

TNF α polymorphism frequencies in HPV-associated cervical dysplasiaA. Kirkpatrick,^a J. Bidwell,^{b,*} A.J.C. van den Brule,^{c,d} C.J.L.M. Meijer,^d
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Abstract

Objectives. Persistent high-risk HPV infection of the uterine cervix is associated with CIN and cervical carcinoma. Women with a reduced pro-inflammatory response to HPV are likely to be susceptible to viral persistence, and therefore, potentially more vulnerable to cervical neoplasia. In this study, we investigate whether nucleotide sequence polymorphisms in the TNF α (*TNFSF2*) gene (which can modify gene transcription up to 9-fold) might influence susceptibility to, or evolution of, CIN.

Methods. Induced heteroduplex analysis was used to identify polymorphisms at positions TNF α –308 and –238 in women with normal cervical cytology and with cervical disease. Patients with low-grade disease were HPV typed using general primer GP5+/6+ PCR/EIA and reverse line blotting, and were reassessed for disease status at 6 and 24 months.

Results. CIN patients as a group had a significantly higher frequency of TNF α –308 low-secreter genotypes (GG) compared to controls, and this effect was most pronounced in the CIN1 group ($P = 0.01$ and $P = 0.004$, respectively). TNF α polymorphism frequencies at position –238 were similar for patients and controls. Neither polymorphism was associated with the presence of HPV infection at recruitment or disease outcome at 6 or 24 months.

Conclusions. These findings support the hypothesis that susceptibility to CIN is influenced by TNF α –308 polymorphism.

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Introduction

Many human papillomaviruses (HPVs) infect the lower female genital tract, and are classified into low-risk (LR) and high-risk (HR) types based on phylogenetic data and their presence in benign or malignant cervical disease [1,2]. Cervical intra-epithelial neoplasia (CIN, grades 1–3), the asymptomatic precursor of squamous cervical cancer, usually presents cytologically as a positive cervical smear. Although many HR HPV infections are transient and inconsequential, HR-HPVs are strongly associated with both CIN and invasive disease [3], and persistent infection seems a pre-requisite for the neoplastic process.

Factors determining the outcome of early HPV infection and low-grade dysplasia (CIN1) are incompletely understood. Although it is estimated that 10% of CIN 1 progresses to high-grade dysplasia (CIN 2/3) and 1% to carcinoma, the majority of lesions regress or remain unchanged (60% and 30%, respectively) [4], and lesion outcome cannot be predicted. A pronounced shift from a Th1 (pro-inflammatory) cytokine production to Th2 (anti-inflammatory) cytokine production has been observed in CIN patients with extensive HPV infection [5], suggesting that the cytokine response to HPV infection may be influential in determining disease outcome. In addition, there is evidence that reduced Th1 [6] cytokine levels and increased Th2 [7] levels are associated with poor prognosis in cervical cancer. Both qualitative and quantitative variations in cytokine production can result in impairment of immune function [8]. In HPV-associated CIN, individual cytokine gene polymorphisms might potentially affect the disease process

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by several mechanisms, including moderation of cytokine production in response to HPV.

TNF α is a potent pro-inflammatory (Th1) cytokine. Single base polymorphisms occur within the TNF gene that appear to be biologically important, and a G to A transition at position –308 of the TNF α gene increases in vitro transcription by some 6- to 9-fold [9,10], and may affect disease susceptibility. A second G to A transition at position –238 is associated with various diseases, including endometrial carcinoma [11,12], but has not been directly linked with in vitro expression. Our hypothesis is that these two polymorphisms might influence susceptibility to CIN. In this study, we investigate associations between constitutional polymorphisms in TNF α positions –238 and –308, low-grade cervical disease, HPV infection and disease outcome.

Materials and methods

Patients

Patients with either low-grade cervical smear abnormalities (borderline changes or mild dyskaryosis) or high-grade CIN were recruited from the colposcopy clinic at St. Michael's Hospital, Bristol. Patients were only included in the study if there was no discrepancy between their presenting cytological abnormality and the histological diagnosis made at the first visit. All patients were initially colposcopically assessed and a further cervical smear taken. Histological diagnosis was obtained by histopathological examination of either a punch biopsy or (in cases of high-grade dyskaryosis) Loop Excision of the Transformation Zone (LETZ biopsy). A blood specimen was taken by standard venepuncture technique from all patients for TNF α polymorphism analysis. Follow-up colposcopy visits were scheduled at 6-month intervals. Cytological smears were taken at each visit, and biopsy was performed if colposcopically indicated. Patients with diagnosed CIN2/3 were offered treatment by LETZ biopsy and those that had colposcopically and cytologically returned to normal had a further follow up smear at 6 months and then 12 months later.

