68.57 vs. 6.66%; $p<0.05$). In the study group, HPV-positive smokers recurred with more advanced grades (CIN III and microinvasive carcinoma/MIC; $p<0.01$). In non smokers, the severity of recurrence was not statistically correlated with HPV positivity.*

Conclusion: *Persistent HPV infection, smoking associated with HPV infection and more advanced age were demonstrated to be of statistical significance for CIN recurrence. Parity, use of oral contraceptives, Chlamydia infection, and smoking as independent etiologic factors were not significantly associated with CIN relapse.*

Key words: cervical intraepithelial neoplasia, human papillomavirus, relapse

Introduction

The epidemiology of preinvasive mucous lesions of the uterine cervix has demonstrated that these changes tend to appear 10-15 years earlier compared to invasive lesions and that they are more common in younger women. The prevalence of premalignant mucous changes CIN I and II is 25.7 per 1000 women aged 25-29 years, while in those over 50 years of age the prevalence is 9 per 1000. The highest prevalence of CIN III and carcinoma *in situ* (CIS) is encountered among women aged 35-39 years (4.6 per 1000). The average age of those with more serious intraepithelial changes (CIN III and CIS) is shifting towards younger women, even those below 30 years of age. Sadegh et al. stated that the prevalence of intraepithelial lesions in the age group 15-19 was 16.2/1000, being the highest in the 24-29 years age group (29/1000) [1].

The percentage of young women treated with conization or some other surgical technique for premalignant and malignant cervical diseases has been rising with the rise of incidence of these neoplasias. Since these are young women in their fertile years of age, it is vital to timely detect and predict possible recurrence, because any subsequent surgical intervention can disturb their fertility status. All the patients who had some form of treatment for CIN have 5-fold risk of recurrence even up to 25 years after, compared to other women [2].

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The percentage of recurrence of intraepithelial neoplasias after treatment ranges from 8-19% [3]. In the paper by Gerrard and Moritz, recurrences were recorded in up to 12%, out of which 70% with positive cone margins. All the recurrences were HPV-positive [4].

The aim of this study was to investigate the association of some risk factors with recurrence of intraepithelial neoplasias in patients treated with uterine cervix conization who had negative cone margins.

Methods

From March 2006 to March 2009 a clinical study was performed at the Clinic of Obstetrics and Gynecology. The first 35 patients with recurrence after conization of some of the stages of CIN were included into the study group; the control group comprised the first 30 patients who were free from recurrence for more than a year after conization.

Samples for HPV typization were taken using sterile swabs with tube from both exo- and endocervix, and sent to the Laboratory for Molecular Biology and Cytogenetics of the Clinical Centre Nis, where further isolation and identification of malignant types of HPV took place, using the PCR method. Comparison of the obtained findings in the study and control groups was carried out, and the results were correlated with possible known risk factors and anamnestic information. All the results were statistically processed and illustrated in tables.

Statistical analysis

The study used x^2 test for analysis. The frequency distribution test and contingency tables were used depending on whether assessment included the significance of one or more features. Empirical and theoretical distribution of frequency was compared and the degree of their agreement and disagreement was measured by x^2 test.

The empirical value of x^2 test measures the agreement of frequency. It is square because it cannot have a negative value. Pearson's formula is used to calculate empirical value: the quotient of square difference between empirical and theoretical frequency divided by theoretical frequency.

The degree of freedom is obtained from the formula: DF = r – 1. Here, (r) represents the number of members, i.e. gradations (the number of frequency groups) in a series, whereas 1 stands for the number of conditions limiting free variations of the members of the series.

The theoretical value of the x^2 test is the maximum value of the x^2 for which the null hypothesis is still valid, concerning the degree of freedom and the level of significance. This value is obtained from the table of limit values of x^2 test. The empirical and theoretical values of x^2 are compared. If the empirical value is less than or equal to the theoretic value for the calculated degree of freedom and risk probability of 0.05, the null hypothesis is accepted, the working hypothesis is rejected and the conclusion is that the difference in frequency is not statistically significant ($p > 0.05$). If the empirical value is greater than the theoretical for the calculated degree of freedom and level of significance ($p = 0.05$ or $p = 0.01$), the null hypothesis is rejected, the working hypothesis is accepted and the conclusion is that difference in frequency is statistically significant ($p < 0.05$) or highly significant ($p < 0.001$).

Results

Table 1 displays the distribution of histopathologic findings of recurrences by age of patients from the study group. In women over 40 years of age there was a significantly higher percentage of cases with more advanced pathologic stage (CIN II-III and MIC compared to younger ones (66.66 vs. 33.33%; $p<0.01$).

