Genetic variation in pro-inflammatory cytokines (interleukin-1β, interleukin-1α and interleukin-6) associated with the aggressive forms, survival, and relapse prediction of breast carcinoma

Kaouther Snoussi[1], A. Donny Strosberg[2], Noureddine Bouaouina[1,3], Slim Ben Ahmed[4], Lotfi Chouchane[1]

[1] Laboratoire d'Immuno-Oncologie Moléculaire, Faculté de Médecine de Monastir, Université de Monastir, 5019 Monastir, Tunisia
[2] Department of Infectology, Scripps-Florida USA
[3] Department of Cancérologie Radiothérapie CHU Farhat Hached, Sousse, Tunisia
[4] Service de Carcinologie Médicale, CHU Farhat Hached, Sousse, Tunisia

Correspondence : Pr L. Chouchane
<lotfi.chouchane@planet.tn>

This work was supported by the ministère de la Recherche Scientifique et de Technologie et du Développement des Compétences, by the ministère de l'Enseignement Supérieur, and by the ministère de la Santé Publique de la République Tunisienne

ABSTRACT. ***Objectives.*** **Interleukin-1 (IL-1) and interleukin-6 (IL-6) are determining factors in the immune and inflammatory responses to tumors cells. Experimental data suggest that interleukin-1 and interleukin-6 play important roles in the development and progression of breast cancer. We designed a broad study to investigate the susceptibility and prognostic implications of the genetic variation in IL-1*α*, IL-1β and IL-6 in breast carcinoma.** ***Experimental design. We*** **used the polymerase chain reaction and restriction enzyme digestion to characterize the genetic variation of IL-1α, IL-1β and IL-6 in 305, unrelated Tunisian patients with breast carcinoma and 200 healthy control subjects. Associations between the genetic markers and the clinicopathological parameters, the specific overall survival rate (OVS) of breast carcinoma and the disease free-survival rate (DFS) were assessed using univariate and multivariate analyses.** ***Results.*** **Both IL-6** ***(-597) GA*** **and IL-6** ***(-174) GC*** **heterozygous genotypes were found to be significantly associated with breast carcinoma (OR = 1.59, p = 0.024 and OR = 1.61, p = 0.022 respectively). A highly significant association was found between the** ***(+3954) T*** **allele of IL1-*B* gene and the aggressive phenotype of breast carcinoma as defined by the high histological grade, axillary lymph node metastasis and large tumor size. The IL-1α** ***(-889) TT*** **homozygous genotype showed a significant association with reduced disease-free survival and/or overall survival rate. The IL-1β** ***(+3954) TT*****, IL-6** ***(-597) GG*** **and IL-6** ***(-174) GG*** **homozygous genotypes were found to be associated with reduced DFS but not with overall survival.** ***Conclusions.*** **The polymorphisms in the promoter region of the IL-6 gene may represent a marker for the increased risk of breast carcinoma. Genetic variations in IL-1*α*, IL-1*β* and IL-6 may predict the clinical outcome of breast carcinoma.**

Keywords: breast carcinoma, polymorphism, prognosis, susceptibility, interleukin-1α, interleukin-1*β*;, interleukin-6

The role of genetic factors in the epidemiology and pathogenesis of both sporadic breast carcinoma and hereditary breast carcinoma are now well established [1, 2]. Only a small minority (± 5%) of patients with breast carcinoma develop the disease as a result of inheritance of germline mutations in dominant, highly penetrant susceptibility genes such as BRCA1 and BRCA2. However, polymorphisms in genes involved in the complex mechanisms of carcinogenesis may confer low penetrant susceptibility to breast carcinoma in a significant proportion of the remainder of patients [2-4].

Multifunctional cytokines, interleukin-1 (IL-1) and interleukin-6 (IL-6), are involved in the development of inflammatory and immunological responses which play a crucial role in the pathogenesis of autoimmune and malignant diseases.

The IL-1 gene cluster on chromosome 2q14.2 contains three related genes within a 430 kb region: IL-1α, IL-1β, and IL-*RN*, which encode the pro-inflammatory cytokines IL-1α, IL-1β as well as their endogenous receptor antagonist IL-1ra, respectively [5]. These cytokines are produced by a variety of cell types, including monocytes, macrophages, and epithelial cells and have multiple biological

effects [6]. The role of IL-1 in carcinogenesis has been investigated extensively. Experimental data support the crucial role for these cytokines as autocrine or paracrine stimuli in murine and human carcinogenesis [7-9].

In breast carcinoma, secreted IL-1α of tumor cell origin was shown to induce expression of prometastasic genes in the malignant cells (IL-6 and IL-8) and in stromal fibroblasts (IL-6, IL-8 and MMP-3), in an autocrine and paracrine manner, respectively [10]. IL-1β was found to combine with oestrogen receptor (ER)α, resulting in transcriptional activation in breast cancer cells [11]. The concentration of IL-1β was reported to be higher in invasive breast carcinoma tissue than in benign lesions [12].

Several polymorphisms have been reported for the three IL-1 members [13, 14]. Among those, IL1-*A (-889) C-T* and IL1-*B (+3954) C-T* are reported to be functional [14, 15]. The polymorphism *(-889) C-T* of IL-1α corresponds with altered IL-1α protein expression *in vivo* and *in vitro*, the presence of the *T* allele has been associated with increased IL-1α secretion [15, 16].

The polymorphism at position +3954 in exon 5 of the IL-1β gene, has been widely investigated for its effect on protein production. The presence of the *T* allele at this site has been associated with increased IL-1β secretion *in vitro* [14]. IL1-*B* polymorphisms have been reported to be associated with pancreatic cancer risk [17].

IL-6 is another pleiotropic pro-inflammatory cytokine that may be involved in the host response to cancer. Experimental findings suggest that IL-6 acts as a potent stimulator of metastasis by up-regulating the expression on endothelial cells of adhesion receptors, such as intercellular adhesion molecule-1 and leukocyte adhesion molecule-1, and also by stimulating the production of growth factors such as hepatocyte growth factor and vascular endothelial growth factor [18-20].

