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RESEARCH

Tumor Necrosis Factor Alpha (−238 / −308) and TNFRII-VNTR (−322) Polymorphisms as Genetic Biomarkers of Susceptibility to Develop Cervical Cancer Among Tunisians

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Abstract Host genetic factors may confer susceptibility to Cervical Cancer. TNF-α as pro-inflammatory cytokine participates in the maintenance of immune homeostasis. Allelic variation of immuno-modulatory genes is associated with alteration in immune function. This study investigated the associations between TNF-α-308G>A, −238G>A, and TNFRII - VNTR-322 and cervical cancer in Tunisian women. Genotypes of those polymorphisms were detected in 130 cases and 260 controls. The variant heterozygote −308 G/A was associated with a 41 % decreased risk of cervical cancer (GG vs A/A; p=0.002; OR = 0.41; 95 % CI =0.23–0.76). Furthermore, compared with dominant variant G/G, the (G/A+A/A) genotypes was significantly associated with a decreased risk of CC (GG vs G/A+A/A; p=0.026; OR = 0.62; 95 % CI = 0.40–0.97). The FIGO stratified analysis showed that the minor variant A/A and combined G/A+A/A of TNFα-238 G>A and TNFα-308 G>A increased the risk of the tumor evolution, respectively, (P=0.011; OR = 2.98; 95 % CI = 1.16–7.72) (P=0.008; OR = 2.76; 95 % CI = 1.20–6.41), (P=0.000; OR = 16.33; 95 % CI = (5.10–55.23) (P=0.000; OR = 7.54; 95 % CI = 2.68–22.29). There was statistically significant relationship between the incidence of the TNF-α mutations and the clinical progression of cancer according to the FIGO classification. In our study, the haploview analysis revealed no LD between rs1800629 and rs361525. TNF-α and TNFRII polymorphisms might be genetic risk factors for cervical cancer inTunisian population

Keywords Tumor necrosis factor · Polymorphisms · Cervical cancer · Tunisians · Haplotypes

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Introduction

Cervical cancer (CC) is third most common cancer in both incidence and mortality in females; accounting for 9 % of the total new cancer cases and 8 % of the total cancer deaths among women [28]. Although HPV infection is a necessary agent of CC development, it is not a sufficient cause for the malignancy [10]. In fact, there are a large number of females infected with HPV but never develop CC, suggesting that immunological, environmental and genetic factors are also involved in the progression of cervical precancerous lesions to invasive CC [44, 25, 15, 39, 14].

Several cytokines modulating the immunologic control have been implicated in the development of cancer [19]. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that plays a critical role in the development of the immune response and there is an increasing evidence that TNF-α mediates carcinogenesis through induction of invasion, proliferation, and metastasis of tumor cells [41, 45]. Additionally, increased TNF-α blood level are observed in solid tumors [3].

It was proposed that 60 % of the variation in TNFα production is genetically determined thus suggesting a possible genetic predisposition to develop the disease [49]. This variability may be explained by a perturbation in the production or a decreased functionality of their receptors; among normal healthy individuals, significant variations between individuals in the production of TNF-α protein have been interpreted as inherited inter-individual differences [26].

Several common single-nucleotide polymorphisms (SNPs) have been identified in the promoter region of the TNF-α gene (TNFA) which can regulate the transcription and the production of TNF-α such as TNFα-308 G/A (rs1800629) and −238 G/A (rs361525) polymorphisms [5, 36, 33, 38]. The −308A allele was shown to increase the transcriptional activity of the TNFA [50] while a putative repressor site located in a 25-bp stretch included the position G-238A [21].

Previous studies reported that there was a possible association of the TNFα-238 G>A and −308 G>A polymorphisms with CC, but the findings were conflicting [47, 8, 6, 30, 23, 20, 42, 22].

In addition, TNFα has different structures and is activated by two distinct cell-surface receptor molecules; TNF receptor I TNFRI [p55] and TNF receptor II TNFRII [p75]. TNFRs give rise to a similar proinflammatory response and have been shown to play a critical role in many pathogenesis [2, 9, 43].

Many polymorphisms in the promoter TNFR may alter the regulation of the expression and levels of TNF-α and thus modulate the manifestation of pathology [46, 4, 43].

However, the role of the TNF-α and its soluble receptors in HPV-induced immunopathogenesis of pre-malignant and malignant diseases are not yet completely investigated.

