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IL-10 Promoter nt −1082A/G Polymorphism and Human Papillomavirus Infection in Cytologic Abnormalities of the Uterine Cervix

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ABSTRACT

The role of A/G polymorphism at nucleotide −1082 in the interleukin-10 (IL-10) promoter was assessed by following the disease course of 253 patients who had had a routine diagnostic hybrid capture human papillomavirus (HPV) test because of cytologic or colposcopic abnormalities of the uterine cervix. At baseline, 97 (78%) of the 125 high-risk HPV-positive and 83 (65%) of the 128 HPV-negative patients had equivocal cytologic atypia classified as P3 by the Papanicolaou classification, and the rest of the patients had mild colposcopic atypia with cytologic results of no oncogenic significance. In the high-risk HPV-infected patients, the frequency distribution of the nt −1082 genotypes (A/A: 28%; A/G: 52%; G/G: 20%) did not differ significantly from that in the controls (A/A: 25%; A/G: 51%; G/G: 24%; $p = 0.70$). On the other hand, the nt −1082 G allele tended to decrease susceptibility to equivocal cytologic atypia unrelated to HPV infection (A/G: OR = 0.56 [95% CI: 0.31–1.02], G/G: OR = 0.27 [95% CI 0.11–0.63], p for trend = 0.05). With respect to the development of high-grade cervical intraepithelial neoplasia (CIN), the established risk factors, such as high-risk HPV infection (RR = 104.6, 95% CI: 14.2–769.9) and cytologic atypia (RR = 9.6, 95% CI: 2.34–39.7) but not the various nt −1082 genotypes (A/A: reference; A/G: RR = 1.11 [95% CI: 0.59–2.08]; G/G: RR = 0.62 [95% CI: 0.25–1.50]) were found to increase the risk for high-grade CIN. In conclusion, the nt −1082 polymorphism had no influence on the early phase of cervical carcinogenesis but may determine different susceptibilities to cervical abormalities unrelated to HPV infection.

INTRODUCTION

THE ETIOLOGIC ROLE OF HUMAN PAPILLOMAVIRUSES (HPV) in the squamous cell neoplasias of the female genitalia is well established,[1,2] and the high-risk, that is, cancer-associated types of anogenital HPVs, have been identified.[3] However, only a small fraction of high-risk HPV infections will eventually develop into invasive cervical cancer. A key step in papillomavirus-related carcinogenesis is viral persistence,[4,5] which is a relatively rare event, particularly at age <30.[6] After long-term exposure to E6 and E7 viral oncogenes, the infected squamous epithelial cells gradually lose the capability of normal differentiation. They become dysplastic and form premalignant cervical intraepithelial neoplasia (CIN),[7] which is the ultimate point at which cervical cancer can be prevented completely. The atypical squamous cells are detected readily in Pap smears, which yield a remarkable number of equivocal results, however. The detection of high-risk HPV infection in equivocal cytologic atypia provides a sensitive single point identification of patients who are at risk for oncogenic progression.[4,8]

Although high-risk HPV infection is the major etiologic factor of cervical cancer, there are a great number of cofactors that may influence the outcome of the HPV infection. Preceding infections or co-infections with, for example, *Chlamydia trachomatis*, render the cervical epithelium more susceptible to HPV-related neoplasia.[9] On the host side, there are also cofactors for cervical neoplasia, such as smoking and high reproductive activity.[10,11] Cellular immunity plays a pivotal role in controlling HPV infections, and polymorphic HLA alleles are associated with different susceptibility to cervical neoplasia.[12] In the normal transformation zone, from which most cervical neoplasias arise, there is increased expression of transforming

