

INTERLEUKIN-6 POLYMORPHISM IS ASSOCIATED WITH MORE AGGRESSIVE PROSTATE CANCER

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ABSTRACT

Purpose: Interleukin-6 (IL-6) has an important role during prostate cancer progression and IL-6 levels in the serum of patients with hormone refractory and metastatic prostate cancer are significantly increased compared with those in patients with hormone sensitive and localized prostate cancer. The G>C polymorphism at position –174 in the promoter of the *IL-6* gene has been associated with differences in IL-6 transcription in vitro and IL-6 protein levels in vivo. We determined the association of IL-6 polymorphism with prostate cancer progression.

Materials and Methods: We examined the association of IL-6 polymorphism with the risk of advanced disease in 95 patients with different stages of prostate cancer using the tetra-primer polymerase chain reaction genotyping method.

Results: We found that the –174G>C genotype of *IL-6* gene was associated with an overall increased risk of advanced prostate cancer. A strong association between this genotype and Gleason score was observed at the –174G>C locus of the *IL-6* gene ($p < 0.001$). The distribution of this genotype was also significantly different between stages T3-T4 and T1-T2 tumors ($p < 0.001$). In addition, the *IL-6* genotype was linked with vascular invasion ($p = 0.024$), seminal vesicle involvement ($p = 0.006$) and capsular invasion ($p < 0.001$). Furthermore, the –174G>C genotype of the *IL-6* gene was significantly associated with increased serum prostate specific antigen ($p = 0.004$) and with recurrent prostate cancer compared with GG homozygotes ($p = 0.027$).

Conclusions: These data demonstrate a strong association of the –174G>C polymorphism of the *IL-6* gene with the aggressiveness and recurrence of prostate cancer, suggesting that genetic predisposition of genetic differences in the human *IL-6* gene could be linked to the risk of recurrent prostate cancer.

KEY WORDS: prostate; prostatic neoplasms; neoplasm recurrence; interleukin-6; polymorphism, genetic

Interleukin-6 (IL-6) is a glycoprotein consisting of 212 amino acids encoded by the *IL-6* gene localized to chromosome 7p21–14.¹ IL-6 is a pleiotropic cytokine that has a central role in host defense mechanisms by regulating immune responses, hematopoiesis and the induction of acute phase reaction.¹ IL-6 has been implicated in the modulation of growth and differentiation in many malignant tumors and it is associated with poor prognosis in several solid and hematopoietic neoplasms, such as renal cell carcinoma, ovarian cancer, lymphoma and melanoma.¹ The expression of IL-6 and its receptor has been consistently demonstrated, not only in human prostate cancer cell lines, but more importantly in human prostate carcinoma and benign prostate hyperplasia, while tissue specimens from patients with organ confined prostate cancer have higher IL-6 and IL-6 receptor levels.^{2,3} Multiple studies have demonstrated that IL-6 is increased in the serum of patients with metastatic prostate cancer and IL-6 levels correlate with tumor burden as well as with

serum prostate specific antigen (PSA) or clinical evident metastases.^{4,5} In addition, serum IL-6 levels are increased in men with hormone refractory prostate cancer compared with levels in normal controls, and patients with benign prostatic hyperplasia, prostatitis and localized or recurrent disease.⁴ Increased IL-6 levels in prostate cancer could be due to dysregulation of the AP-1 and nuclear factor- κ B pathways.^{6,7} Together these clinical data suggest that increased IL-6 levels are associated with prostate cancer progression to an androgen independent phenotype.

In addition to the clinical data that IL-6 is associated with androgen independent prostate cancer, experimental studies demonstrate that IL-6 has a critical role in prostate cancer cell growth and differentiation. Okamoto et al reported that IL-6 functions as a paracrine growth factor for human LNCaP androgen sensitive prostate cancer cells and as an autocrine growth factor for human DU145 and PC3 androgen insensitive prostate cancer cells.⁸ It has also been indicated that IL-6 can mediate LNCaP cell growth arrest and the induction of neuroendocrine differentiation.⁹ IL-6 activates androgen receptor mediated gene expression by androgen receptor activation through a Stat3 pathway in LNCaP cells.^{10,11} Further studies demonstrated that IL-6 over expression enhances PSA mRNA expression in LNCaP cells, can partially rescue LNCaP cells from growth arrest induced by androgen deprivation and induces androgen independent growth in vivo.¹² Together these findings suggest that IL-6 can regulate the expression of androgen responsive genes in

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an androgen independent manner and it induces the androgen independent growth of androgen dependent human prostate cancer cells.