Here, we analyze the contribution of the TNF-α polymorphisms −308G>A, −238G>A, and the variant VNTR-322 of the TNFRII to the risk of CC in a Tunisian population of 130 Women with CC and 260 control women.

Materials and Methods

Study subjects One hundred and thirty Tunisian women with invasive cervical cancer, confirmed by cervical biopsy, were recruited from Salah Azeiz Oncology Institute (SAI, Tunisia). Cancer diagnosis was established by clinical examination and biopsy findings, and confirmed by two senior SAI pathologists. Tumors were staged according to International Federation of Gynecology and Obstetrics (FIGO) classification [40] (www.figo.org). Control group comprised 260 women free of chronic disease, history of malignancy, drug allergies, hypertension, diabetes, or cardiovascular disease. They were recruited from Tunisian Military Hospital, Regional Hospital of Nefta, and Dispenser of Ettadhamen City. Demographic and clinical data were collected from cases and controls using a unified questionnaire. Written informed consent, approved by local ethics committees, was taken from all study subjects prior to study enrollment.

Blood collection Peripheral venous blood was collected from all participants in EDTA-containing bottles. For patients, blood collection was done prior to radiation or chemotherapy. Genomic DNA was extracted using QIAamp® DNA blood Mini Kit, according to the instructions of the manufacturer (Qiagen GmbH, Hilden, Germany).

Genotyping TNFRII-VNTR-322 was genotyped in subjects using Polymerase Chain Reaction (PCR), TNFα-308G>A (rs1800629) was performed by Amplification Refractory Mutation System Polymerase Chain Reaction method (ARMS-PCR) and TNFα-238G>A (rs361525) was determined by Restriction Fragment Length Polymorphism Polymerase Chain Reaction (RFLP-PCR), with appropriate primers as previously described [37, 16, 29].

Statistical analysis Allele and genotype frequencies were calculated by the gene counting method. The genotype distribution of the tested polymorphisms was consistent with Hardy-Weinberg equilibrium. Statistical analyses were performed by Epi info 7.

Categorical data were analyzed by Pearson chi-square analysis and Fisher's exact test where appropriate. Binary logistic regression analysis and multinomial regression analysis were used for odds ratios (OR) and 95 % confidence intervals (CI) calculation. A P value of <0.05 was considered statistically significant.

Linkage disequilibrium (LD) analysis and haplotype reconstruction was performed using Haploview 4.1 (http://www.broad.mit.edu/mpg/haploview). Logistic regression analysis was performed in order to determine the odds ratios (OR) and 95 % confidence intervals (95%CI).

Ethics

The study protocol was approved by the Ethics Committee at Salah Azeiz Oncology Institute in Tunis, Tunisia.

Results

The observed genotypes distributions of the TNF-α-238G>A, TNF-α-308G>A and TNFRII VNTR-322 polymorphisms in cases and controls conformed to the Hardy–Weinberg equilibrium ($P \geq 0.05$).

Study Design

Demographic and tumor characteristics of the study population are listed in Table 1. The median age, was 52 years for patients with CC and 53 years for healthy controls with a range of 30–81. Among the 130 patients, 124 (95.40 %) are married, 115 (88.50 %) have used hormonal contraceptives and 21 (16.15 %) have a cancer in their family. According to the menopausal status of women with CC, our sample was divided into two groups; 38 (29.25 %) were pre-menopausal and 92 (70.75 %) patients were post-menopausal.

Diagnoses of squamous cell carcinoma were confirmed by histopathological examination as International Federation of Gynecology and Obstetrics, the distribution of the sample according to the FIGO stage is as follow; stage I (30.00 %), II (37.70 %), III (24.60 %) and IV (7.70 %). Three histological types were identified: squamous cell carcinoma (83.08 %), adenocarcinoma (14.61 %) and sarcoma (2.31 %).

Polymorphisms Alleles and Genotypes Analysis

The observed genotype and allele frequency distribution of TNF-α-238, TNF-α-308 and TNFR II-322 gene polymorphisms between cases and controls are depicted in Table 2. No significant differences of allelic and genotypic distributions at TNF-α-238 G>A and TNFRII-322 VNTR were observed between the two groups. In contrast, a significant difference of genotype distribution at the loci TNF-α-308 was revealed. These were summarized as follows. The variant heterozygote −308 G/A was associated with a 41 % decreased risk of cervical cancer (GG vs A/A; p=0.002; OR = 0.41; 95 % CI =0.23–0.76). Furthermore, compared with dominant variant G/G, the (G/A+A/A) genotypes was significantly associated with a decreased risk of CC (GG vs G/A+A/A; p=0.026; OR = 0.62; 95 % CI = 0.40–0.97).