The status of common functional single *G>A* and *G>C* base exchange polymorphisms in the human IL-6 gene promoter (chromosome *7p21*) located respectively at 597 and 174, upstream from the start site of transcription (-597 *G>A* and -174 *G>C* loci), have been reported to influence IL-6 levels *in vitro* and *in vivo* [21-23]. The *G* alleles at (-597) or (-174) increases IL-6 expression, both in basal and stimulated conditions, the highest IL-6 levels in plasma and serum being found in subjects homozygous for the (-597 or -174) *G* allele [21, 22].

Results of subsequent studies of the association of IL-6 *(-174) G/C* polymorphism with a biological phenotype of breast cancer have been controversial. In the Australian population, the IL-6 *(-174) CC* genotype was associated with an aggressive form of breast cancer [24]. In a British study, the heterozygous genotype (*GC*) of this polymorphism was associated with markers of poor prognosis [25]. However, in the American population, the proposed associations have not been confirmed [26].

Based on the abundant evidence for the role of IL-1 and IL-6 in carcinogenesis and the known correlation between polymorphisms of the IL-1α, IL-1β and IL-6 and protein expression, we investigated the potential association of these polymorphisms with the susceptibility, clinicopathological characteristics and prognosis of breast carcinoma.

MATERIALS AND METHODS

Patients and controls

The gene and allele frequencies of the genes for IL-1α, IL-1β and IL-6 were determined in a group of 200, unrelated, control subjects and 305, unrelated patients with breast carcinoma. The control and patient groups were selected from the same population, living on the coast of Tunisia.

Clinical follow-up data were collected in the cohort of the 305 patients recruited from the department of Radiation Oncology and Medical Oncology of Sousse Hospital (Sousse, Tunisia) between 1991 and 2003.

All patients included in this study had primary breast carcinoma, with unilateral breast tumors. The patients (301 females and 4 males) had a mean age of 50 ± 24 years. The median of follow-up was 36 months (range, 1-120 months). At the time of analysis, 65 patients experienced recurrence (local or distant). Among them, 18 patients died from breast carcinoma (27.7%). A detailed description of the clinicopathological characteristics of this cohort has been reported elsewhere [27]. *Table 1* shows the treatment description of all patients.

Control subjects (191 females and 9 males) having a mean age of 46 ± 12 years, were healthy blood donors, having no evidence of any personal or family history of cancer (or other serious illnesses). Written informed consent was obtained from all subjects.

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes by a salting procedure [28]. Briefly, 10 mL of blood were mixed with triton lysis buffer (0.32M sucrose, 1% Triton X-100, 5mM $MgCl_2$, H_2O, 10mM Tris-HCl, PH 7.5). Leukocytes were spun down and washed with H_2O. The pellet was incubated with proteinase K at 56°C and subsequently salted out at 4°C using a substrate NaCl solution. Precipitated proteins were moved by centrifugation. The DNA in supernatant fluid was precipitated with ethanol. The DNA pellet was dissolved in 400 µl H_2O.

Table 1
Treatment description of the 305 patients with breast carcinoma

	Surgery	No surgery
Radiotherapy	33	16
Chemotherapy	23	17
Radiotherapy + Chemotherapy	133	25
Radiotherapy + endocrine therapy	14	0
Chemotherapy + endocrine therapy	6	6
Radiotherapy + Chemotherapy + endocrine therapy	32	0

Polymorphism analysis of the IL-1α gene

Based upon the method described by Tarra *et al*. [29], a polymerase chain reaction (PCR), followed by digestion with the endonuclease *Nco*I was used to detect the *C* to *T* transition polymorphism at position -889 of IL-1α gene. Two sequence specific oligonucleotide primers were used for the PCR: the 3'-primer (5'- GGGGGCTTCACTATGT-TGCCCACACTGGACTAA - 3') was used with the 5'-primer (5'- GAAGGCATGGATTTTTACATATGACCT-TCCATG - 3'). Thirty microliters of PCR reaction mixture comprised genomic DNA samples (100ng), 200 µmol/L dNTPs, 1.5 mM $MgCl_2$, 1x Taq polymerase buffer, 50 pmol of each primer, and 0.5 unit of Taq DNA polymerase (Amersham, Paris, France). Reaction conditions used with the thermal cycler (Biometra, GÖttingem, Germany) were as follows: an initial incubation at 94°C for 4 minutes, followed by 30 cycles of incubation at 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds and followed by a final incubation at 72°C for 5 minutes. The amplified fragments (300 bp) were digested with *Nco*I, fragments were analysed by agarose-gel electrophoresis. The *T* allele remained uncut, while the *C* allele was cut into 2 fragments of 280 and 20 bp.

Polymorphism analysis of the IL-1β gene

A 249 bp fragment of the fifth exon encoding IL-1β (position +3816 to 4066) was amplified using the following specific oligonucleotide primers: the 5' primer: 5'-GTTGTCATCAGACTTTGACC-3' was used in combination with the 3' primer: 5'- TTCAGTTCATATGGAC-CAGA -3' in a 25 µL reaction mixture containing genomic DNA samples (100ng), 200 µmol/L dNTPs, 1.5 mM $MgCl_2$, 1x Taq polymerase buffer, 50 pmol of each primer, and 0.5 unit of Taq DNA polymerase (Amersham, France). Amplification was accomplished by initial incubation at 96°C for 5 minutes followed by 3 cycles of 96°C for 90 seconds, 53°C for 90 seconds; 35 cycles of 96°C for 60 seconds, 53°C for 60 seconds, 72°C for 60 seconds respectively, and a final extension at 72°C for 10 minutes.
To assess the polymorphism of the IL-1β at position 3954, the corresponding PCR products were digested with *Taq*I. The presence of a *Taq*I site, which corresponds to the *C* allele, was indicated by the cleavage of the 249 bp amplified product to yield fragments of 135 and 114 bp. The *T* allele corresponds to the uncut fragment.