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growth factor-β1 (TGF-β1) and interleukin-10 (IL-10), which can suppress cell-mediated immunity locally.[13] Although the level of IL-10 production is constant during the physiologic ovulatory cycle,[14] host factors and infections cause a remarkable interindividual variation in the genital cytokine pattern.[15,16] In high-grade CIN lesions, interferon-γ (IFN-γ) expression is decreased and IL-10 expression is increased.[13,17] In these patients, the systemic cytokine profile is shifted into the type 2 direction.[18,19] IL-10 has pleiotropic immunomodulatory effects.[20] Basically, it is a type 2 cytokine that inhibits the antigen-presenting function[21,22] and renders $CD4^+$ T lymphocytes anergic.[23] Thereby, IL-10 inhibits the initiation of cell-mediated immune responses. However, once $CD8^+$ cytotoxic T lymphocytes (CTLs) are activated, IL-10 has a direct stimulatory effect on the maintenance and augmentation of the activated state. This phenomenon is observed after both polyclonal[24] and HPV-specific stimulation.[25] The expression of IL-10 receptors is inducible in keratinocytes, which are the host cells of HPVs.[26,27]

The 5′ flanking region of the IL-10 gene is mapped at >4 kb in length.[28] In its proximal part, there are single nucleotide polymorphisms at nucleotide positions −1082(A/G), −819(C/T), and −592(C/A) relative to the transcriptional start.[29] Because of a strong genetic linkage, there are three common haplotypes, GCC, ACC, and ATA, in the Caucasian population.[30] All three single nucleotide polymorphisms map to putative transcription factor binding sites.[28] The net −1082 alleles are shown to have different binding abilities to a transcription factor, most probably an ETS transcription factor,[31] and these alleles also influence the transcriptional activity of the IL-10 promoter.[31,32] The nt −1082 polymorphism[33] but not the other two polymorphisms[34] were found to determine different risks for cervical cancer. However, these studies were performed in human populations with an nt −1082 A allele frequency greatly exceeding the G allele frequency, which resulted in a low percentage of non-A/A genotypes.

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In this study, the impact of IL-10 promoter nt −1082 polymorphism on cervical abnormalities was evaluated in a Caucasian population, in which the A and G alleles tend to be equally frequent.[31] Clinical data were collected from a cohort of patients having had a routine diagnostic HPV test because of squamous cell abnormalities of the uterine cervix. We analyzed the association of IL-10 promoter nt −1082 polymorphism with the baseline data of HPV infection and cervical abnormalities and with the outcome of the cervical disease. The last was evaluated by following the patients until the end point of histologic diagnosis of CIN, and patients who did not develop CIN were censored at their last registered visit. The nt −1082 G allele was found to protect against HPV-unrelated cervical abnormalities, but it affected neither the HPV infection nor the development of CIN.

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MATERIALS AND METHODS

Study group

This study was performed on epithelial cells scraped from the uterine cervix for routine HPV testing. In cervical screening, HPV testing was used as a secondary screening for patients with cervical abnormalities as detected by cytologic or colposcopic test. Digene's hybrid capture tube test (Digene, Madison, MA) was used to detect the low-risk and the high-risk HPV infections separately. From our hybrid capture specimen collection, we identified the first 140 high-risk HPV-positive and the first 140 HPV-uninfected cervical specimens of patients who had no previous history of CIN or worse. A high-risk HPV-positive result by the hybrid capture test is interpreted as an infection by one or more of the following HPV types: 16, 18, 31, 33, 35, 51, 52, and 56. The clinical data of the patients were collected from the electronic patient registry of the Medical Center.

HPV genotyping

Genotyping within the high-risk HPV group was done by RFLP analysis of the MY09–MY11 PCR products. DNA was extracted from the specimens processed previously for the routine hybrid capture test and was subjected to MY09–MY11 PCR amplification and typing as described previously.[35]

IL-10 promoter nt −1082 typing

The same cervical DNA samples were used to analyze the polymorphic −1082 nt of the IL-10 promoter with allele-specific PCR using the primers described by Perrey et al.[36] After an initial denaturation at 95°C for 2 min, 35 PCR cycles were done with the following profile: 95°C for 30 sec, 64°C for 1 min, 72°C for 1 min. PCR products were detected by ethidium bromide staining after agarose gel electrophoresis.