Among the three polymorphisms, no significant association was observed upon carrier analysis using the dominant model.

Association of TNF-α-238, TNF-α-308 and TNFR II-322 Gene Polymorphisms with Demographic and Tumour Characteristics of Cervical Cancer

A case-only, analysis was carried out to investigate whether any possible association exists between TNF-α-238, TNF-α-308 and TNFR II-322 gene polymorphisms and stages of CC; early stage (stages I + II ; n=88 cases) and advanced stage (stages III + IV ; n=42 cases). Our data demonstrated that the minor variant A/A and combined G/A+A/A; increased the risk of the tumor evolution for TNFα-238 G>A and TNFα-

Table 1 Demographic and tumour characteristics of the study subjects

	Patients n (%)	Controls n (%)
All women	130	260
Age on diagnostic (year)		
30–40	17 (13.08 %)	87 (33.46 %)
41–50	33 (25.38 %)	93 (35.77 %)
51–60	34 (26.15 %)	53 (20.38 %)
61–70	24 (18.46 %)	24 (9.23 %)
71–81	22 (16.93 %)	3 (1.16 %)
Married status		
+ / −[1]	124 (95.40 %) /6 (4.60 %)	257 (98.00 %) /3 (2.00 %)
Hormonal contraception		
+ / −[2]	115 (88.50 %) /15 (11.50 %)	220 (84.00 %) /40 (16.00 %)
Status of menopause		
Premenopausal	38 (29.25 %)	67 (26 %)
Postmenopausal	92 (70.75 %)	193 (74 %)
FIGO staging		
Stage I	39 (30.00 %)	
Stage II	49 (37.70 %)	
Stage III	32 (24.60 %)	
Stage IV	10 (7.7 %)	
Histological type		
Squamous cell carcinoma	108 (83.08 %)	
Adenocarcinoma	19 (14.61 %)	
Sarcoma	3 (2.31 %)	

+ / −[1] ; (+) Married ; (−) Single, + / - [2] ; (+) Yes ; (−) No, FIGO = International Federation of Gynecology and Obstetrics; n: Number of women

Table 2 Genotype and allele frequency distribution of TNF-α-238, TNF-α-308 and TNFR II-322 polymorphisms in the study subjects

Polymorphisms		study subjects		P_{Value}	OR (95 % CI)
Alleles Genotypes	Change	Cases (n=130)	Controls (n=260)		
TNF-α-238	G>A				
G		143 (55.00 %)*	304 (58.50 %)	Reference	–
A		117 (45.00 %)*	216 (41.50 %)	0.757	1.05 (0.77–1.44)
G/G		60 (46.15 %)*	132 (50.80 %)	Reference	–
G/A		23 (17.70 %)*	40 (15.40 %)	0.420	0.79 (0.42–1.47)
A/A		47 (36.15 %)*	88 (33.80 %)	0.742	1.08 (0.65–1.80)
G/A+A/A		70 (53.85 %)*	128 (49.20 %)	0.886	0.97 (0.62–1.51)
TNF-α-308	G>A				
G		143 (55.0 %)	317 (61.0 %)	Reference	–
A		117 (45.0 %)	203 (39.0 %)	0.110	0.78 (0.57–1.07)
G/G		55 (42.3 %)	141 (54.2 %)	Reference	–
G/A		33 (25.4 %)	35 (13.5 %)	0.002*	0.41 (0.23–0.76)
A/A		42 (32.3 %)	84 (32.3 %)	0.314	0.78 (0.47–1.30)
G/A+A/A		75 (57.7 %)	119 (45.8 %)	0.026*	0.62 (0.40–0.97)
TNFR II −322	VNTR				
1		124 (45.3 %)	285 (54.8 %)	Reference	–
2		118 (54.7 %)	235 (45.2 %)	0.357	0.87 (0.63–1.19)
1/1		58 (44.6 %)	118 (45.4 %)	Reference	
1/2		26 (35.4 %)	49 (18.8 %)	0.792	0.93 (0.50–1.71)
2/2		46 (35.4 %)	93 (35.8 %)	0.979	0.99 (0.60–1.64)
1/2+2/2		72 (55.4 %)	142 (54.6 %)	0.885	0.97 (0.62–1.51)