Polymorphism analysis of the IL-6 gene

The two polymorphic sites (-174 and -597) of the promoter of IL-6 gene were analyzed. A 198 bp fragment covering the (-174) polymorphic site of the IL-6 gene was amplified using the following primers: 5'- TGACTTCAGCTTTACT CTTTGT - 3' and 5'- CTGATTGGAAACCTTATTAAG - 3' in a 20 µl reaction mixture containing genomic DNA samples (100ng), 200 µmol/L dNTPs, 1.5 mM $MgCl_2$, 1x Taq polymerase buffer, 0.5 µmol/L of each primer, and 0.5 unit of *Taq* DNA polymerase (Amersham, France). PCR amplification were performed with an initial denaturation temperature of 94°C for 10 minutes, followed by 35 cycles of 96°C for 60 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, and a final extension at 72°C for 10 minutes.
PCR products were digested with *Nla*III restriction enzyme at 37°C overnight and electrophoresed on a 2% agarose gel. *Nla*III RFLP was detected by ethidium bromide staining. The presence of an *Nla*III site was indicated by the cleavage of the 198 bp amplified product to yield 140 bp and 58 bp. The *G* allele corresponds to the uncut fragment and the *C* allele to the presence of *Nla*III site.
The analysis of the (-597 G/A) polymorphism of the IL-6 gene was performed using the following primers 5'-GGAGACGCCTTGAAGTAACTGC - 3' and 5'-GAGTTT CCTCTGACTCCATC - 3' to generate a PCR fragment of 163 bp. Genotyping was resolved by PCR product digestion with the *Fok*I enzyme. The rare IL-6 *(-597) A* allele has a *Fok*I restriction cutting site. The resulting fragments have the size of 116 and 47 bp.
To assess genotyping reliability PCR products for the IL-1α, IL-1β and IL-6 polymorphisms were analyzed by direct sequencing.

Statistical analyses

The allele frequencies of IL-1α, IL-1β or IL-6 were tested for the Hardy-Weinberg equilibrium for both patient and control groups using the Chi-Square test. The same test was used to evaluate for significant association between disease (breast carcinoma against controls) and IL-1α, IL-1β or IL-6 genotypes. Relative risk of breast carcinoma associated with a particular genotype was estimated by the odds ratio (OR).
Disease-free survival (DFS) was defined as the time from the date of diagnosis to the first local or distant recurrence or to last contact. Breast carcinoma-specific overall survival (OVS) was defined as the time from the date of diagnosis to death if the patient died from breast carcinoma or to last contact. Six-year survival rates were estimated, and survival curves were plotted according to Kaplan and Meier [30]. Differences between groups were calculated by the log rank test [31]. Clinicopathological parameters were dichotomized as follows: nodal status (≥1 *versus* no positive lymph node), SBR (Scarff, Bloom and Richardson) tumor grade (1-2 *versus* 3), clinical tumor size (T_1-T_2 *versus* T_3-T_4). Statistics were performed using SEM-STATISTIQUES software (Centre Jean Perrin, Clermont-Ferrand, France).

RESULTS

Polymorphisms in the IL-1α, IL-1β and IL-6 genes as risk factors for breast carcinoma

Table 2 shows genotype frequencies for IL-1α, IL-1β and IL-6 in patients with breast carcinoma and in the control group. The genotype distributions of IL-1α, IL-1β and IL-6 genes were in Hardy-Weinberg equilibrium for both patient and control groups.
No significant differences in IL-1α genotype distribution were seen between the patients and controls. The frequency of the IL-1β *(+3954) TT* homozygous genotype was higher in the patient group than in the control subjects, but the difference reached only borderline significance (0.111 *versus* 0.070, OR = 1.86, p = 0.06).
A significant increase in the frequency of the IL-6 *(-597) GA* heterozygous genotype was observed in the patient

Table 2
IL-1α, IL-1β and IL-6 genotypes frequencies in control subjects and in patients with breast carcinoma

Genotype	Patients n = 305		Controls n = 200		OR	Confidence interval	p-value
	n	*f*	*n*	*f*			
(-889) IL-1 α							
C/C	116	(0.380)	83	(0.415)	1		
C/T	132	(0.433)	82	(0.410)	1.15	[0.76-1.74]	NS
T/T	57	(0.187)	35	(0.175)	1.17	[0.68-2]	NS
(+3954) IL-1β							
C/C	157	(0.515)	120	(0.60)	1		
C/T	114	(0.374)	66	(0.330)	1.32	[0.88-1.98]	NS
T/T	34	(0.111)	14	(0.070)	1.86	[0.91-3.82]	0.06
(-597) IL-6							
G/G	197	(0.646)	149	(0.745)	1		
G/C	99	(0.324)	47	(0.235)	1.59	[1.04-2.44]	0.024
C/C	09	(0.030)	04	(0.020)	1.70	[0.47-6.70]	NS
(-174) IL-6							
G/G	199	(0.653)	150	(0.750)	1		
G/C	98	(0.321)	46	(0.230)	1.61	[1.05-2.47]	0.022
C/C	08	(0.026)	04	(0.020)	1.51	[0.40-6.07]	NS

The $\chi 2$ test used to determine whether significant differences (p-value) were observed when the patient group was compared with the control group; NS: not significant.

group compared to the control group (0.324 *versus* 0.235, OR = 1.59, p = 0.024).
The frequency of the IL-6 *(-174) GC* heterozygous genotype was also higher in patients than in controls (0.321 *versus* 0.230, OR = 1.61, p = 0.022). In agreement with a previous report [32], the two polymorphisms were found in tight linkage disequilibrium.