Cross-sectional study

Patients were classified by the results of the routine HPV testing and the concurrent cytologic results. The diagnosis of equivocal cytologic atypia was established if the cytologic result was P3 (mild to severe dyskaryosis) according to the Papanicolaou classification. If colposcopic atypia was detected with no recent history of cytologic abnormalities, a cytologic smear was taken routinely before HPV sampling. Several patients with mild colposcopic atypia, such as mosaic, punctuation or acetowhite, had concurrent cytologic results of no oncogenic significance. The IL-10 promoter nt −1082 genotype frequencies of the patient groups were compared with those of the control group of 168 blood donors.

Follow-up study

The baseline data used for follow-up were the patient data used in the cross-sectional study. Time zero was set at the visit when the baseline abnormality was first detected. The patient data were followed until the histologic diagnosis if the cervical lesions were removed surgically. The surgical excisions were made by either cold knife conization or the large loop electrocautery excision technique. A histologic diagnosis of CIN2 or worse (high-grade CIN) was considered to be of oncogenic significance and was used as the end point of the follow-up. Patients not requiring surgical excision were given conservative patient care and in the analysis were censored at the last registered visit. For these patients, the typical screening intervals recommended by gynecologists were 3, 6, or 12 months, depending on the gynecologic status.

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Statistical analysis

Frequency distribution of genotypes was compared by chi-square statistics. The association between the respective genotypes and the baseline patient data was tested by logistic regression analysis. For the follow-up, multivariate Cox's proportional hazards regression analysis was carried out to estimate the relative risks and 95% confidence interval (95% CI) of developing high-grade CIN according to IL-10 nt −1082 polymorphism and the baseline gynecologic data.

RESULTS

Study group

Using the nt −1082 allele-specific PCR methodology, IL-10 promoter-specific sequences could be amplified from 125 high-risk HPV-positive and 128 HPV-negative cervical abnormalities. At entry, the ages of the women with high-risk HPV-positive cervical abnormalities ranged from 18 to 56 years (median 31). Among these patients, 97 (78%) had equivocal cytologic atypia, and the rest of the patients had mild colposcopic atypia with cytology of no oncogenic significance. We could determine the exact type of the infecting high-risk HPVs in 120 patients. The most common high-risk HPV type, HPV16 was found in 63 (50.4%) of the high-risk HPV-positive patients. The other HPV types with remarkable prevalence were type 33 (12.6%), type 31 (10%), and type 18 (6%). The HPV types with low prevalence were type 35, 45, 52, 53, 56, 58, 66, 72, and MM4. In the 5 untyped samples, the infecting HPVs were other than type 16. Infections with multiple HPV types (16/31, 16/33, 16/52, 16/53, 18/58, 31/52, 31/56, 33/54, 33/66, 45/6, 52/56, 56/55, 58/6) were detected in 15 (12%) patients. The ages of the patients with HPV-negative cervical abnormality ranged from 19 to 61 years (median 35), and 83 (65%) patients had equivocal cytologic atypia.

IL-10 polymorphism in relation to baseline HPV and gynecologic status: cross-sectional study

At the polymorphic nucleotide −1082 of the IL-10 promoter, the A and G allele frequencies of the control group were 0.51 and 0.49, respectively, and the frequency distribution of the genotypes (Table 1) showed good fit to the Hardy-Weinberg equilibrium ($p = 0.95$). Similar results (A/A: 23%; A/G: 56%; G/G: 23%) were obtained also when the nt −1082 polymorphism was determined in the cervical scrapes of 44 healthy pregnant women from a previous study.[37] Among the patients with cervical abnormalities, the allele-specific PCR analysis revealed an altered frequency distribution of genotypes ($p = 0.05$). A/A genotype was detected in 88 cases (35%), A/G genotype in 123 cases (49%), and G/G genotype in 42 cases (16%). The altered frequency distribution of the nt −1082 genotypes was, however, not associated with the high-risk HPV-positive cervical abnormalities. The frequencies of A/A (28%), A/G (52%), and G/G (20%) genotypes were not significantly different from those of the controls ($p = 0.70$). Stratifying the high-risk HPV-infected patients by the cytologic result (Table 1) also did not reveal significant differences in the allele and genotype frequencies. Among the high-risk HPVs, type 16 has the highest prevalence worldwide[38] and the strongest association with cervical cancer,[3] which suggested an analysis of this particular HPV type. Nevertheless, the frequency distribution of nt −1082 genotypes among HPV16-infected patients was also not different from that of the controls ($p = 0.61$).