There are several common single nucleotide polymorphisms (SNPs) that have been identified in the *IL-6* gene promoter, namely -597G>A, -572G>C and -174G>C.¹³ Although the functional effect of these polymorphisms is incompletely understood, the G>C polymorphism at position -174 in the promoter of the *IL-6* gene has been found to directly affect the *IL-6* transcription rate in vitro and *IL-6* levels in vivo.¹⁴ Additional studies suggest that -174G>C promoter polymorphism is associated with the risk several diseases, including osteoporosis,¹⁵ systemic lupus erythematosus¹⁶ and ovarian cancer.¹⁷

Since *IL-6* levels in serum are highly increased in patients with advanced prostate cancer and the -174G>C polymorphism of the *IL-6* promoter actually causes variations in the transcription and expression of *IL-6* in vivo, we hypothesized that this polymorphism might affect individual susceptibility to advanced prostate cancer. We evaluated the association of the -174G>C polymorphism of the *IL-6* promoter with the risk of recurrent prostate cancer.

MATERIALS AND METHODS

Study population. This study is a retrospective analysis of patients diagnosed with prostate carcinoma at our institution between 1992 and 2003. Eligibility requirements were primary prostate cancer, surgical resection as the initial treatment modality and adequate tumor tissue in the archival histology blocks. A total of 95 patients were included in this study. Data collected included patient age, date of initial diagnosis, histopathological diagnosis, Gleason score, tumor volume and pathological tumor stage. These tissues were archived and de-identified in the Translational Research Tissue Support Resource of Pathology under Institution institutional review board protocols CIC91-10 and I05003. Clinical data were collected under Institutional Existing Data Review EDR 12203.

Histological examination, tissue assessment and procurement. Tissue specimens obtained from diagnostic or therapeutic procedures were fixed in neutral buffered formalin (10% volume/ formalin in water, pH 7.4) and embedded in paraffin wax. Serial sections 5 μ m thick were cut and mounted on charged Superfrost™ Plus glass slides. Sections were stained with hematoxylin and eosin, and reviewed for confirmation of the histopathological diagnosis and tissue adequacy for study. Histological diagnosis and differentiation grade (Gleason score) were determined by examining conventional hematoxylin and eosin stained slides. Key pathological variables, including the presence or absence of perineural invasion, capsular invasion, vascular invasion, seminal vesicle involvement and lymph node metastasis, were recorded.

To procure relatively pure cell populations for *IL-6* polymorphism analysis specific areas on the slide were identified and verified by microscopic inspection and marked for manual dissection. Tumor cells and adjacent benign cells were harvested separately.

Genotyping methodology. Genomic DNA was extracted from tumor cells and adjacent benign cells using phenol extraction methods. Polymorphism analysis was performed in duplicate according to protocols based on the tetra-primer amplification refractory mutation system (ARMS)-polymerase chain reaction (PCR)¹⁸ using 2 primer pairs to amplify the 2 alleles of SNP, respectively, in a single PCR reaction. The PCR primers used were forward inner primer (G allele) 5'-GCACTTTTCCCCCTAGTTG-TGTCTTCCG, reverse inner primer (C allele) 5'-ATTGTGCAATGTGACGTCCTTTAGCTTG, forward outer primer 5'-GACTTCAGCTTTACTCTTTGTCAAGACA and

reverse outer primer 5'-GAATGAGCCTCAGACATC-TCCAGTCCTA. Each PCR reaction was performed in a total volume of 10 μ l, containing 100 ng genomic DNA, 10 pmol of each inner primer, 1 pmol of each outer primer, 200 μ M deoxynucleoside triphosphate, 2.5 μ M MgCl₂ and 2.5 U Taq polymerase in storage buffer B (Promega, Madison, Wisconsin). PCR cycling conditions for the assay were 95C for 2 minutes, followed by 35 cycles of touchdown reactions at 95C for 1 minute and 72C for 1 minute for the first cycle, decreasing by 1C per cycle until annealing temperature reached to 63C and then continuing at 63C in the annealing step of the remaining cycles with extension at 72C for 1 minute and a final extension step at 72C for 5 minutes. After PCR amplification a 5 μ l aliquot of PCR products was resolved by 5% nondenaturing polyacrylamide gel electrophoresis and visualized by ethidium bromide staining.