Prognostic significance of polymorphisms in the IL-1α, IL-1β and IL-6 genes

Table 3 shows the distributions of IL-1α, IL-1β and IL-6 polymorphisms according to the clinicopathological indices of breast carcinoma severity. The *T (+3954)* allele of the IL-1β gene was found in 63.75% of patients with large tumors (T3-T4). The same allele was found in 66.66% of patients having tumors with high SBR tumor grade (grade 3). In addition, the *T (+3954)* allele was found in 61.11% of patients with lymph node metastases. Taken together, these results suggest that the *T (+3954)* allele of the IL-1β gene is highly associated with the aggressive forms of breast carcinoma.
Figure 1 shows breast carcinoma-specific overall survival (OVS) and disease-free survival (DFS) in patients according to the presence or absence of the IL-1α *(-889) TT* homozygous genotype. The estimated 3- and 6-year breast carcinoma-specific OVS rate in the group of patients carrying or not carrying the IL-1α *(-889) TT* homozygous genotype was, respectively, 75% and 36.8% *versus* 94.9% and 70.6% (log rank test, $p < 0.01$). The 3-year DFS rate in the group of patients with IL-1α *(-889) TT* genotype was 80.9% and 98.5% in the group of patients without IL-1α *(-889) TT* genotype (log rank test, $p < 10^{-5}$).
A remarkably strong association between DFS and IL-1β *(+3954) TT* genotype was found in all the 305 patients *(figure 2)*. The estimated 3-year DFS rate in the group of patients with IL-1β *(+3954) TT* genotype was 79.4% and 98.5% in the group of patients without the IL-1β *(+3954) TT* marker (log rank test, $p < 10^{-3}$). No statistical difference in the OVS was observed between the two groups of patients.
As shown in *figure 3*, the breast carcinoma-specific DFS was significantly shorter in the group of patient with the IL-6 *(-597) GG* and IL-6 *(-174) GG* genotypes. The estimated 3-year DFS rate in the groups of patients with *(-597) GG* or *(-174) GG* genotypes was 92% and 99% in that of patients without IL-6 *(-597) GG* or *(-174) GG* markers (log rank test, $p < 0.02$). No association was found between IL-6 *(-597) GG* and IL-6 *(-174) GG* genotypes and the OVS rate in this population of patients with breast carcinoma (data not shown).
Multivariate analyses were undertaken to evaluate the importance of IL-1 and IL-6 markers in the risk of the recurrence and death compared with the clinicopathological parameters. Introducing the genetic and clinicopathological parameters bearing prognostic significance tested the Cox model. No genetic or clinicopathological parameters were selected for OVS and DFS.

DISCUSSION

Given the important role of IL-1 and IL-6 in cancer pathogenesis, IL-1α, IL-1β and IL-6 can be regarded as candidate genes for cancer.
The present case-controlled study revealed no significant differences in IL-1α and IL-1β genotype distributions between patients with breast carcinoma and the control subjects. These results suggest that these genetic variations in IL-1α and *-B* are unlikely to play an important role in the genetic predisposition to breast carcinoma. In the Tunisian population, the frequency of IL-1α *(-889) TT* genotype (17.5%) was higher compared to Italian (2.8%) and the American (11.8%) populations [33].
The assessment of the prognostic value of the genetic markers IL-1α and *–B* in breast carcinoma indicated that the IL-1α *(-889) TT* homozygous genotype is associated with reduced overall and disease-free survival, and therefore with a poor prognosis in breast carcinoma.

Table 3
Genotype frequencies of IL-1α, IL-1β and IL-6 polymorphisms in relation to pathological indices of breast cancer severity

	n (%)		p value
IL-1α-889 polymorphism	C/C	C/T +T/T	
Tumor size			
T1-T2	72 (38.1)	117 (61.9)	0.83
T3-T4	29 (36.7)	50 (63.3)	
Histological grade			
1-2	62 (36.26)	109 (63.74)	0.9
3	30 (37.03)	51 (62.97)	
Lymph node metastases			
Negative	59 (41.85)	82 (58.15)	0.19
Positive	56 (34.57)	106 (65.43)	
IL-1β+3954 polymorphism	**C/C**	**C/T +T/T**	
Tumor size			
T1-T2	111 (58.73)	78 (41.27)	**0.0007**
T3-T4	29 (36.25)	51 (63.75)	
Histological grade			
1-2	99 (57.9)	72 (42.10)	**0.0002**
3	27 (33.33)	54 (66.66)	
Lymph node metastases			
Negative	93 (65.96)	48 (34.04)	**0.000002**
Positive	63 (38.89)	99 (61.11)	
IL-6 -597 polymorphism	**G/G**	**G/A +A/A**	
Tumor size			
T1-T2	117 (61.9)	72 (38.1)	0.18
T3-T4	55 (70.51)	23 (29.49)	
Histological grade			
1-2	110 (63.95)	62 (36.05)	0.45
3	55 (68.75)	25 (31.25)	
Lymph node metastases			
Negative	93 (65.49)	49 (34.51)	0.75
Positive	102 (63.75)	58 (36.25)	
IL-6 -174 polymorphism	**G/G**	**G/C+C/C**	
Tumor size			
T1-T2	117 (62.23)	71 (37.77)	0.07
T3-T4	58 (73.41)	21 (26.59)	
Histological grade			
1-2	111 (64.91)	60 (35.09)	0.31
3	57 (71.25)	23 (28.75)	
Lymph node metastases			
Negative	91 (64.54)	50 (35.46)	0.81
Positive	106 (65.84)	55 (34.16)	

The IL-1β *(+3954) TT* homozygous genotype was specifically associated with reduced DFS but not with OVS. More interestingly, we showed that the *(+3954) T* allele was highly associated with aggressive forms of breast carcinoma as defined by large tumor size, high grade and lymph node metastases.

Several studies have shown that IL-1β promotes invasiveness, including tumor angiogenesis and also induces immune suppression in the host [34]. Moreover, IL-1β is expressed in 90% of invasive breast carcinoma and to lesser extent in ductal, *in situ* breast carcinoma and benign lesions [12]. In advanced breast carcinomas, high IL-1β correlated well with other parameters of aggressive tumors (high tumor grade, presence of p53, absence of bcl2), and expression of other pro-inflammatory cytokines such as IL-8 [12, 35].

Most studies indicate that IL-1α production by tumor cells increases invasiveness [36-38]; only rarely were anti-tumor effects observed [39]. In breast cancer cell lines and in malignant breast tumors, expression of IL-1α is associated with both a more malignant phenotype and ERα negativity [37]. Additionally, it has been shown that IL-α promotes tumor growth and cachexia in the MCF-7 xenograft model of breast cancer [38].