On the other hand, the nt −1082 allele frequencies were altered among the patients with HPV-negative cervical abnormalities. With regard to the genotypes, the frequency distribution differed significantly from that of the controls in patients with HPV-negative equivocal cytologic atypia (Table 1). In the analysis of the disease association of the nt −1082 genotypes, we took into account that the A allele is much more frequent than the G allele in most non-Caucasian populations. Therefore, A/A was regarded as the reference genotype, and the relative disease association of A/G and G/G genotypes was ex-

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Table 1. Frequency Distribution of IL-10 Promoter nt −1082 Genotypes in Patients with Cervical Abnormalities[a]

		HPV-negative patients		*HPV-positive patients*	
	Controls n = *168*	*NOS* n = *45*	*P3* n = *83*	*NOS* n = *28*	*P3* n = *97*
Genotype					
A/A	42 (25%)	18 (40%)	35 (42%)	8 (29%)	27 (28%)
A/G	86 (51%)	19 (42%)	39 (47%)	13 (46%)	52 (54%)
G/G	40 (24%)	8 (18%)	9 (11%)	7 (25%)	18 (18%)
Allele	A: 0.51	A: 0.63	A: 0.66	A: 0.52	A: 0.55
frequency	G: 0.49	G: 0.37	G: 0.34	G: 0.48	G: 0.45
		$p = 0.13$[d]	$\mathbf{p = 0.006}$[d]	$p = 0.89$[d]	$p = 0.60$[d]

[a]Cross-sectional study.
[b]Cytologic result of no oncogenic significance.
[c]Equivocal cytologic result (Papanicolaou classification).
[d]Chi-square test for difference in the frequency distributions of genotypes between the patients and the controls.

TABLE 2. ODDS RATIOS[a] (OR) AND 95% CONFIDENCE INTERVALS FOR ASSOCIATIONS BETWEEN IL-10 PROMOTER NT −1082 GENOTYPES AND HPV-NEGATIVE CERVICAL ABNORMALITIES[b]

Genotype	*Controls* n = *168*	*HPV-negative NOS[c] patients* n	*OR (95% CI)*	*HPV-positive P3[d] patients* n	*OR (95% CI)*
A/A	42	18	1.0	35	1.0
A/G	86	19	0.56 (0.26–1.23)	39	0.56 (0.31–1.02)
G/G	40	8	0.50 (0.19–1.30)	9	**0.27 (0.11–0.63)**
			$p = 0.13$[e]		***p* = 0.05**[e]

[a]Odds ratios are adjusted to age (groups) below and above 35 years.
[b]Cross-sectional study.
[c]Cytologic result of no oncogenic significance.
[d]Equivocal cytologic result (Papanicolaou classification).
[e]*p* for trend.

pressed by calculating the odds ratios (OR) and their 95% CI, which were adjusted for age to rule out the confounding effect of having age-unmatched controls. The resistance to HPV-negative cytologic atypia (P3) was significant in patients with G/G genotype and borderline significant in patients with A/G genotype (Table 2). The distribution of nt −1082 genotypes was not different between the older (≥35) and the younger (<35) patients, as using A/A genotype as a reference, the ORs for A/G and G/G were 1.18 (95% CI: 0.71–1.97) and 0.78 (95% CI: 0.41–1.50), respectively.

