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TNF-α 308 G/A Polymorphism and Cervical Intraepithelial Neoplasia

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Abstract. *Aim: The purpose of the present study was the analysis of the relationship between tumor necrosis factor-α (TNF-α) -308 G/A and cervical intraepithelial neoplasia (CIN). Materials and Methods: A prospective case−control study was performed, enrolling 78 cases of cervical intraepithelial neoplasia and 107 controls. Every patient had a complete gynecological examination with cervical sampling and colposcopy and TNF-α 308 G/A genotyping. Results: The homozygous AA genotype was extremely rare in the study group. The GG genotype was the one most frequently encountered in all classes of cervical intraepithelial neoplasia (CIN) and controls. No statistical differences were found in global comparison between cases and controls [odds ratio (OR)=3, p=0.09], nor between well-documented cases of more evolved high-grade squamous cervical intraepithelial lesion versus controls (OR=1.2, p=0.68). However, the results were significant for invasive carcinoma (OR=10.8261, 95% confidence interval=1.0748-109.0511, p=0.0433). Conclusion: The presence of an A allele at -308 TNF-α represents a risk for invasive carcinoma.*

Cervical cancer still represents an important proportion of morbidity and mortality in women, being the third most frequent malignancy in women and the seventh malignancy overall (1). The data emerging from large vaccinated populations are promising (2-5). Unfortunately Romania still occupies the first position in incidence (1), due probably to opportunistic screening. Genetic susceptibility might also be involved in the increased incidence in Romania than in countries with similar screening conditions (*e.g.* Romania 23.9:100000 and Moldavia 17.1:100000) (1).

The most important risk factor for cervical cancer is persistent Human Papiloma Virus (HPV) infections (6). HPV has been isolated from virtually all cervical cancer cases but only a small minority of HPV-infected women will develop cervical cancer (6). HPV is considered the most prevalent sexually-transmitted disease (7-8), having two incidence peaks: one in teenagers and a second one around the age of forty in both females and males (9). In most patients, HPV infections resolve spontaneously due probably to an appropriate immune response.

TNF-α is known to exert anti-tumor and anti-viral effects and to participate in the regulation of the immune response (10). In the presence of an HPV infection, the components of the adaptive immune response will secrete type 1 cytokine including interleukine-12 (IL-12) and interferon-γ (IFN-γ) and pro-inflammatory cytokines: IL6, IL-8 and TNF-α in order to attract activated leukocytes in the infected tissue (11). TNF-α is produced mainly by activated macrophages, but also by the HPV-16 infected keratinocytes. It exerts an antiproliferative effect upon HPV16-infected cells that involves growth arrest in G_0-G_1 phase of the cell cycle (11, 12). TNF-α has a repressive effect upon E6 and E7 expression at the transcriptional level in HPV-16-immortalized human keratinocyte cell line (13), both oncoproteins being as key factors of carcinogenesis. Experimental studies have demonstrated that HPV-16 infected cells not only produce biologically active TNF-α in an autocrine manner, but also that it exerts an autocrine growth-inhibitory effect that may represent one of the key self-limiting regulatory mechanisms inhibiting the development of HPV-induced neoplasia (10).

Genetic polymorphisms are responsible for genetic variability (14). The promoter region represents a key region for gene replication; a polymorphism located in the promoter region can have a major impact on gene transcription, enhancing, inhibiting or even silencing a particular gene

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(14). Several polymorphisms have been described at the promoter level of TNF: -238G/A,-308G/A, -375 -857C/T and -863C/A (15), the most frequently studied being -308 G>A.

The aim of this study was to analyze the relationship between TNF-α promoter polymorphism -308 G>A and cervical intraepithelial neoplasia.

Materials and Methods

The present study was conceived as a prospective transversal case-report study. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy (no 491/12.12.2011). Every patient willing to participate in the study signed an informed consent form. In total, 185 patients (78 cases and 107 controls) were recruited between 1st of January 2012 and 30th of April 2013 at the First Obstetrics and Gynecology Clinic, County Emergency Hospital Cluj-Napoca, Romania.