Our findings, which show the strong association between IL-1α and *-B* gene polymorphisms and the poor prognosis in breast carcinoma, along with those showing that these genetic variations in the IL-1 genes are functionally important elements influencing IL-1 production [10-12], suggest that the genetic basis of the potential tumor promoter role attributed to IL-1 may result from IL-1α and *-B* polymorphisms. Therefore, these polymorphisms could be causal, predisposing to a poor prognosis in breast carcinoma.

The genotype frequencies of IL-6 determined for the current cohort indicated a significant increase of IL-6 *(-597) GA* and – *(-174) GC* heterozygous genotypes in patients

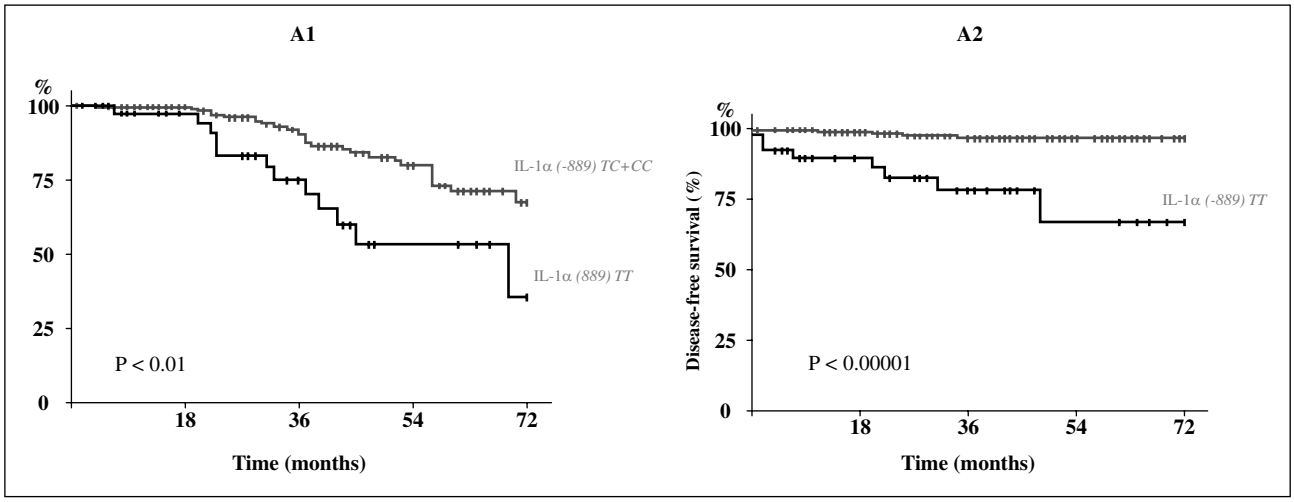

Figure 1
Breast carcinoma specific overall survival (**A**) and specific disease-free survival (**B**) of 305 breast carcinoma patients according to the presence or absence of IL-1α *(-889) TT* homozygous genotypes (p denotes the log-rank test value).

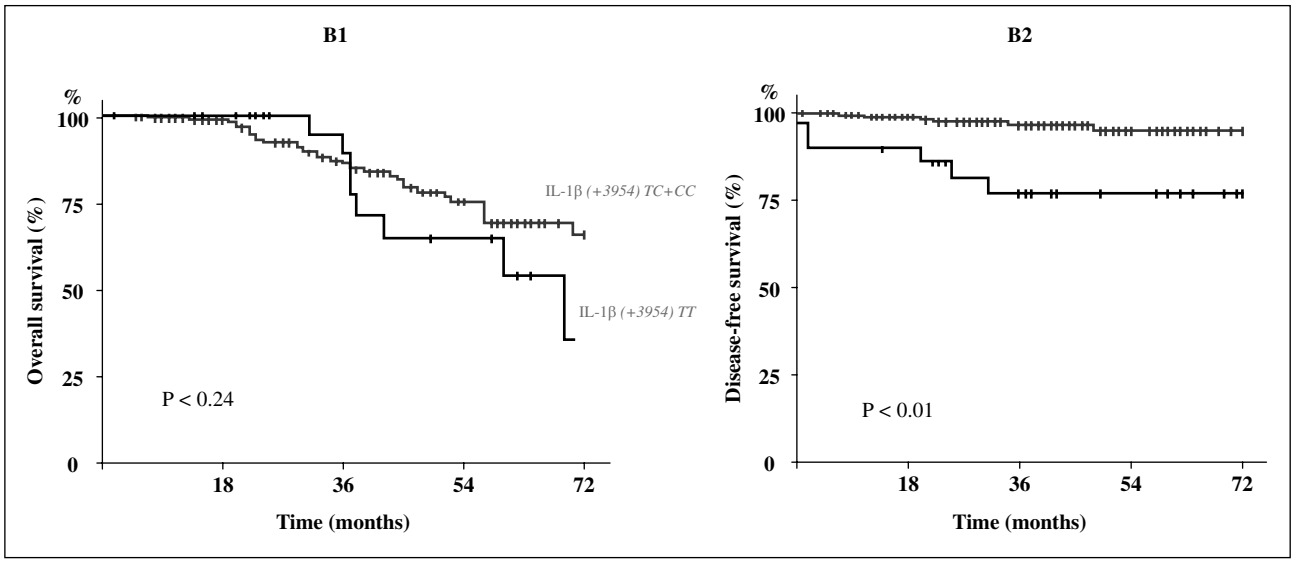

Figure 2
Breast carcinoma specific overall survival (**A**) and specific disease-free survival (**B**) of 305 breast carcinoma patients according to the presence or absence of IL-1β *(+3954) TT* homozygous genotypes (p denotes the log-rank test value).

with breast carcinoma compared to controls. The frequency of the IL-6 *(-597) A* and *(-174) C* alleles was higher in the patient group compared to the controls, but the differences did not reach statistical significance. It has been reported that the polymorphisms of cytokine genes differ considerably among different populations, particularly polymorphisms in the IL-6 gene. In the Tunisian population, we showed that the frequencies of the IL-6 *(-597) A* and IL-6 *(-174) C* alleles are lower compared to other populations: (13% versus 35%). Our data provided evidence of an association between the IL-6 *(-597) GA* and IL-6 *(-174) GC* genotypes and an increased risk of breast cancer in the Tunisian population, but not with the IL-6 *(-597) A* or IL-6 *(-174) C* alleles; this was probably because of the low number of IL-6 *(-597) AA* and IL-*6 (-174) CC* patients in the Tunisian population.