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IL-10 polymorphism and outcome of cervical abnormalities: follow-up study

The outcome of the baseline cervical abnormalities was evaluated longitudinally during a cumulative follow-up of 5432 patient months. Fifty-seven patients underwent surgical excision in the first 6 months of the follow-up, and 36 of them were diagnosed with high-grade CIN2 or worse, and 3 had CIN1. For the patients followed longer than 6 months, the median number of visits was 6 (range 2–12), the median screening interval was 5.7 months (range 1.5–24), and the surgically excised biopsies of 30 patients revealed 17 high-grade CIN and 4 CIN1 lesions. Altogether, 53 high-grade CIN cases were identified, of which 50 were detected in patients with high-risk HPV-positive equivocal cytologic atypia. Absolute and relative risks (RR) for high-grade CIN were calculated (Table 3). As expected, high-risk HPV infection (RR 104.6) and the presence of equivocal cytologic atypia (RR 9.6) were independent risk factors for high-grade CIN. Although the point estimate of absolute risk for high-grade CIN was somewhat higher in A/G heterozygotes than in the other two genotypes, the confidence intervals overlapping to a great extent argued against a true biologic difference. Relative risks were calculated by Cox's proportional hazards regression model to rule out possible confounding effects of the other risk factors and the individual differences in follow-up time. The relative risks also did not reveal any significant difference between the IL-10 nt −1082 genotypes, indicating that this polymorphism does not influence the progression to high-grade CIN.

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TABLE 3. ABSOLUTE AND RELATIVE RISKS (WITH 95% CONFIDENCE INTERVALS) FOR HIGH-GRADE CIN ACCORDING TO IL-10 PROMOTER NT −1082 POLYMORPHISM, HPV INFECTION, AND CYTOLOGY[a]

Risk factor	*High-grade CIN*	*Absolute risk*	*Relative risk[b]*	*Follow-up (patient months)*
nt − 1082 genotype				
A/A (*n* = 88)	15	0.17 (0.10–0.26)	1.0	1943
A/G (*n* = 123)	30	0.24 (0.17–0.33)	1.11 (0.59–2.08)	2597
G/G (*n* = 42)	8	0.19 (0.09–0.34)	0.62 (0.25–1.50)	892
HPV infection				
No (*n* = 128)	1	0.008 (<0.04)	1.0	3770
High-risk HPV (*n* = 125)	52	0.42 (0.33–0.50)	**104.6** (14.2–769.9)	1662
Cytology[c]				
NOS (*n* = 73)	2	0.03 (<0.10)	1.0	2038
P3 (*n* = 180)	51	0.28 (0.22–0.36)	**9.6** (2.3–39.6)	3394
Age, years				
<35	28	0.19 (0.13–0.25)	1.0	3203
≥35	25	0.24 (0.16–0.33)	**2.27** (1.30–3.99)	2229

[a]Follow-up study.
[b]Relative risks are adjusted to the other risk factors shown in the table.
[c]NOS, cytologic result of no oncogenic significance; P3, equivocal cytologic result (Papanicolaou classification).

DISCUSSION

This study focused on whether the IL-10 promoter nt −1082 polymorphism can influence the early phase of cervical carcinogenesis. The rationale to investigate this polymorphism was that because of a strong linkage between the polymorphic nucleotides, the nt −1082 alleles are associated with a limited number of haplotypes with different IL-10-producing ability.[29,30] The study groups consisted of patients participating in routine gynecologic care, and we used HPV testing as a secondary screening method for patients with equivocal cytologic or colposcopic abnormalities. This means that the nt −1082 polymorphism was analyzed together with the major risk factors for cervical neoplasia. Because of the routine protocol of gynecologic care, single HPV testing was done in most women at the first diagnosis of cervical abnormality. Nonetheless, the cervical abnormalities not requiring surgical excision in the short term were controlled regularly by cytologic and colposcopic testing and occasionally by late histologic excision. These data revealed that the baseline HPV testing provided a good assessment for involvement of high-risk HPVs in cervical abnormalities, as most high-grade CIN lesions were detected in patients with high-risk HPV-positive equivocal cytologic atypia. As HPV infection not only precedes but also persists longer than cytologic atypia,[39] a negative HPV result was presumed to indicate HPV-unrelated atypia. Also, the outcomes supported that these abnormalities were generally free of HPV. As the applied routine HPV testing method, the hybrid capture HPV test, provides bulk information on the infecting HPV types, we developed a simple method to make the archived hybrid capture specimens suitable for PCR amplification in order to determine the exact HPV type.[35] This method also enabled us to perform nt −1082 allele-specific PCR amplification from the same samples.

