−174 G>C Polymorphism of *Interleukin 6* Gene Promoter Affects Interleukin 6 Serum Level in Patients with Colorectal Cancer

Claudio Belluco,[1] Fabiola Olivieri, Massimiliano Bonafè, Simona Giovagnetti, Enzo Mammano, Romano Scalerta, Alessandro Ambrosi, Claudio Franceschi, Donato Nitti, and Mario Lise

Department of Oncological and Surgical Sciences, University of Padova, Padova 35128 [C. B., E. M., R. S., A. A., D. N., M. L.]; Italian National Research Center on Aging, Ancona [F. O., S. G., C. F.]; and Department of Experimental Pathology, University of Bologna, Bologna [M. B., C. F.], Italy

ABSTRACT

Purpose: **Experimental data suggest that interleukin 6 (IL-6) plays an important role in the development and progression of metastasis from colorectal cancer (CRC), and *−174 G>C* polymorphism has been identified recently in the *IL-6* gene promoter. Therefore, the aim of the present study was to investigate the significance of this type of polymorphism in patients with CRC.**

Experimental Design: **Using enzyme immunoassay, IL-6 concentrations were measured in preoperative serum samples from 65 stage I-IV CRC patients. DNA was extracted from peripheral blood mononuclear cells, and *−174 G>C* polymorphism detected using PCR, followed by *Nla*III restriction enzyme digestion and electrophoresis.**

Results: **The median IL-6 serum level was 0.14 pg/ml in patients with stage I-III disease *versus* 0.41 pg/ml in patients with stage IV disease ($P < 0.001$). DNA amplification was possible in 62 cases. On grouping genotypes at the *−174 G>C* locus as C+ (*CC* and *CG*) and C− (*GG*), a significant association was observed between the type of polymorphism and IL-6 serum level: the median value for IL-6 was 0.14 pg/ml in C+ patients ($n = 32$) and 0.32 pg/ml in C− patients ($n = 30$; $P = 0.034$). Moreover, in patients with hepatic metastasis the median level of IL-6 was 0.23 pg/ml in C+ patients ($n = 9$) and 0.96 pg/ml in C− patients ($n = 9$; $P = 0.004$).**

Conclusions: **In patients with CRC, the *−174 G>C* polymorphism status of the *IL-6* gene promoter affects the IL-6 serum level, particularly in the presence of hepatic metastasis.**

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[1] To whom requests for reprints should be addressed, at Department of Oncological and Surgical Sciences, University of Padova, Via Giustiniani, 2, 35128 Padova, Italy. Phone: 390498212056; Fax: 39049651891; E-mail: claudio.belluco@unipd.it.

INTRODUCTION

IL-6,[2] a M_r 25,000 glycoprotein growth factor, has multiple stimulatory effects on inflammation and cell growth (1). 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 by stimulating the production of growth factors such as hepatocyte growth factor and vascular endothelial growth factor (2–4). Several clinical studies have reported that in different tumor types, a high IL-6 serum level is associated with advanced stage disease (5–8) and a worse outcome (5, 9, 10). In particular, we reported elsewhere that a high preoperative IL-6 serum level is associated with the presence of metastasis and is a negative prognostic factor in patients with CRC (11).

The status of a common functional single *G>C* base exchange polymorphism in the human *IL-6* gene promoter (chromosome *7p21*) located at *174 bp*, upstream from the start site of transcription (*−174 G>C* locus) (12), has been reported to influence IL-6 levels *in vitro* and *in vivo* (13, 14). The *G* allele 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 *G* allele (13, 14). Moreover, it has been reported that *−174 G>C* polymorphism is involved in several chronic conditions, such as insulin and noninsulin-dependent diabetes mellitus, juvenile chronic arthritis, coronary heart disease, dementia, peripheral artery occlusive disease, and postmenopausal osteoporosis (14–21). However, to our knowledge, no studies have been conducted to evaluate the effect of *−174 G>C* polymorphism on IL-6 serum levels in patients with solid tumors. Therefore, the aim of the present study was to investigate the influence of *−174 G>C* polymorphism on IL-6 serum levels in patients with CRC, particularly those with hepatic metastasis.