Total number of chromosomes in cases = 260 and controls = 520; OR: age-adjusted odds ratio; CI: confidence interval; * cases data are reported from our previous study [26, 53]

308 G>A, respectively, (P=0.011; OR = 2.98; 95 % CI = 1.16–7.72) (P=0.008; OR = 2.76; 95 % CI = 1.20–6.41), (P= 0.000; OR = 16.33; 95 % CI = (5.10–55.23) (P=0.000; OR = 7.54; 95 % CI = 2.68–22.29). There was statistically significant relationship between the incidence of the TNF-α mutations and the clinical progression of cancer according to the FIGO classification. However, any significant association of TNFRII-VNTR-322 was identified Table 3.

Otherwise, using the same approach, we observed that there was no significant association between the analyzed polymorphisms and modulation of cervical cancer risk due to married and menopause status, hormonal contraception and histological type (data not shown).

TNF-α Haplotype Analysis

In our study, haplotype analysis including the two TNF-α SNPs studied showed that the prevalence of all the haplotypes constructed (TNF-α-308A/TNF-α-238A; TNF-α-308A/ TNF-α-238G; TNF-α-308G/TNF-α-238A ; TNF-α-308G/ TNF-α-238G) was comparable between cases and controls (Table 4).

In our study, the haploview analysis revealed no LD between rs1800629 and rs361525.

Discussion

It was established that cytokines have a pivotal role in tumors progression and are considered as the first candidates for being genetic host markers in the susceptibility to development of different cancers including CC [7]. Among them, TNF-α and his receptors are potent cell mitogens [5, 11, 51].

TNF-α is one of the primary cytokines released in the place of HPV infection providing a particular growth advantage for abnormal cervical cells in vivo [19]. This cytokine play a critical role in up-regulating the expression of antigen-processing and -presentation pathway components for class I HLA [24, 32].

Thus, the antigen presentation could be affected by the level of expression of this cytokine. Additionally, in high concentrations in patients presenting certain types of cancers with viral or bacterial etiology like CC, the genetic programmed ability to produce increased levels of this cytokine could present risk for the development of cancer [13]. Several studies have proved that the level of transcription of this cytokine is highly regulated by a polymorphic promoter in the −308 and −238 region of the gene [50].

In this context, we assessed the association of two common polymorphisms of TNFα gene and a VNTR polymorphism of TNFRII gene with CC susceptibility.

Table 3 Correlations between TNF-α-238, TNF-α-308 and TNFR II-322 polymorphisms with early (I + II) vs advanced (III + IV) stages of cervical cancer

		FIGO stages		P_{value}	OR (95 %CI)
		Early stages I + II n (%)	Advanced stage III + IV n (%)		
TNF-α-238	G/G	51 (58 %)	14 (33 %)	–	Reference
	G/A	15 (17 %)	10 (24 %)	0.076	2.43 (0.80–7.37)
	A/A	22 (25 %)	18 (43 %)	**0.011**	2.98 (1.16–7.72)
	G/A + A/A	37 (42 %)	28 (67 %)	**0.008**	2.76 (1.20–6.41)
TNF-α-308	G/G	49 (56 %)	6 (14 %)	–	Reference
	G/A	25 (28 %)	8 (19 %)	0.097	2.61 (0.72–9.73)
	A/A	14 (16 %)	28 (67 %)	**0.000**	16.33 (5.10–55.23)
	G/A + A/A	39 (44 %)	36 (86 %)	**0.000**	7.54 (2.68–22.29)
TNFRII-322	1/1	40 (45 %)	18 (43 %)	–	Reference
	1/2	19 (22 %)	7 (17 %)	0.703	0.82 (0.26–2.55)
	2/2	29 (33 %)	17 (40 %)	0.525	1.30 (0.53–3.20)
	1/2+2/2	48 (55 %)	24 (57 %)	0.780	1.11 (0.50–2.49)

Total number of cervical cancer cases of early stages (I + II) = 88 and of advanced stages (III + IV) = 42

OR: age-adjusted odds ratio ; CI: confidence interval

In our finding, no significant association between TNF-α-238 G>A polymorphism and CC was identified.

Similar results were reported in seven CC case-contol studies [48, 30, 17, 22, 42, 12, 27]. However, the overall results of a recent meta-analysis in which data from those studies were pooled, the -238A allele was associated with a decreased risk of CC [31].