A questionnaire was completed for every patient regarding age, medical history and previous pap smears. For each patient, a complete gynecological examination, followed by colposcopy was performed and biopsy when needed. During the physical examination, a cervical sample was obtained using a cervical brush (Cytobrush®,Virofem, Wiesbaden, Germany) that was later immersed in a liquid-based cytology vial (Cytoprep®, Virofem, Wiesbaden, Germany); at the end of the visit, an EDTA vial of peripheral blood (2 ml) was drawn. When indicated for patients diagnosed with high-grade disease or carcinoma in situ, the cervix was removed either by cold-knife conization, or by hysterectomy (in the case of a concomitant medical condition with indication for hysterectomy). Both cervical vial and blood were stored at 4-8˚C.

All cervical probes were processed in the Laboratory Department of the County Emergency Hospital. Cervical cytology results were formulated according to Bethesda nomenclature 2001 (16). One cytologist examined all samples after preliminary Papanicolaou staining.

The DNA extraction and TNF-α-308 genotyping took place at the Department of Medical Genetics of the Iuliu Haţieganu University of Medicine and Pharmacy, Cluj Napoca, Romania. DNA extraction was performed using a commercial kit (Wizard Genomic DNA Purification Kit, Promega®, Madison, Wisconsin, USA) according to manufacturer instructions. The extracted DNA was then stored at −20˚C, until analysis.

TNF-α-308 genotyping was performed following a (PCR-RFLP) protocol described by Ishii *et al*. (17). One microliter (10 μM) of each primer (Eurogentec, Seraing, Belgium®) with the following sequences: forward primer 5'-TCCCCAAAAGAAATGG AGGCAA TA-3', reverse primer 5'-GGTTTTGAGGGCCATGAGACGTCTG CTGGCTGGGTG-3', 2 μL of DNAgenomic DNA were used, 12.5 μL 2x PCR master mix (Thermo Fischer Scientific Inc.®, Madison, Wisconsin, USA), 1 micro l BSA 20 mg/ml (Thermo Fischer Scientific Inc.®, Madison, Wisconsin, USA), and 7.5 μL nuclease-free water in a total volume of 25 μl. For the PCR reaction, a Mastercycler Gradient (Eppendorf®, Hamburg, Germany) Thermal cycle was used. Following an initial denaturation step at 95˚C for 12 min, 35 cycles of PCR were carried-out (denaturation at 95˚C for 30 s, annealing 60˚C for 30 s, elongation at 72˚C for 60 s), which were followed by one cycle of elongation at 72˚C for 5 min (17).

Enzymatic digestion was then performed on the amplification products using 5 UI NcoI® (Thermo Fischer Scientific Inc.®, Madison, Wisconsin, USA) and 1x buffer overnight at 37˚C. Electrophoresis using 3% agarose gel (MetaPhor® Agarose, Lonza, Basel, Swizerland Inc.) was performed for the enzymatic digestion products followed by an UV examination of the gel using a transiluminator (Vilber-Lourmat®, Marne La Vallée France). Electrophoretic analysis defines three distinct banding patterns, each corresponding to thus three possible genotypes: GG wild-type homozygous genotype (326 and 36 bp fragments), AG heterozygous genotype (362, 326 and 36 bp fragments) and AA mutant homozygous genotype (362 bp undigested fragment). A gel fragment from this study is presented as an example in Figure 1.

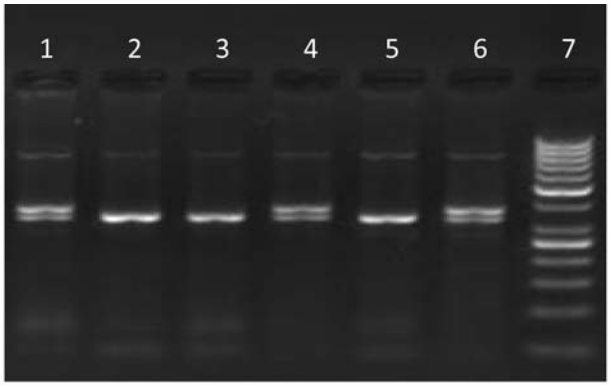

Figure 1. *Electrophoretic analysis for -308A/G polymorphism of tumor necrosis factor-α gene. Lanes 1, 4, 6: Heterozygous AG genotype; lanes 2, 3, 5: homozygous AA genotype; lane 7: 50-bp reference marker.*

Resulting data were analyzed using Graph Pad Prism 6® statistical software package(free trial, La Jolla, CA, USA). Categorical variables were reported as numbers and were compared using Chi-square test and odds ratio (OR). A two-tailed *p*-value <0.05 was considered statistically significant.