IL-6 is a pleiotropic cytokine that has been shown to be important in the activation of the host anti-tumor response. *In vitro* studies have shown that IL-6 acts as a late-acting killer factor (KHF) in the differentiation of cytotoxic T-lymphocytes, and it augments the activities of NK-cells [40]. Low expression of IL-6 can affect the immune system.

Several studies have shown that the IL-6 *(-597) G-A* and *(-174) G-C* SNPs are functional alterations that affect serum levels of IL-6 [21, 32]. Recent data from Belluco *et al.* demonstrate that circulating IL-6 levels are significantly higher in IL-6 *(-174) GG* homozygotes with colon cancer compared with carriers of a *C* allele [40]. Thus, we hypothesized that carriage of the mutant IL-6 allele influences the genetic susceptibility to breast carcinoma.

More interestingly, we showed that the IL-6 *GG* genotype homozygous for both positions, known to be genetic factors dictating the over-production of IL-6, are associated with reduced disease-free survival. Recently it has been reported that the IL-6 *(-174) G* allele is significantly associated with increased risk of recurrence compared with those of the *C* allele in patients with high risk, node-

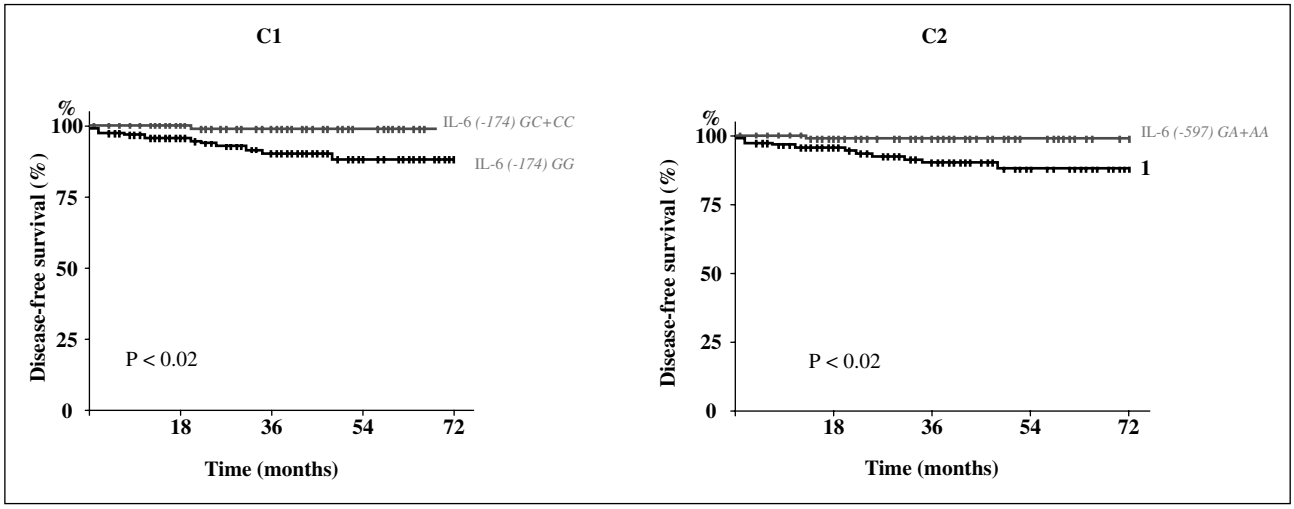

Figure 3
Breast carcinoma specific disease-free survival of 305 breast carcinoma patients according to the presence or absence of IL-6 *(-174) GG* (**A**) and IL-6 *(-597) GG* (**B**) homozygous genotypes (p denotes the log-rank test value).

positive breast cancer [26]. This result is in agreement with our findings.

Several reports highlighted the role of IL-6 in cancer pathogenesis and disease progression. It has been shown that in different tumor types, a high IL-6 serum level is associated with advanced stage disease [41-44] and a worse outcome [41, 42, 45, 46]. Regarding breast carcinoma, it has been reported that a high IL-6 serum level is associated with poor response to cancer therapy and reduced survival [47-49].

The results of the current study, which show the association between the IL-6 polymorphisms and both susceptibility and disease-free survival associated with breast carcinoma, suggest the genetic basis for the various roles of IL-6 in tumor development, and clinical outcome may result from IL-6 polymorphisms.

In conclusion, this study suggests that variation in genes for IL-1 and IL-6 represent attractive factors for the prognosis of breast carcinoma. The role of the pro-inflammatory cytokines as genetic markers of breast cancer can be complemented with other SNPs and haplotype analysis. Extension of the findings of the current study to other malignant tumors will be of use in determining whether these genetic markers are specific to breast carcinoma.