CIN I recurrence was more frequent in patients aged 20-29 years (50 vs. 21.42%; $p<0.01$).

Table 2 illustrates the distribution of both study subjects and controls by their parity status. The difference in parity was not statistically significant ($p>0.05$).

Table 1. Distribution of pathologic findings of recurrences related to patient age in the study group

Age (years)	*CIN I n (%)*	*CIN II n (%)*	*CIN III n (%)*	*MIC n (%)*	*Total n*
20-29	7 (50)	1 (20)	1 (7.69)	0	9
30-39	4 (28.57)	3 (60)	6 (46.15)	1 (33.33)	14
40-49	3 (21.42)	1 (20)	6 (46.15)	2 (66.66)	12
Total	14 (100)	5 (100)	13 (100)	3 (100)	35

CIN: cervical intraepithelial neoplasia, MIC: microinvasive carcinoma. Age in relation to stage of recurrence, $p<0.01$

Table 2. Distribution of parity in the study and control groups

No. of deliveries	*Study group n (%)*	*Control group n (%)*
0	12 (34.28)	0 (0)
1-2	8 (22.85)	10 (33.33)
3-4	11 (31.42)	15 (50)
>4	4 (11.42)	5 (16.66)
Total	35 (100)	30 (100)

No significant difference between groups ($p>0.05$)

Table 3 presents the distribution of patients of the study and control group by smoking. Women in the control group were more commonly non-smokers, compared to the study group (56.66 vs. 40%; p>0.05).

We could not demonstrate statistically significant difference in cases with advanced pathology of relapsed disease and smoking (50 vs. 76.92%; p>0.05) (Table 4).

Table 5 shows the relationship of the number of smoked cigarettes with the advanced grade lesions of the study group. Analysing only advanced grade recurrences (CIN II-III and MIC), a statistically significant correlation emerged between the number of smoked cigarettes with more advanced grades (80 vs. 20%; p<0.05).

Table 6 demonstrates the distribution of smoked cigarettes of the study and control subjects, for which we did not find any statistically significant difference (69.23 vs. 71.42%; p>0.05).

The percent of incidence of HPV-positive patients among study and control subjects with and without recurrence is shown in Table 7. Patients with recurrences were more commonly HPV-positive compared to controls (68.57 vs. 6.66%; p<0.05).

The distribution of HPV-positive and negative cases by grade of recurrences is shown in Table 8. Significantly more patients with intraepithelial neoplasias of more advanced grades recurred vs. those with lower grade lesions (90.47 vs. 35.7%; p<0.01).

In the study group, HPV-positive patients who were smokers suffered from recurrence of more advanced stages (CIN III), compared to smokers who were HPV-negative (60 vs. 16.66%; p<0.01) (Table 9). In non smokers, the severity of recurrence grade was not statistically correlated with HPV positivity. It thus seemed that smoking could be regarded as a co-carcinogen, acting as one of the promoters of HPV infection. On the other hand, as an independent factor, smoking without HPV infection was not shown to be sufficiently significant etiologic factor in the etiology of recurrence of intraepithelial neoplasias.

Table 3. Distribution of smoking status in the study and control groups

Smoking status	*Study group n (%)*	*Control group n (%)*
Non smokers	14 (40)	17 (56.66)
Smokers	21 (60)	13 (43.33)
Total	35 (100)	30 (100)

No significant difference between groups (p>0.05)

Table 4. Distribution of pathologic findings of recurrent disease in the study group related to smoking status

Smoking status	*CIN I n (%)*	*CIN II n (%)*	*CIN III n (%)*	*MIC n (%)*	*Total*
Non smokers	7 (50)	3 (60)	3 (23.07)	1 (33.33)	14
Smokers	7 (50)	2 (40)	10 (76.92)	2 (66.66)	21
Total	14 (100)	5 (100)	13 (100)	3 (100)	35

For abbreviations see footnote of Table 1.
Stage of recurrent disease: smokers vs. non smokers, p>0.05

Table 5. Distribution of pathologic findings of recurrent disease in smokers in the study group related to the number of smoked cigarettes per day

No. of cigarettes smoked	*CIN I n (%)*	*CIN II n (%)*	*CIN III n (%)*	*MIC n (%)*	*Total n (%)*
Up to 10	3 (42.85)	1 (50)	2 (20)	0	6 (28.57)
1 pack or more	4 (57.14)	1 (50)	8 (80)	2 (100)	15 (71.42)
Total	7 (100)	2 (100)	10 (100)	2 (100)	21 (100)

For abbreviations see footnote of Table 1.
CIN III up to 10 cigarettes vs. ≥ 1 pack, p<0.05