Results

A total of 78 cases and 107 controls were included in the study. The cases were represented by minor cytological anomalies: atypical cells of undetermined significance (ASC-US), 11 patients; atypical squamous cells–cannot exclude high-grade squamous intraepithelial lesion (HSIL) (ASC-H), 15 patients; low-grade squamous intraepithelial lesion (LSIL), seven patients; HSIL, 36 patients; and five patients with invasive carcinoma. The cases and controls were age-matched with no statistically significant differences in mean age distribution (the data are shown in Table I). All cases tested positively for HPV; negative HPV cases were excluded from the study.

In all classes of cervical intraepithelial neoplasia, GG genotype was the most frequently encountered (data are presented in Table II). The AA genotype, the presence of two variant alleles, was extremely rare detected, in only one patient from the control group from a total of 185 Romanian women.

Table I. *Analysis of patients' age.*

Cervical cytology results	Patient age (years)			
	Average	Minimum	Maximum	Standard deviation
ASCUS	37.70	27.00	52.00	8.14
ASC-H	45.50	31.00	77.00	12.62
LSIL	34.33	20.00	53.00	11.20
HSIL	37.38	21.00	66.00	11.17
CIS	34.00	23.00	42.00	8.04
Invasive carcinoma	38.50	23.00	46.00	10.47
HSIL/CIS/invasive	36.44	20.00	66.00	10.80
Controls	41.25	20.00	71.00	11.71
Overall CIN	38.68	20.00	77.00	11.11

Age-related differences	*p*-Value
Overall CIN *versus* controls	0.1206
LSIL/HSIL	0.5125
LSIL *versus* HSIL/CIS/invasive	0.6227

Abreviations: ASCUS - Atypical Squamous Cells of Undetermined Significance; Atypical squamous cells- cannot exclude HSIL; LSIL-Low-grade Squamous Intraepithelial Lesion; HSIL - High-grade Squamous Intraepithelial Lesion; CIS- in situ carcinoma; CIN - cervical intraepithelial neoplasia.

Table II. *The frequency of tumor necrosis factor-α genotype detection among cases and controls.*

	Genotype (no.of cases)			Total (no.of cases)
	AA	AG	GG	
ASCUS	0	5	6	11
ASC-H	0	4	11	15
LSIL	0	3	4	7
HSIL	0	9	27	36
CIS	0	1	3	4
Invasive carcinoma	0	4	1	5
HSIL/CIS/invasive	0	12	33	45
Controls	1	23	83	107

The analysis of the relationship between the TNF-α 308 genotype between cases and controls is given in Table III. No statistical significant differences were found in the global comparison between cases and controls (Chi-square=1.94, p=0.16; OR=3, p=0.09). The stratified analysis for each class of abnormal Pap smear result *versus* control failed to reach statistical significance, except for the invasive-carcinoma group. The presence of the A allele involves an significant increased risk for for invasive carcinoma (OR=10.8261, 95% confidence interval (CI)=1.0748-109.0511, p=0.0433).

Discussion

The extreme rarity of this genotype in the Romanian population has been previously described by Petrisor *et al*. (18), who reported no AA genotype in 226 patients, both men and women. In different studies analyzing the relationship between specific pathology and TNF-α-308 in different populations, the AA genotype is not that rarely encountered: 21,62% in an Egyptian study (19), 1.6% German study (20) or 4.1% American study (21). Of course due to the fact that our study group was not a representative sample of the Romanian population, but still valuable for this type of study, we can only speculate that the AA genotype is less frequently encountered than in the rest population and clearly further study is necessary.