Statistical analyses. The 2-sided t test with Satterwaite's approximation was used to analyze relationships between *IL-6* polymorphism, and PSA levels and patient age. The 2-sided Fisher exact test was performed to determine the relationships between *IL-6* polymorphism and other clinicopathological features, including Gleason score, tumor stage, vascular invasion, seminal vesicle involvement, capsular invasion, perineural invasion, periprostate adipose invasion and surgical resection margin. Survival probabilities were estimated by the Kaplan-Meier method and the difference in 2 survival curves was tested by the log rank test. Statistical analysis was performed using SAS software (SAS Institute, Cary, North Carolina) with p <0.05 considered statistically significant.

RESULTS

Since *IL-6* is significantly increased in the serum of patients with hormone refractory prostate cancer and metastatic prostate cancer compared with that of men with androgen dependent and localized prostate cancer, we hypothesized that *IL-6* variant allele genotypes modulate the risk of advanced prostate cancer. In this study the *IL-6* genotype was evaluated in patients with high and low grade prostate cancer to determine whether an association exists between the *IL-6* genotype and prostate cancer aggressiveness. This study is a retrospective analysis of 95 patients diagnosed with prostate carcinoma at our institution between 1992 and 2003. Median patient age \pm SD was 62.4 \pm 6.5 years (range 43 to 80). Average PSA at initial diagnosis was 10.5 ng/ml. Disease-free survival (DFS) was defined as months from patient received surgery to PSA recurrence. As of September 2003, 30 patients (33%) had PSA recurrence of prostate cancer, while 54 (57%) remained disease-free. The distribution of race/ethnicity in study patients was 89% white, 6% black and 5% other.

IL-6 polymorphism analysis was performed using genomic DNA isolated from tumor cells and adjacent benign cells in duplicate according to protocols based on tetra-primer ARMS-PCR¹⁸ using 2 primer pairs to amplify the 2 alleles of an SNP, respectively, in a single PCR reaction. Three representative genotypes derived from the -174 site of the *IL-6* gene polymorphism were identified, namely the C/C homozygous (326 plus 176 bp bands), the G/G homozygous (326 plus 205 bp bands) and the C/G heterozygote (326 plus 205 plus 176 bp bands) (fig. 1). The -174 site of *IL-6* genotypes were identical in tumor cells and adjacent benign cells from the same patient, suggesting that the -174 site of the *IL-6* gene polymorphism originated from germline DNA.

The association between *IL-6* polymorphism and the histological degree of prostate cancer was determined in patients with prostate cancer with low and high grade tumors. For analysis due to the small number of patients with the C/C genotype the G/C and C/C genotypes were pooled and compared with G/G homozygotes. A strong association be-

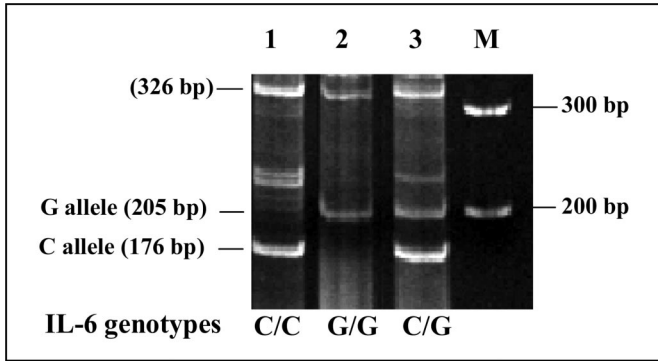


FIG. 1. Tetra-primer ARMS-PCR genotyping for -174 IL-6 polymorphism in patients with prostate cancer. Banding patterns in gels indicate 3 genotypes. Lane 1, C/C homozygote. Lane 2, G/G homozygote. Lane 3, C/G heterozygote.

tween genotypes and Gleason score was observed at the IL-6 -174 locus (table 1). The distribution of -174 IL-6 genotypes in high grade tumors (Gleason 8 to 10) differed significantly from that in low (Gleason 2 to 6) and intermediate (Gleason 7) grade tumors (GC/CC 87% vs 32% and 50%, respectively, $p < 0.001$).