We found no association between IL-10 nt −1082 polymorphisms and HPV-related cervical abnormalities, which indicates that this polymorphism does not influence the HPV infection itself. Consistently, the patients with different nt −1082 genotypes did not have different risks for developing premalignant cervical lesions. Thus, IL-10 promoter nt −1082 polymorphism was not found to be a cofactor in the early stage of cervical carcinogenesis. With regard to invasive cervical cancer, an association with the A/G genotype was found previously,[33] which, however, was not confirmed in a recent study.[40]

On the other hand, we found an inverse association between the nt −1082 G allele and HPV-unrelated cytologic atypia, which does not increase the risk for oncogenic progression. The effect of nt −1082 alleles and other polymorphisms on the IL-10-producing ability has been studied extensively. At the transcriptional level, the nt −1082 alleles seem to regulate differently in the different cell types. In the U937 monocytic cell line, the G allele has higher transcriptional activity,[32] whereas in a cell line of B lymphocyte origin, the A allele has higher transcriptional activity.[31] The IL-10-secreting ability also depends on the cell type. In concanavalin A (ConA)-stimulated mononuclear cells, the nt −1082 G allele is associated with increased IL-10 production,[29] whereas the nt −1082 polymorphism has no effect on the IL-10-producing ability of whole blood samples after lipopolysaccharide (LPS) treatment.[41] The IL-10 level in genital secretions does not correlate with the plasma level, indicating the dominance of local cytokine production.[42] Because the majority of the infiltrating mononuclear cells are T lymphocytes in the genital epithelium[12] and ConA directly stimulates T lymphocytes, we suppose that there is increased local IL-10 production in women carrying the nt −1082 G allele. In the background of equivocal cytologic atypia, there can be inflammatory and regenerative processes as well as dysplastic cellular changes. The mechanism by which increasing IL-10-producing ability can protect against cellular changes reactive to inflammation can be explained easily by the anti-inflammatory properties of IL-10. If IL-10 also negatively regulates the regenerative and dysplastic cellular changes in the squamous epithelium, this effect has to be mediated through the IL-10 receptor of the keratinocytes. However, the direct effects of IL-10 on keratinocytes are still poorly known, hindering postulating other mechanisms possibly involved in the inverse association between IL-10-producing ability and HPV-unrelated cervical abnormalities.

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This and other studies[33,34,40] assessed the risk associated with genetic background, a risk factor stable during a lifetime. However, the relation between genotype measures and the actual cytokine measures at the uterine cervix is unknown. On the other hand, the IL-10 levels at the uterine cervix are influenced by numerous cofactors associated with the patient's age and lifestyle.[16] Thus, a limitation to this study is that the retrospective data did not provide information about such cofactors. Nevertheless, including the age in the statistical analysis must have made a surrogate assessment for the effect of some confounding factors. The age distribution was different among the study groups, which is in good agreement with the observation that HPV infection has its highest prevalence in young adults.[43] Adjustment for age in the multivariate regression models had only a slight effect on the risk assessment of the other factors tested in this study.

In conclusion, the IL-10 nt −1082 polymorphism demonstrated no effect on cervical carcinogenesis in the studied population. On the other hand, women carrying the nt −1082 G allele in the IL-10 promoter demonstrated less susceptibility to cytologic atypia unrelated to HPV infection; that is, in the cervical screening programs, the ratio between the HPV-positive and HPV-negative cytologic abnormalities may increase with the G allele frequency of the population.

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