Acknowledgements. *We gratefully acknowledge the technical assistance of S. Gabbouj. We thank the staff of the Departments of Radiation Oncology and Medical Oncology of CHU F. Hached, Sousse, for providing samples and clinical information.*

REFERENCES

1. Greene MH. Genetics of breast cancer. *Mayo Clin Proc* 1997 ; 72 : 54.
2. Dunning AM, Healey CS, Pharoah PD, *et al.* A systematic review of genetic polymorphisms and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* 1997 ; 8 : 843.
3. Coughlin SS, Piper M. Genetic polymorphisms and risk of breast cancer. *Cancer Epidemiol Biomarkers Prev* 1999 ; 8 : 1023.
4. Weber BL, Nathanson KL. Low penetrance genes associated increased risk for breast cancer. *Eur J Cancer* 2000 ; 36 : 1193.
5. Taylor SL, Renshaw BR, Garka KE, *et al.* Genomics organization of the interleukin-1 locus. *Genomics* 2002 ; 79 : 726.
6. Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood* 1996 ; 87 : 2095.
7. Apte RN, Douvdevani A, Zoller M, White RM, Dvorkin T, Shimoni N, Fima E, Hachamj M, Huleilhel M, Benharroch D. Cytokine-induced tumor immunogenecity : endogenous interleukin-1 alpha expressed by fibrosarcoma cells confers reduced tumorigenecity. *Immunol Lett* 1993 ; 39 : 45.
8. Castrilli G, Tatone D, Diodoro MG, Rosini S, Piantelli M, Musiani P. Interleukin-1α and interleukin-6 promote the in vitro growth of both normal and neoplastic human cervical epithelial cells. *Br J Cancer* 1997 ; 75 : 855.
9. Glaccum MB, Stocking KL, Charrier K, Smith JL, Willis CR, Maliszewski C, Livingston DJ, Peschon JJ, Morrisey PJ. Phenotypic and functional characterization of mice that lack the type I receptor for IL-1. *J Immunol* 1997 ; 159 : 3364.
10. Nozaki S, Sledge GWJR, Nakshatris H. Cancer cell-derived interleukin 1 α contributes to autocrine and paracrine induction of pro-metastatic genes in breast cancer. *Biochem Biophys Res Commun* 2000 ; 275 : 60.
11. Speirs V, Kerin MJ, Newton CJ, Walton DS, Green AR, Desai SB. Evidence for transcriptional activation of ERα by IL-1β in breast cancer cells. *Int J Oncol* 1999 ; 15 : 1251.
12. Jin L, Yuan RQ, Fuchs A, Yao Y, Joseph A, Schwall R. Expression of interleukin-1β in human breast carcinoma. *Cancer* 1997 ; 80 : 421.
13. Bailly S, di Giovine FS, Blakemore AIF, Duff GW. Genetic polymorphism of human Interleukin1 α. *Eur J Immunol* 1993 ; 23 : 1240.
14. Pociot F, Molvig J, Wogensen L, Worsaae H, Neup JA. TaqI polymorphism in the human interleukin1-beta (IL-1βeta) gene correlates with IL-1 beta secretion in vitro. *Eur J Clin Invest* 1992 ; 22 : 396.
15. Shirodaria S, Smith J, McKay IJ, Kennett CN, Hughes FJ. Polymorphisms in the IL-1α gene are correlated with levels of interleukin-1 alpha protein in gingival crevicular fluid of teeth with severe periodontal disease. *J Dent Res* 2000 ; 79 : 1864.
16. Dominici R, Cattaneo M, Malferrari G, Archi D, Mariani C, Grimaldi LM. Cloning and functional analysis of the allelic polymorphism in the transcription regulatory region of interleukin-1 alpha. *Immunogenetics* 2002 ; 54 : 82.
17. Barber MD, Powell JJ, Lynch SF, Fearson KC, Ross JA. A polymorphism of interleukin-1 beta gene influences survival in pancreatic cancer. *Br J Cancer* 2000 ; 83 : 1443.

18. Hutchins D, Steel CM. Regulation of ICAM-1 (CD54) expression in human breast cancer cell lines by interleukin 6 and fibroblast-derived factors. *Int J Cancer* 1994 ; 58 : 80.

19. De Jong KP, van Gameren M, Bijzet E, Limburg PC, Sluiter WJ, Slooff JH, de Vries EGE. Recombinant human interleukin-6 induces hepatocyte growth factor production in cancer patients. *Scand J Gastroentero* 2001 ; 36 : 636.

20. Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ. Interleukin-6 induces expression of vascular endothelial growth factor. *J Biol Chem* 1996 ; 271 : 736.

21. Gemma V, José L, San M, José S, Héctor F. The -597 G/A and -174 G/C Polymorphisms in the Promoter of the IL-6 Are Associated with Hyperandrogenism. *J Clin Endocrinol Metab* 2002 ; 87 : 1134.

22. Fishman D, Faulds G, Jeffery R, Mohammed-Ali V, Yudkin JS, Humphries S, Woo P. The effect of novel polymorphism in the interleukin-6 (IL-6) gene on IL-6 transcription and serum levels, and association with systemic-onset juvenile chronic arthritis. *J Clin Investig* 1998 ; 102 : 1369.

23. Bonafe M, Olivieri F, Cavallone L, Giovagnetti S, Marchegiani F, Cardelli M, Pieri C, Marra M, Antonicelli R, Lisa R, Rizzo MR, Paolisso G, Monti D, Franceshi CA. Gender-dependent genetic predisposition to produce high levels of IL-6 is determinal for longevity. *Eur J Immunol* 2001 ; 31 : 2357.

24. Iacopetta B, Grieu F, Joseph D. The -174 G/C gene polymorphism in interleukin-6 is associated with aggressive breast cancer phenotype. *Br J Cancer* 2004 ; 90 : 419.

25. Smith KC, Bateman AC, Fussell HM, Howell WM. Cytokine gene polymorphisms and breast cancer susceptibility and prognosis. *Eur J Immunogent* 2004 ; 31 : 167.

26. DeMichele A, Martin AM, Mick R, Gor P, Wray L, Klein-Carbal M, Athanasiadis G, Collingan T, Stadtmauer E, Weber B. Interleukin-6 -174 G→C Polymorphism Is Associated with Improved Outcome in High risk Breast Cancer. *Cancer Res* 2003 ; 63 : 8051.

27. Ben Ahmed S, Aloulou S, Bibi M, Landolsi A, Nouira M, Ben Fatma L, Kallel L, Gharbi O, Khairi H, Kraiem C. Breast cancer detection in Tunisian women : an analysis of a hospital trial involving 729 patients. *Sante Publique (Bucur)* 2002 ; 14 : 231.

28. Olerup O, Zetterquiest H. HLA-DR typing by PCR amplification with sequence specific primers (PCR-SSP) in two hours : an alternative to serological DR typing in clinical practice including donor recipient matching in cadaveric transplantation. *Tissue Antigens* 1992 ; 39 : 225.