The distribution of genotypes was significantly different between stages T3-T4 and T1-T2 tumors ($p < 0.001$). Of 22 samples with T3-T4 tumors 21 (95%) had GC/CC genotypes. In contrast, 31 of 64 samples with T1-T2 tumors (48%) had GC/CC genotypes. In addition, IL-6 GC/CC genotypes were linked with vascular invasion ($p = 0.024$), seminal vesicle involvement ($p = 0.006$), capsular invasion ($p < 0.001$) and surgical resection margin ($p = 0.039$, table 1). There was no statistically significant difference in regard to perineural invasion ($p = 0.067$) or periprostate adipose invasion ($p = 0.083$). Together these data suggest that GC/CC IL-6 genotypes closely correlate with prostate cancer aggressiveness.

The relationship of IL-6 genotypes to serum PSA levels at diagnosis (before treatment) was analyzed. Mean serum PSA in patients with prostate cancer in the GG IL-6 genotype

group was 6.0 ng/ml, whereas serum PSA level in patients with prostate cancer in the GC/CC IL-6 genotype group was 10.5 ng/ml. PSA in patients with GC/CC IL-6 genotypes was significantly higher than in those with the GG IL-6 genotype ($p = 0.004$).

The relationship of IL-6 genotypes with DFS was analyzed. Of the 29 patients homozygous for the G allele at the IL-6 promoter locus 62% remained disease-free 78 months following surgery, whereas 42% of the 40 patients with GC/CC IL-6 genotypes remained disease-free 78 months following surgery (fig. 2). Patients with GC/CC IL-6 genotypes were at significantly increased risk for recurrent prostate cancer compared with those homozygous for the G allele ($p = 0.027$, fig. 2). To identify the variables of potential prognostic significance as potential predictors for PSA relapse, recurrent prostate cancer, univariate analysis of Gleason score, IL-6 polymorphism and PSA levels at initial diagnosis was performed in relation to PSA relapse DFS (table 2). Of patients with the GG IL-6 genotype 19% were diagnosed with recurrent disease during the 12-year followup, whereas 51% of those with GC/CC IL-6 genotypes were diagnosed with recurrent disease during the same followup ($p = 0.003$). In addition, higher Gleason score was significantly associated with the risk of PSA relapse, recurrent prostate cancer ($p = 0.0019$). However, serum PSA at initial diagnosis did not significantly correlate with PSA relapse, recurrent prostate cancer ($p = 0.274$).

DISCUSSION

In this study we examined the relationship of IL-6 polymorphism and the risk of recurrent prostate cancer. We found that IL-6 polymorphism is significantly associated with the risk of more aggressive prostate cancer.

The -174G>C IL-6 genotype appears to be biologically and clinically important. There is increasing evidence that IL-6 gene polymorphism is associated with altered levels of IL-6 expression. The association between the -174G>C IL-6 genotype and increased IL-6 levels was recently reported in a sample of patients with aortic aneurysms who had evidence of a chronic, low grade systemic inflammatory response.¹⁴

TABLE 1. IL-6 polymorphisms and clinicopathological variables

Variables	GG	GC/CC	Total	p Value
No. Gleason score:				
2-6	17	8	25	
7	10	10	20	
8-10	4	26	30	<0.001
Au PSA (ng/ml)	6.1	10.5		0.004
No. tumor stage:				
T1-T2	33	31	64	
T3-T4	1	21	22	<0.001
No. vascular invasion:				
Yes	1	11	12	
No	31	39	70	0.024
No. seminal vesicle involvement:				
Yes	0	10	10	
No	32	40	72	0.006
No. capsular invasion:				
Yes	8	32	40	
No	24	17	41	<0.001
No. perineural invasion:				
Yes	14	33	47	
No	18	17	35	0.067
No. periprostate adipose invasion:				
Yes	1	8	9	
No	31	42	73	0.083
No. surgical resection margin:				
Pos	0	7	7	
Neg	32	43	75	0.039
Mean age	60	61		0.820