In theory, CIN can be detected cytologically, colposcopically or histologically but there is only partial correspondence between the disease states identified by each of these. In order that the diagnostic criteria were as rigorous as possible, we studied only women in whom there is full concordance of the three evaluation modalities. Hence, of 209 patients referred with borderline change or mild dyskaryosis, only 62 with a histological diagnosis of koilocytic change or CIN1 were entered into the study. At the time of this study, local policy was referral to colposcopy after three borderlines or two mildly abnormal smears taken 6 months apart, and hence patients would have had a cytological abnormality for a minimum of 9 months before recruitment.

In all 43 patients with koilocytic change, 19 patients with CIN 1 and 58 patients with CIN 2/3 met these diagnostic

criteria and successfully underwent TNF α polymorphism analysis. To assess the influence on disease evolution in patients with confirmed koilocytosis or CIN 1 (low grade dysplasia), this group were HPV typed from an additional cervical Cytobrush smears taken at recruitment, and were followed up colposcopically, cytologically and histologically at 6 and 24 months. In addition, 46 women with a normal smear history were recruited as age-matched controls from Family Planning clinics at Central Health Clinic, Bristol. All women gave written consent for participation in the study, which had been approved by the Ethics Committee of the United Bristol Hospitals Trust. Pearson's χ^2 test was used for statistical analysis.

DNA polymorphism analysis

Genomic DNA from whole blood, collected into sodium citrate anticoagulant, was extracted by standard techniques [13]. PCR amplification [14] was as previously described: oligonucleotide primers and IHG longmers were synthesised in-house on a PerSeptive Biosystems 8900 Expedite oligonucleotide synthesiser. Primer sequences were:

TNF α –308 polymorphism

Forward: 5'-TCCTGCATCCTGTCTGGAAG-3'

Reverse: 5'-GTCTTCTGGGCCACTGACTG-3'

TNF α –238 polymorphism

Forward: 5'-GTTCAGCCTCCAGGGTCCTACACA-3'

Reverse: 5'-GGGATTGGAAGTTGGGGACACA-3'

Polymerase chain reaction (PCR) amplification was carried out in 50 μ l volumes containing 0.5 μ M each of forward and reverse primers, 2.5 mM MgCl₂, 200 μ M each of dNTP, 1 \times Taq polymerase buffer (75 mM Tris–HCl pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% V/V Tween), 0.5 unit Taq polymerase (Advanced Biotechnologies) and either diluted IHG or 500 ng genomic DNA. PCR parameters were: initial denaturation at 95°C for 5 min; 25 cycles of: 95°C for 1 min, 59°C for the –308 polymorphism, 58°C for the –238 polymorphism for 1 min, 72°C for 1 min; final extension at 72°C for 5 min.

Construction of induced heteroduplex generators (IHG)

IHG for the TNF α polymorphisms were synthesised as single long oligonucleotides using 0.2 μ mol membrane. Following synthesis, deprotection and precipitation, the oligonucleotides were amplified by PCR using appropriate primers (see above) and if necessary were purified by preparative polyacrylamide gel electrophoresis.

Heteroduplex analysis

Equal volume aliquots (7.5–10 μ l) of amplicons from genomic DNA and IHGs were mixed, denatured at 95°C for

5 min and allowed to cool slowly from 95°C to 37°C over a 30-min period.

Electrophoresis

Heteroduplexes were resolved by electrophoresis for 90 min at 200 V in 15% non-denaturing polyacrylamide mini-gels. Gels were stained for 5 min in 1× TBE containing 0.5 µg/ml ethidium bromide and examined using a Glyko FACE digital imager.

Human papillomavirus typing

Cervical smears were obtained using Cytobrushes and the cellular content suspended in phosphate-buffered saline with 0.05% Merthiolate. HPV typing was undertaken at the Department of Pathology, Vrije Universiteit Medical Center, Amsterdam using a previously reported consensus primer assay, GP5+/6+ PCR/EIA [15]. Using cocktail probes, HPV types were then classified as high risk or low risk. Subsequent typing into individual HPV types (high risk: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68; or low risk: HPV 6, 11, 40, 42, 43, 44, 82 (MM4), 83 (MM7), 84 (MM8), Iso39, 71 (CP8061), CP6108, 81 (CP8304), 26, 34, 53, 54, 55, 57, 61, 70, 72, and 73) was performed using the recently described Reverse Line Blotting [16] of the GP5+/6+ PCR products obtained.