29. Tarra L, Mcdowell JA, Symons RP, Øystein F, Gordon WD. A Genetic Association between Juvenile Rheumatoid Arthritis and a Novel Interleukin-1 α Polymorphism. *Arthritis Rheum* 1995 ; 38 : 221.

30. Kaplan EL, Meir PO. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958 ; 53 : 457.

31. Peto R, Pike MC, Armitage P, Breslow NE, Cox DR, Howard SV, Mantel N, McPherson K, Peto J, Smith PG. Design and analysis of randomised clinical trials requiring prolonged observation of each patient. *Br J Cancer* 1977 ; 35 : 1.

32. Brull DJ, Montgomery J, Sanders S, Dhamrait L, Luong L, Rumley A, Lowe GDO, Humphries S. Interleukin-6 Gene -174G>C and -572 G>C Promoter Polymorphisms are strong predictors of plasma interleukin-6 levels after coronary artery bypass surgery. *Arterioscler Thomb Vasc* 2001 ; 21 : 1458 ; (Biol).

33. Seripa D, Matera MG, Dal Forno G, Gravina C, Masullo C, Daniele A, Binetti G, Bonvicini C, Squitti R, Palermo MT, Davis DG, Antuono P, Wekstein DR, Dobrina A, Gennarelli M, Fazio VM. Genotypes and haplotypes in the IL-1 gene cluster : analysis of two genetically and diagnostically distinct groups of Alzheimer patients. *Neurobiol Aging* 2005 ; 26 : 455.

34. Song X, Vornov E, Dvorkin T, Fima E, Cagnano E, Benharroch D, Shendler Y, Bjorkdahl O, Segal S, Dinarello CC, Apte RN. Differential effects of IL-1 alpha and IL-1 beta on tumorigenecity patterns and invasiveness. *J Immunol* 2003 ; 15 : 6448.

35. Miller LJ, Kurtzman SH, Anderson K, Wang Y, Stankus M, Renna M, Linfquist R, Barrows J, Kreutzer DL. Interleukin-1 family expression in human breast cancer : interleukin-1 receptor antagonist. *Cancer Invest* 2000 ; 18 : 293.

36. Furuya Y, Ichikura T, Mochizuki H. Interleukin-1 alpha concentration in tumors as a risk factor for liver metastasis in gastric cancer. *Surg Today* 1999 ; 29 : 288.

37. Singer C, Kronsteiner N, Hudelist G, Marton E, Walter I, Kubista M, Czerwenka K, Shreiber M, Seifert M, Kubista E. Interleukin-1 system and sex steroid receptor expression in human breast cancer : interleukin-1α protein secretion is correlated with malignant phenotype. *Clin Cancer Res* 2003 ; 9 : 4877.

38. Kumar S, Kishimoto H, Chua HL, Badve S, Miller KD, Bigsby RM, Nakshatri H. Interleukin-1 alpha promotes tumor growth and cachexia in MCF-7 xenograft model of breast cancer. *Am J Pathol* 2003 ; 163 : 2531.

39. Naume B, Espevik T. Immunoregulatory effects of cytokines on natural killer cells. *Scand J Immunol* 1994 ; 40 : 128.

40. Belluco C, Olivieri F, Bonafe M, Giovagnetti S, Mammano E, Scalerta R, Ambrosi A, Franceschi C, Nitti D, Lise M. 174 G>C polymorphism of interleukin 6 gene promoter affects interleukin 6 serum level in patients with colorectal cancer. *Clin Cancer Res* 2003 ; 9 : 2173.

41. Zhang GJ, Adachi I. Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. *Anticancer Res* 1999 ; 19 : 1427.

42. Shariat SF, Andrews B, Kattan MW, Kim J, Wheeler TM, Slawin KM. Plasma levels of interleukin-6 and its soluble receptor are associated with prostate cancer progression and metastasis. *Urology* 2001 ; 58 : 1008.

43. Oka M, Yamamoto K, Takahashi M, Hakozaki M, Abe T, Izuka N, Hazama S, Hirazawa K, Hayashi H, Tangoku A, Hirose K, Ishihara T, Suzuki T. Relationship between serum levels of interleukin-6, various disease parameters, and malnutrition in patients with esophageal squamous cell carcinoma. *Cancer Res* 2001 ; 19 : 1457.

44. Mouawad R, Benhammouda A, Rixe O, Antoine EC, Borel C, Weil M, Khayat D, Soubrane C. Endogenous interleukin-6 levels in patients with metastatic malignant melanoma : correlation with tumor burden. *Clin Cancer Res* 1996 ; 2 : 1405.

45. Berek JS, Chung C, Kaldi K, *et al.* Serum interleukin-6 levels correlate with disease status in patients with epithelial ovarian cancer. *Am J Obestet Gynecol* 1991 ; 164 : 1038.

46. Wu CW, Wang SR, Caho MF, Watson JM, Knox RM, Martinez-Maza O. Serum interleukin-6 levels reflect disease status of gastric cancer. *Am J Gastroenterol* 1996 ; 91 : 1417.

47. Hakan B, Gulcan U, Mustafa S, Mustafa Y, Tomris O, Mehmet A, Hasan A, Ilhan A, Burhan S. Tumour necrosis factor- alpha, interleukin-6, and fasting serum insulin correlate with clinical outcome in metastatic breast cancer patients treated with chemotherapy. *Cytokine* 2004 ; 27 : 58.

48. Yokoe T, Iino Y, Morishita Y. Trends of IL-6 and IL-8 levels in patients with recurrent breast cancer : preliminary report. *Breast Cancer* 2000 ; 7 : 187.

49. Nishimura R, Nagao K, Miyayama H, Mastsuda M, Baba K, Mastsuoka Y, Yamashita H, Fukuda M, Mizumoto T, Hamamoto R. An analysis of serum interleukin-6 levels to predict benefits of medroxyprogesterone acetate in advanced or recurrent breast cancer. *Oncology* 2000 ; 59 : 166.