In the literature, a large number of genetic polymorphisms have been associated with cervical cancer. The presence of an A allele at -308 of TNF-α was shown by Wilson *et al*. to be associated with an increased level of secretion, acting mainly as a potent transcriptional activator (22). Moreover, A allele binds to a different nuclear transcription protein than allele G, leading to a two-fold increase in TNF-α transcription (23). On the other hand, persistent elevation of TNF is characteristic of a longer HPV clearance interval (24), persistent HPV infection being known as the most important risk factor for cervical intraepithelial neoplasia (25). Several studies have shown a higher level of TNF-α from cervicovaginal washes in patients with cervical cancer *versus* controls (26, 27). Interestingly the study by Azar *et al*. found increased levels of TNF-α in HSIL compared to inflammatory cervicitis or normal Pap smear of HPV-positive patients (28). We did not study the levels of TNF-α, but the calculated OR was higher for invasive carcinoma than for HSIL (OR=10.8261, 95% CI=1.0748-109.0511, p=0.0433 *versus* OR=1.2029, 95% CI=0.4967-2.9133, p=0.6823). A possible explanation for this is the demonstrated secretion of TNF by HPV-16-infected keratinocytes as well as macrophages and dendritic cells, but also the possibility of associated high TNF-producing polymorphism associated with a higher risk of developing cervical cancer (11, 12).

Due to the location of the *TNF-α* gene on chromosome 6, within the major histocompatibilty complex (MHC), between HLA class I and II regions, TNF-α-308 genetic polymorphism has been previously studied in relation with immune response to infection and for its association with autoimmune disorders [rheumatoid arthritis (29), Takayasu's arteritis (30), primary Sjogren syndrome (31), sarcoidosis (32) and asthma (33)]. This also might explain the association of HLA-DR with autoimmune disorders (22). A genetic susceptibility to cervical cancer (HLA-B*07 and HLA-DQB1*0302, DQB1*0301) and a protective effect (HLA-DRB1*1301) have been previously described for this polymorphism (34-36). In our study, autoimmune disorders were not more frequent in cases than

Table III. *Risk assessment for AG tumor necrosis factor-α genotype.*

	OR	CI 95%	z	*p*-Value
HSIL *versus* controls	1.2029	[0.4967 ;2.9133]	0.409	0.6823
CIS *versus* controls	1.2029	[0.1194;12.1168]	0.157	0.8754
Invasive carcinoma *versus* controls	10.8261	[1.0748;109.0511]	2.021	0.0433
HSIL/CIS/invasive *versus* controls	1.3123	[0.5860;2.9384]	0.661	0.5088
Overall CIN *versus* controls	1.6039	[0.8234;3.1241]	1.389	0.1649
HSIL/LSIL	0.4444	[0.0831;2.3759]	0.948	0.343
			Chi-square	*p*-Value
Overall CIN/controls			1.944411	0.16323

in controls. Interestingly, TNF-α polymorphism alone was not associated with cervical cancer by one study, but it was significantly associated with cervical cancer among HPV16-positive cases and HLA DR15-DQ6 (B*0602) (36). In order to analyze TNF polymorphisms and their association with cervical cancer, it is probably important to keep in mind a possible interaction with neighboring MHC.

The presence of an A allele has been reported to be associated with a poor response to anti-viral therapy in the case of hepatitis C infection (36) and moreover, with development of liver carcinoma and cirrhosis (37), increased vulnerability for encephalitis in Japanese (38) and increased susceptibility for tuberculosis (39), but no association with septic shock (40). Altered levels of TNF-α may influence the immune response to pathogens and contribute to an individual's susceptibility to disease, including HPV infection, which is probably the key to the association of its polymorphisms with cervical dysplasia and cancer.

Our data suggest an association between the presence of an A allele and cervical neoplastic pathology (OR=1.6039, 95% CI=0.8234-3.1241, p=0.1649), the association being stronger for invasive cancer (OR=10.8261, 95% CI=1.0748-109.0511, p=0.0433). Overall, results from the literature are conflicting, disparate studies showing an increased risk for cervical cancer (41, 42), or no link (43). Two meta-analyses (to our knowledge) with concordant results to the present study had already confirmed that the presence of A allele (A *vs*. G: OR=1.43, 95% CI=1.00-2.03) and especially AA genotype (AA *vs*. GG: OR=2.09, 95% CI=1.34-3.25 respectevely AA *vs*. GG: OR=1.41, 95% CI=1.03-1.92, p=0.033) are associated with cervical cancer (44, 45) which brings us one step further in detecting the population susceptible to developing cervical cancer.

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