Table 6. Distribution of the number of daily smoked cigarettes in smokers in both study and control groups

	Up to 10 cigarettes n (%)	*1 pack or more n (%)*	*Total n (%)*
Control group	4 (30.76)	9 (69.23)	13 (100)
Study group	6 (28.57)	15 (71.42)	21 (100)
Total	10 (29.41)	24 (70.58)	34 (100)

No significant difference in relation to the number of smoked cigarettes (p>0.05)

Table 7. Distribution of HPV typization in the study and control groups related to the presence or absence of recurrence

	HPV positive n (%)	*HPV negative n (%)*	*Total n (%)*
With recurrence (study group)	24 (68.57)	11 (31.42)	35 (100)
Without recurrence (control group)	2 (6.66)	28 (93.33)	30 (100)
Total	26 (40)	39 (60)	65 (100)

HPV: human papillomavirus.
HPV positive vs. HPV negative, p<0.05

Table 8. Distribution of HPV typization in the study group with CIN II + CIN III recurrence related to CIN I recurrence

Histology of recurrence	*HPV-positive n (%)*	*HPV-negative n (%)*	*Total n (%)*
LSIL (CIN I)	5 (35.71)	9 (64.28)	14 (100)
HSIL (CN II+III)	19 (90.47)	2 (9.52)	21 (100)
Total	24 (68.57)	11 (31.42)	35 (100)

LSIL: low grade squamous intraepithelial lesion, HSIL: high grade squamous intraepithelial lesion, HPV: human papillomavirus.
CIN vs. more advanced grades, p<0.01

Table 9. Distribution of pathologic findings of recurrence in smokers in the study group related to their HPV test results

	CIN I n (%)	*CIN II n (%)*	*CIN III n (%)*	*MIC n (%)*	*Total n (%)*
Smokers HPV$^+$	2 (13.33)	2 (13.33)	9 (60)	2 (13.33)	15 (100)
Smokers HPV$^-$	5 (83.33)	0	1 (16.66)	0	6 (100)
Total	7 (33.33)	2 (9.52)	10 (47.61)	2 (9.52)	21 (100)

HPV: human papillomavirus, CIN: cervical intraepithelial neoplasia, MIC: microinvasive carcinoma.
HPV positive smokers vs. HPV negative smokers, $p<0.01$

Table 10. Distribution of pathologic findings of recurrence related to high risk (smokers, HPV$^+$, >40 years of age) and low risk of recurrence (non smokers, HPV$^-$, <40 years of age)

	CIN I n (%)	*CIN II n (%)*	*CIN III n (%)*	*MIC n (%)*	*Total n (%)*
High risk	0	1 (16.66)	3 (50)	2 (33.33)	6 (100)
Low risk	4 (66.66)	1 (16.66)	1 (16.66)	0	6 (100)
Total	4 (33.33)	2 (16.66)	4 (33.33)	2 (16.66)	12 (100)

For abbreviations see footnote of Table 1.
High risk vs. low risk patients, $p<0.01$

Based on all the above established risk factors, two subgroups of the study group patients were defined: those with high risk (HPV-positive, smokers, and elderly) and those with lower risk of recurrence (without any risk factor). Table 10 depicts the distribution of recurrence stages in these two groups. In the high risk group, more advanced stages of recurrence of intraepithelial neoplasia tended to occur in a significantly higher percentage (50 vs. 16.66%; $p<0.01$).

Discussion

Park et al. have investigated all known predictors for CIN recurrence. Age above 50 years, involvement of conization margins, and pre-cone high-risk-HPV viral load >300 were statistically significant predictors of recurrence of intraepithelial neoplasias. Postmenopausal status was near the cut-off value of statistical significance, while parity (more than 3), glandular involution, pre-treatment grade of neoplasia and conization method (knife conization or large loop excision) were statistically insignificant predictors of residual disease [5].

Two independent studies in 5 USA cities and 4 countries of Latin America have demonstrated an independent effect of parity on the occurrence intraepithelial and invasive cervical changes. Linear rise of incidence of these changes with the number of deliveries was shown to be significant for the Latin America group. These findings support the fact that parity is not an independent factor, but that it could be a co-factor in the etiology of premalignant and malignant changes. Parity as a risk factor should be considered in the context of other important factors, such as nutritional, hormonal, traumatic, and immunologic ones [6].

The significance of persistent HPV infection in patients with CIN recurrence compared to controls was established by Bodner et al. Three months after conization, there was HPV negativity in 73% of the patients. In this control group, the percentage of detected recurrences was only 4%. In the experimental group (27% of the patients) with persistent HPV infection, CIN recurrences were detected in 50% of the cases. Experimental cases with persistent HPV infection were mostly older compared to controls (36 vs. 34 years) [7].