This discrepancy is likely attributed to the simple size analyzed in each study. In addition, Zuo et al. and Barbasian et al. have also reported a luck of association between TNF-α-238 G>A polymorphism and CC in Southwest of China and in Argentina, respectively, [54, 8]. Larger prospective studies investigating the association of TNF-α-238 G>A with CC are needed to get better picture of the role of this polymorphism with CC.

TNF-α-308G/A is a promoter SNP related with the TNF expression and is a widely investigated variant with risk of CC with conflicting results. Here, we demonstrate that carriers of G/A and G/A + A/A genotypes have a decreased risk of CC. In contrast, three meta-analysis, including respectively, eight, twelve and fifteen case–control studies have suggested that TNF-α-308G/A polymorphism is associated with an increased risk of CC [18, 31, 52]. Subgroup analysis by ethnicity further showed that there was a significant association of this polymorphism and increased risk of CC in Asians [52] and in Caucasian and African populations [35]. However, a large number of studies have been performed to evaluate TNF-α-308G/A polymorphism as risk factor for CC and the findings were inconclusive. These controversial results could be explained by differences in ethnic compositions; genetic background and sizes of samples between analyzed populations. It is evident that more extensive studies with larger samples and using patients with different genetic makeup should provide additional insights and improve our understanding of TNF-α-308G/A variant in etiology of cervical cancer.

Moreover, our results showed an association that the A/A and combined G/A + A/A genotypes increased the risk of the

Table 4 Haplotype analysis of SNPs TNF-α-308 G>A (rs1800629) and TNF-α-238 G>A (rs361525) for association with cervical cancer cases in Tunisians

Haplotype	Freq (cases)	Freq (ctrlols)	χ^2	*p* Fisher's	*p* Pearson's	*p* (Fisher) MonteCarlo	*p* (Pearson) MonteCarlo	OR (95%CI)
AA*	0.1750	0.1527	0.6421	0.4230	0.4229	0.9500	0.9500	1.1772 (0.789–1.755)
AG*	0.2173	0.2550	1.3417	0.2468	0.2467	0.8200	0.8200	0.8111 (0.568–1.156)
GA*	0.2712	0.2339	1.2988	0.2545	0.2544	0.8200	0.8200	1.2188 (0.867–1.713)
GG*	0.3365	0.3584	0.3652	0.5456	0.5456	0.9600	0.9600	0.9079 (0.663–1.242)
Global	–	–	2.7716	0.4283	0.4282	0.9500	0.9500	–

Global chi2 is 2.7716 while df = 3 (frequency < 0.03 in both control & case has been dropped.)

tumor evolution for TNF-α-238 G>A and TNF-α-308 G>A. In this context, Ahmed et al. showed that TNF-α levels were correlated to advanced tumor stage [1]. Correlation with tumor stage indicates that TNF-α might have a role in tumor development and progression [34].

We found no association between TNFR II −322 VNTR and CC risk. This finding suggests that this polymorphism may not impact CC pathogenesis. To the best of our knowledge this is the first report that investigated the possible association between the TNFR II −322 VNTR and the risk of CC in Tunisia and worldwide. Additional studies investigating the association of this polymorphism with CC and other tumors are needed to get a better picture on the role of TNFR II −322 VNTR in cancer.

This was the first study to investigate the association of TNF-α-238G/A, TNF-α-308G/A and TNFR II −322 VNTR polymorphisms in Tunisian CC patients. The study has some strengths but also limitations. The study subjects enrolled were of similar ethnicity (both cervical cancer patients and controls were Tunisians), thereby minimizing the possibility of racial differences inherent in genetic association studies. In addition, questionnaire-based personal interviews were conducted for assessment of demographic data, and cancer staging of patients. However, the HPV status of the study population was not determined; biopsy immunochemical studies were not done. Serological levels of TNF-α were not measured. Detailed stage-specific molecular and cellular expression studies in biopsy specimens of CC might help in determining the functional consequences of TNF-α and TNFR II gene polymorphisms, thereby drawing definitive conclusions regarding the potential link between TNFRs and cervical cancer.

In conclusion, our results demonstrate a decreased risk for CC with TNFα-308G/A AA and AG + AA genotypes. Future studies using patients with different ethnic backgrounds should provide additional insights and improve our understanding of the TNF-α and TNFR gene variants in cervical carcinogenesis, which may in future lead to better prediction of individuals who are at risk of CC.

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Conflicts of interest No competing financial interests exist.

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