Results

TNFα polymorphism frequencies and cervical disease

Table 1 shows the TNFα polymorphism frequencies at positions –308 and –238 for women with normal cervical cytology compared to women with cervical disease. It can be seen that all categories of CIN are associated with TNFα –308 low secretor phenotype GG compared to women with

Table 1
TNFα –308 and –238 polymorphisms in women with cervical disease

Polymorphism	Normal	Cervical diagnosis ^a		
		Koilocytes	CIN 1	CIN 2/3
<i>TNFα-308 (secretor phenotype)</i>				
GG (low)	24 (52%)	28 (65%)	18 (95%)	41 (71%)
GA/AA (high)	22 (48%)	15 (35%)	1 (5%)	17 (29%)
Total (<i>n</i>)	46	43	19	58
<i>P</i> value	reference group	0.31	0.004	0.05
<i>TNFα-238</i>				
GG	41 (89%)	38 (88%)	17 (89%)	50 (86%)
GA/AA	5 (11%)	5 (12%)	2 (11%)	8 (14%)
Total (<i>n</i>)	46	43	19	58
<i>P</i> value		0.83	0.69	0.89

^a Koilocytes (koilocytic change) and CIN 1 corresponds to low grade dysplasia and CIN2/3 to high grade dysplasia.

Table 2

Incidence of high-risk HPV types at recruitment in 56 women of known TNFα polymorphism with a histological diagnosis of koilocytic change or CIN 1^a

High-risk HPV type	No	%	TNFα polymorphisms			
			–308		–238	
			GG	GA/AA	GG	GA/AA
16	13	23.5	2	1	12	1
18	7	12.5	5	2	7	0
31	7	12.5	4	3	6	1
33	5	8.9	3	2	5	0
35	2	3.6	1	1	2	0
39	7	12.5	6	1	6	1
45	1	1.8	0	1	1	0
51	2	3.6	1	1	2	0
52	6	10.7	6	0	6	
56	4	7.2	1	3	4	0
58	3	5.4	2	1	3	0
59	3	5.4	3	0	3	0
66	6	10.7	5	1	5	1
68	0	0.0	0	0	0	0
2 or more	18	32.1	14	4	17	1
HR-HPVs						
No HR-HPV ^b	14	25.0	10	4	0	4

^a No HPV result was obtained in six patients recruited with koilocytic change/CIN1.

^b All 14 HR-HPV negative patients where LR-HPV negative; however, 7 of the 42 HR-HPV positive patients were also LR-HPV positive.

normal cervical cytology. The association is strongest for CIN1, and 95% of CIN1 patients (18/19) are GG low secretors ($P = 0.004$), but only just reaches significance for the CIN2/3 group ($P = 0.05$). In contrast, there is no apparent association between the koilocytic change group and TNFα –308 secretor phenotypes ($P = 0.31$). No correlations were found between TNFα –238 polymorphisms and any of the cervical disease groups.

Incidence of high risk HPV types at recruitment in women with CIN1 or koilocytic change

About 75% (42) patients had HR-HPV infection and 32.1% (18) were positive for more than one HR-HPV type. 16.6% of women (7) were both HR- and LR-HPV-positive. 25% (14) were HR-HPV-negative, and all HR-HPV-negative women were found to be also LR-HPV-negative (Table 2).

TNFα –308 and –238 polymorphism frequencies and presence of high-risk HPV infection at recruitment in women with low-grade cervical disease (koilocytes or CIN1)

No significant associations were found between TNFα polymorphisms and initial HPV status in women with koilocytes or CIN1. In patients, who were high risk HPV-positive, 74% (31/42) had the TNFα –308 low secretor phenotype (GG genotype), which is comparable to 71% (10/14) in the high-risk HPV-negative group with the same phenotype ($P = 0.87$).

TNFα polymorphism frequencies and disease outcome

Analysis of disease outcome was performed at 6 and 24 months in patients presenting with CIN I or koilocytic change. At 6 months, 58 patients remained in the study (four lost to follow up). There was a relatively high persistence/progression rate in the high secretor phenotype group (73% (11/15) vs. 47% (20/43) in the low secretor group), but the results did not reach significance for the number of patients examined ($P = 0.15$). At 24 months, 42 patients remained in the study but there was no demonstrable effect of TNFα −308 secretor phenotype on disease outcome: 26/31 (84%) women with the low secretor phenotype and 8/11 (82%) with the high secretor phenotype had regressed ($P = 0.345$).