PATIENTS AND METHODS

Patients. The study population consisted of 65 fully informed consent patients (42 men and 23 women; mean age 66 years; range, 43–83 years), who underwent surgery for colorectal adenocarcinoma at our institution between January 2001 and December 2001. Patients with hereditary CRC, inflammatory bowel disease, and obstructing or perforated tumor were excluded from the study. None of the patients underwent preoperative chemoradiotherapy. The tumor was located in the colon in 47 cases (72%) and in the rectum in 18 cases (28%). The histological grade was assessed according to WHO criteria (22): 13 tumors (20%) were well differentiated, 34 (53%) mod-

[2] The abbreviations used are: IL, interleukin; CRC, colorectal cancer; C, cytosine; G, guanine; TNM, tumor-node-metastasis; NF, nuclear factor.

erately differentiated, and 18 (27%) poorly differentiated. Following the International Union Against Cancer classification and TNM staging system (23), 20 of the tumors (31%) were stage I, 14 (21%) stage II, 13 (20%) stage III, and 18 (28%) stage IV. All of the patients with stage IV disease had hepatic metastasis, which involved <50% of the liver in 10 patients (56%) and >50% of the liver in 8 patients (44%). Four of the patients with hepatic metastasis (22%) underwent resection, 8 (45%) systemic chemotherapy, 2 (11%) locoregional treatment, and 4 (22%) were treated by supportive care only.

Blood samples were obtained from fasting patients immediately before anesthesia and surgery.

Serum IL-6 Levels. Blood, collected in pyrogen-free glass tubes, was centrifuged at 600 rpm for 10 min. The serum was removed and stored at −80°C in pyrogen-free plastic tubes until analysis. Serum IL-6 levels were measured using a commercially available dual antibody sandwich enzyme-linked immunoassay (BioSource Cytoscreen human IL-6 UltraSensitive kit; Biosource International, Camarillo, CA). Using this kit, which has been used in previous studies (13, 24), the minimum detectable dose of IL-6 was 0.10 pg/ml, and no cross-detection of other cytokines was observed. All of the samples were thawed only once and assayed in duplicate. The intra-assay coefficient of variation was <10%.

Determination of *−174 G>C* Polymorphism. DNA, extracted from peripheral blood mononuclear cells using phenol-chloroform following standard procedures, was amplified by primers designed for the promoter region of the *IL-6* gene, as described previously (12). The primers used were: TTG TCA AGA CAT GCC AAG TGC T (forward) and GCC TCA GAG ACA TCT CCA GTC C (reverse).

PCR products were digested with the *Nla*III restriction enzyme overnight and electrophoresed on 2% agarose gel. The presence of a cytosine (*C* allele) at nucleotide *−174* was revealed by the presence of the *Nla*III cutting site.

The genotypes identified at the *−174 G>C* locus were classified as *G/G* (homozygotes for the *Nla*III cutting site absence), *G/C* (heterozygotes for the *Nla*III cutting site absence and presence), and *C/C* (homozygotes for the *Nla*III cutting site presence). On the basis of our previous experience (13), genotypes were subsequently grouped as C+ (*CC* and *CG*) and C− (*GG*).

Statistical Analysis. Because of the great departure from the normality of log-transformed data, a comparison was made between serum IL-6 levels in relation to genotype and clinicopathologic variables using the Mann-Whitney and Kruskall-Wallis nonparametric rank tests (25). *P*s < 0.05 were considered significant. The data are reported as medians with the first and the third quartiles, the latter representing variability within each group. All of the analyses were computed with S-Plus software (StatInc., Seattle, WA).

RESULTS

Correlation between IL-6 Serum Level and Clinical-Pathologic Variables. Serum IL-6 levels were not significantly correlated with sex, age, or tumor site and grade. However, in patients with stage I-III disease ($n = 47$) the median IL-6 serum level was 0.14 pg/ml (0.24–2.34), whereas in patients with hepatic metastasis ($n = 18$) the median IL-6 serum level was 0.41 pg/ml (0.10–0.37; $P < 0.001$). No statistically significant difference was found between the IL-6 serum level of patients with stage I-II and that of patients with stage III disease (Table 1).

Table 1 Serum concentrations of IL-6 in 65 patients with CRC according to clinicopathological variables

IL-6 serum levels are reported in pg/ml.

Variable	*n*	IL-6 serum level median (1st–3rd quartile)	Statistical significance
Sex			
Male	42	0.15 (0.10–0.42)	NS[a]
Female	23	0.22 (0.10–0.62)	
Age			
<75 years	50	0.17 (0.10–0.39)	NS
≥75 years	15	0.25 (0.12–0.79)	
Tumor location			
Colon	47	0.20 (0.10–0.62)	NS
Rectum	18	0.14 (0.10–0.32)	
Tumor grade			
G1	13	0.19 (0.10–0.40)	
G2	34	0.15 (0.10–0.41)	NS
G3	18	0.27 (0.10–0.96)	
TNM stage			
I	20	0.10 (0.10–0.20)	
II	14	0.21 (0.12–0.77)	
III	13	0.14 (0.10–0.31)	$P < 0.001$[b]
IV	18	0.41 (0.24–2.34)	

[a] NS, not significant.
[b] Stage I–III *versus* stage IV.