One of more recent papers has also demonstrated the association of HPV positivity with post-conization recurrence of any stage of intraepithelial neoplasia in 60% of the patients, while for higher grade disease (CIN II-III) the percentage was 90% [8].

A group of authors from the Netherlands has assessed the predictive value of HPV positivity and recurrences. After conization, 43 patients were tested for HPV. In 16 of them (37.20%) the findings demonstrated disease recurrence. In 12 (44.44%) of 27 patients in whom residual disease was not found, HPV typization findings were normal too. In 55.55% of the cases HPV typization findings were false positive. Out of 16 patients with recurrence, HPV typization was positive in 10 (62.5%) [9]. Persistent HPV infection after treatment does not always mean disease recurrence. These are the reasons why many authors have investigated possible cofactors of influence on viral persistence and disease recurrence.

Commonly mentioned co-carcinogens in the evolution and progression of intraepithelial neoplasias are oral contraceptives. One of the more recent studies dealing with this cofactor was performed in 2008. Frega et al. [10] processed a large sample of 1320 HPV positive patients, monitored for a minimum of 5 years after ablative and excisional therapies. The experimental group consisted of 650 HPV positive individuals using oral contraceptives and treated for intraepithelial neoplasias. The control group consisted of 670 HPV positive patients treated for intraepithelial neoplasias, who did not use oral contraceptives. Both groups were monitored by colposcopy, cytology, and biopsy, if necessary. The conclusion, obtained by statistical processing of data, was that use of oral contraception did not significantly increase the persistence of HPV positivity, nor the recurrence of intraepithelial neoplasias after ablative and excisional surgical therapies [10].

In our study, the percentage of the study group sub-

jects who gave the information about the use of oral contraception for at least a year was 20% (7 women), while among controls 13.33% (4 women) used oral contraceptives. The difference was not statistically significant.

Data from the literature about the significance of oral contraception as a cofactor of carcinogenesis demonstrated a degree of significance for the development of intraepithelial neoplasias but not for the development of invasive cancer. These results were explained by more frequent visits to gynecologists of the patients using contraception, in whom intraepithelial changes were detected earlier in their course. Sexual activity of the patients using contraceptives was of influence too. On the other hand, a study from 2003 [11] pooling 28 studies, failed to demonstrate any statistically significant association of oral contraceptives with intraepithelial neoplasias and cancer. The suggested explanation was the aggregation, i.e. the cumulative effect of the factor. In patients who repeatedly used contraceptives there was a cumulative positive trend of the occurrence of neoplasias, especially in the presence of some other risk factor, like malnutrition or avitaminosis [11].

In numerous papers the importance of inadequate nutrition and avitaminoses in the development of intraepithelial and malignant cervical lesions differs considerably. Malnutrition and inadequate diet in economically underdeveloped countries are well known risk factors of recurrence after conization. We should not neglect also the various diets younger women are intentionally exposed to. The lack of beta carotene and the antioxidant vitamins A and E, is especially important. On the other hand, the protective effects of vitamin A as a food ingredient and as topical treatment for CIN is well known. In certain studies an inverse relationship of serum lycopene, serum alpha carotene and tocopherol in patients with the diagnosis of CIN and cancer was demonstrated [12].

Coinfection with *Chlamydia trachomatis* and herpes simplex virus type 2 (HSV 2) in HPV positive patients with intraepithelial neoplasias was an issue studied by Paba et al. [13]. The percentage of HPV positive patients with intraepithelial neoplasia and carcinoma was 91.27% in their paper. In the group of HPV positive patients, the percentage of those with positive test to *Chlamydia trachomatis* was 23.52%. The percentage of HPV treated, *Chlamydia trachomatis* positive patients with intraepithelial neoplasia was statistically significant ($p=0.001$). The percentage of HPV positive, HSV 2 positive patients treated for intraepithelial neoplasia was 21.32%, and the value was at the cut off level of statistical significance ($p=0.053$). Except for positivity for these two coinfection factors, these authors investigated the impact of another 13 biomarkers of persistence of HPV infection. Only 3 biomarkers were demonstrated to be statistically significant, with survivin being the only overexpressed biomarker in HSV 2 and HPV infection. In conclusion, Paba and workers stressed that concomitant infection with *Chlamydia* favored the onset and persistence of HPV infection, enabled better cellular integration of the virus, inhibited apoptosis, enabled overexpression of E6/E7 oncogene, and cellular transformation [13].