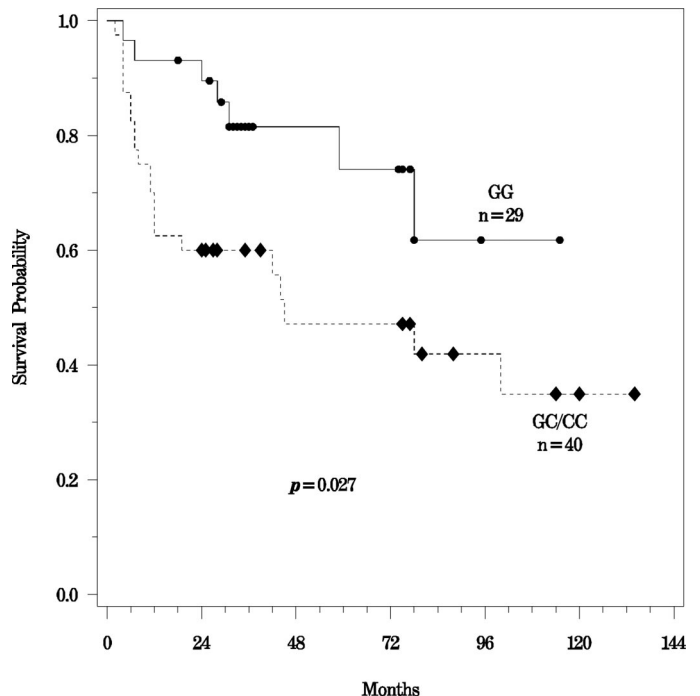


FIG. 2. PSA-DFS in patients with prostate cancer, as grouped by their genotypes for -174 IL-6 polymorphism.

TABLE 2. Correlation with PSA-DFS

	DFS p Value (PSA relapse)
Gleason score	0.0019
-174 G/C IL6 polymorphism	0.0032
PSA at diagnosis	0.274

The association of the -174G>C *IL-6* genotype with various diseases has been demonstrated. The -174G>C *IL-6* genotype has been shown to be significantly associated with disease states, including osteoporosis and systemic lupus erythematosus.^{15,16} The -174G>C *IL-6* genotype is also associated with the risk of development of ovarian and breast cancer.^{17,19} A recent study indicated that cytokine genes, including *IL-8*, *IL-10* and *VEGF* polymorphisms, influence the development of prostate cancer.²⁰ The results of this study establishing the relationship between this *IL-6* polymorphism and advanced prostate cancer provide additional evidence, further suggesting that cytokine polymorphisms, possibly associated with differential cytokine production, are risk factors for recurrent prostate cancer.

Because *IL-6* functions as autocrine and paracrine growth factor for prostate cancer, we compared the frequency of variant alleles of *IL-6* in patients with different stages of prostate cancer. Our results indicate that high grade prostate cancer is strongly associated with the -174 polymorphism in the promoter region of the human *IL-6* gene. The -174G>C *IL-6* genotype, associated with altered *IL-6* production, was significantly over represented in high grade prostate cancer (Gleason 8 to 10) compared with low (Gleason 2 to 6) or intermediate (Gleason 7) tumors ($p < 0.001$). The -174G>C *IL-6* genotype was also strongly associated with tumor stage, vascular invasion, seminal vesicle involvement and capsular invasion, suggesting that *IL-6* polymorphisms are associated with aggressive prostate cancer phenotypes. In addition, we found that *IL-6* polymorphism was associated with increased serum PSA ($p = 0.004$) and the risk of PSA relapse prostate cancer ($p = 0.027$). This study linked *IL-6* polymorphism with the risk of recurrent prostate cancer, providing an additional mechanism of *IL-6* mediated prostate cancer growth and androgen independent progression.

This study was designed to study the association of *IL-6* polymorphism with the risk of advanced prostate cancer. Although our results demonstrate that *IL-6* polymorphism is associated with prostate cancer aggressiveness, higher levels of serum PSA and DFS, due to the relative small sample size these findings must be further evaluated in a larger, prospective cohort. Increased serum *IL-6* has been associated with patients with metastatic and hormone refractory prostate cancer. It would be interesting to examine the relationships between the -174 *IL-6* polymorphism and serum *IL-6* levels. Unfortunately serum was not available and serum *IL-6* measurements could not be performed in this study cohort. Further prospective studies will be required to evaluate the relationships between the -174 *IL-6* polymorphism, serum *IL-6* and the biological phenotype for prostate cancer. In conclusion, this study demonstrates a strong association of the -174G>C polymorphism of *IL-6* gene with the aggressiveness and recurrence of prostate cancer, suggesting that genetic predisposition of genetic differences in the human *IL-6* gene could be linked to the risk of recurrent prostate cancer.

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