High-risk HPV status and disease outcome

In women with CIN1 and koilocytic change, only women who were HR-HPV-positive demonstrated disease progression. For example, at 6 months, no (0/13) HR-HPV-negative patients had disease progression, whereas 3/25 (12%) HR-HPV-positive koilocytic change patients and 5/14 (36%) HR-HPV-positive CIN1 patients had progressed at 6 months. However, in both the HR-HPV-negative and -positive groups, a large proportion of women had regression of disease or no change in disease status (persistence): overall, 15/36 (42%) patients with koilocytes and 9/16 (56%) with CIN1 regressed.

TNFα genotype/secretor phenotype and disease outcome at 24 months in high-risk HPV-positive and -negative patients with koilocytic change/CIN I

The regression and persistence/progression rates (%) are given for the different groupings. No apparent combined effect of high-risk HPV infection or TNFα secretor phenotype on disease outcome was demonstrable (Table 3).

Table 3
Disease outcome at 24 months in women presenting with koilocytic change/CIN1 according to high risk HPV status and TNF-α polymorphism

HPV status at presentation	Disease outcome up to 24 months	TNFα polymorphism			
		−308		−238	
		GG	GA/AA	GG	GA/AA
Positive	regression	17 (81%)	6 (80%)	22 (79%)	1 (100%)
	persistence/progression	4 (19%)	2 (20%)	6 (21%)	0 (0%)
	Total (n)	21	8	28	1
	P value	0.543		0.793	
Negative	regression	7 (100%)	2 (67%)	6 (100%)	3 (75%)
	persistence	0	1 (33%)	0	1 (25%)
	Total (n)	7	3	6	4
	P value	0.300		0.400	
No result	regression	2 (67%)	0	2 (67%)	0
	persistence/progression	1 (33%)	0	1 (33%)	0
	Total (n)	3	0	3	0

Discussion

In this study, we tested the hypothesis that constitutionally determined TNFα polymorphisms might influence response to HR-HPV infection, and therefore, susceptibility to CIN. Our data supports this hypothesis, demonstrating that 95% (18/19) patients with CIN1 have GG (low TNFα secretor phenotype) at −308 compared to 52% (24/46) in the control group, a result that is highly significant ($P = 0.004$, Table 1). This association is present but less strong with CIN2/3 ($P = 0.05$). One interpretation could be that the −308 GG low secretor phenotype, whilst conferring susceptibility to low-grade disease, is relatively protective against disease progression to high-grade CIN 2/3.

A second G to A substitution in the TNFα gene, at position −238, has been shown to be associated with endometrial cancer [12]. Our data does not reveal any association between this polymorphism and cervical pre-cancer or HR-HPV infection, nor does this polymorphism appear to affect the evolution of low-grade dysplasia over a 24-month period. However, this may be due to the inadequate power of our study to identify significant association between TNFα −238 phenotypes and cervical disease.

Although a significant proportion of HR-HPV-positive lesions regress in the short term, the presence of high-risk HPV appears to be a requirement of disease progression regardless of the TNFα −308 or −238 genotype (Table 3): no HR-HPV-negative lesions progressed over the 24-month surveillance period.

Cytological assessment alone in low-grade dysplasia is known to have poor specificity, and this study has limited numbers of patients due to rigorous selection procedure of patients at recruitment. Women presenting with borderline/mild dyskaryosis were recruited only if the histological and colposcopic diagnosis was in agreement with cytology, and hence the majority (70%, 147/209) of potentially eligible women with borderline/mild dyskaryosis were excluded in the interest of diagnostic rigor. Although punch biopsy could potentially alter the disease course, this appears not to be the case [17].

Although the majority of recruited women have been followed up for 2 years, default to follow-up is common in patients with borderline/mild dyskaryosis. At 6 months, 4 out of 56 patients (7%) were lost to follow-up and this figure had increased to 14 (25%) at 24 months.

In summary, the data suggests that the TNF −308 GG low secretor phenotype is strongly associated with CIN I, and that susceptibility to CIN may be influenced by inherent individual cytokine polymorphisms. No association between TNFα polymorphism and disease outcome has been demonstrated, but high-risk HPV infection appears to be a requirement for progression from low- to high-grade dysplasia.

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