In our study, we did not find any statistically significant association of *Chlamydia* infection in HPV positive patients with disease recurrence. The percentage of the study group individuals with positive test to *Chlamydia* was as low as 11.42%. Smoking as a risk factor and co-carcinogen in the etiology of CIN is a studied and proven factor.

Except nicotine, with a direct mutagenic action, its metabolites with carcinogenic action too have been isolated in the cervical mucosa. Among them, attention should be paid to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone(NNK) and benzo-(a)-pyrene [14]. Some indirect factors have been demonstrated too, by way of which smoking reduces local immunity and the number of Langerhans cells (LC), assisting thus the carcinogenetic potential of oncogenic HPV types [15].

The results of a more recent, large epidemiologic study of CIN recurrence from 2008, performed on 10,557 women from 4 continents, associated the prevalence and persistence of HPV with smoking (more than 15 cigarettes) and sexual activity (multiple sexual partners) [16].

Sherman et al. have studied the association of high risk HPV infection and smoking with the development of intraepithelial neoplasia. Patients with high grade intraepithelial neoplasias were infected with high risk HPV types in 83%, and in 17% with other viral types. Eighty-one percent of all the examined persons were smokers. There was no statistically significant difference in positivity of high risk HPV between smokers and non smokers. However, if we consider only the group of patients positive for high risk HPV, in whom higher grade intraepithelial neoplasia was detected, then there was a statistically significant higher percentage of smokers (83%) compared to non smokers (59%). Based on the data presented, a conclusion may be drawn that smokers women infected with high risk HPV bear a higher risk of onset and recurrence of intraepithelial neoplasia compared with non smokers, again positive for high risk HPV [17].

It is certain that alongside sexual activity of female patients, sexual activity and infections of their male partners should be considered too. This association of cervical and penile carcinoma was assessed as

being related to smoking too, with established positive predictive value [18].

The significance of LC and their numbers in patients with recurrent intraepithelial neoplasia have been investigated by a group of authors from Brazil in their immunohistochemical study [19]. LC were detected using light microscopy with ×400 magnification. The counting was done in 10 large fields and the results were expressed as the number of LC cells per square mm (LC/mm^2). The number of LC was separately presented for smokers and non smokers among controls and those with intraepithelial neoplasia. Among controls, smokers had fewer LC than non smokers (p=0.045). Lower numbers of these cells were also found in the surrounding intact epithelium of intraepithelial neoplasias related to controls (p=0.004). There was no statistically significant difference between smokers and non smokers in the number of LC of the intact surrounding epithelium in the group with intraepithelial neoplasia and controls (p=0.991). The number of smoked cigarettes reduced LC numbers, without statistical significance though. Based on these results, the authors concluded that the number of LC was reduced in patients with intraepithelial neoplasia but the number of LC was not related directly to smoking [19].

In addition to the reduced numbers of LC, in patients with HPV infection and intraepithelial neoplasia secretory immunoglobulin A (IgA) is also reduced. In the paper by Concalves et al. [20] the concentration of this immunoglobulin was assessed in oral saliva and genital region infected with HPV. Seventy patients with genital HPV infection and 70 controls without HPV infection were studied regarding IgA salivary content. Saliva was centrifuged and treated at -80 degrees, and IgA content was measured using non-nephelometric technique. In addition to this technique, saliva was studied using PCR technique for HPV presence. Oral HPV infection was present in 37.14% of those with HPV infection and in 4.2% of those without genital HPV. IgA titer was markedly lower in patients with positive oropharyngeal HPV compared to HPV negative ones (p=0.0001). The finding of genital HPV infection and smoking are also factors associated with low IgA levels in oral and genital mucosa. Positive oral HPV in non-smokers is related to total IgA reduction [20].

Conclusion

Patients above 35 years of age treated for intraepithelial neoplasias have a higher risk of recurrence and they should be carefully treated and monitored. Parity, *Chlamydia* infection, and use of oral contraception were not demonstrated as statistically significant factors regarding recurrence of CIN.

Smoking, as an independent etiologic factor, was not significant regarding recurrence of CIN.

The number of smoked cigarettes was not an important factor regarding recurrence of lower grades, but an important factor for higher grade lesion recurrence.

Smoking is a statistically significant co-carcinogen in the development of recurrence in HPV positive patients. HPV positive patients who smoke develop recurrence significantly more often, in contrast to HPV positive non smokers.

Patients who remain HPV positive after their treatment for intraepithelial neoplasia have a statistically significant higher risk of developing recurrence.

Recurrences of higher grade intraepithelial neoplasias are significantly more common in patients who remain HPV positive after their treatment.

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