Correlation among *−174 G>C* Polymorphism, IL-6 Serum Level, and Clinicopathologic Variables. The impact of the *−174 G>C* locus on the IL-6 serum level was evaluated in 62 patients (in 3 cases DNA amplification was not possible), of whom 4 were *C/C*, 28 *C/G*, and 30 *G/G*. The genotype distribution among patients was in Hardy-Weinberg equilibrium ($P > 0.05$). The median IL-6 serum levels in the three genotypes were: 0.33 pg/ml (0.15–0.42) in *C/C*, 0.14 pg/ml (0.10–0.22) in *C/G*, and 0.32 pg/ml (0.10–0.94) in *G/G*. On comparing the homozygous group *C/C versus G/G*, and *C/C versus C/G* and *G/G*, no statistically significant difference was found for IL-6 levels; this was probably because of the low number of *C/C* patients. On the other hand, a significant association was observed between C+/C− status and IL-6 serum level: the median IL-6 serum level was 0.14 pg/ml (0.10–0.28) in C+ patients ($n = 32$) and 0.32 pg/ml (0.10–0.94) in C− patients ($n = 30$; $P = 0.034$). No significant association was found between genotype and IL-6 serum level when patients were stratified for sex, age, site, grade, and stage I-III. As shown in Table 2, a strong correlation was found between the genotype and the IL-6 serum level in patients with hepatic metastasis: the median IL-6 serum level was 0.23 pg/ml (0.15–0.30) in C+ patients ($n = 9$) and 0.96 pg/ml (0.45–6.62) in C− patients ($n = 9$; $P = 0.004$).

DISCUSSION

Our findings demonstrate that *−174 G>C* polymorphism in the *IL-6* gene promoter influences the levels of circulating IL-6 in patients with CRC, and to our knowledge, are the first

Table 2 *−174 C>G* polymorphism and IL-6 serum levels in 62 patients with CRC according to clinicopathological variables
IL-6 serum levels are reported in pg/ml.

	−174 C>G polymorphism				
	C+[a]		C−		
Variable	*n*	IL-6 median (1st–3rd quartile)	*n*	IL-6 median (1st–3rd quartile)	Statistical significance
All patients	32	0.14 (0.10–0.28)	30	0.32 (0.10–0.94)	$P = 0.034$
Sex					
Male	23	0.15 (0.10–0.29)	19	0.19 (0.10–1.05)	NS
Female	9	0.14 (0.10–0.22)	11	0.32 (0.18–0.75)	NS
Age					
<75 years	24	0.15 (0.10–0.24)	23	0.31 (0.10–0.70)	NS
≥75 years	8	0.14 (0.10–0.40)	7	0.62 (0.21–1.16)	NS
Tumor location					
Colon	23	0.14 (0.10–0.33)	22	0.32 (0.12–1.08)	NS
Rectum	9	0.15 (0.14–0.26)	8	0.23 (0.10–0.51)	NS
Tumor grade					
G1	4	0.13 (0.10–0.29)	8	0.28 (0.10–0.52)	NS
G2	18	0.14 (0.10–0.22)	14	0.23 (0.11–0.19)	NS
G3	9	0.21 (0.10–0.28)	8	0.47 (0.22–1.42)	NS
TNM stage					
I	9	0.10 (0.10–0.22)	10	0.11 (0.10–0.18)	NS
II	8	0.17 (0.10–0.27)	6	0.58 (0.10–1.08)	NS
III	6	0.12 (0.10–0.15)	5	0.10 (0.10–0.31)	NS
IV	9	0.23 (0.15–0.30)	9	0.96 (0.45–6.62)	$P = 0.004$

[a] C+, *−174* locus *CC* and *CG*; C−, *−174* locus *GG*; NS, not significant.

available evidence of the effect of this genetic variant in patients with solid tumors.

From a molecular viewpoint, the rapid serum clearance of IL-6 circulating levels of this cytokine are mainly regulated at the level of expression, and this could explain the influence on protein levels of *−174 G>C* polymorphism in the *IL-6* gene promoter (26). Altogether, high levels of circulating IL-6 in patients with different tumor types, and the finding of different levels of *IL-6* expression in relation to genetic variants, suggest that this cytokine is mainly produced by host cells rather than by cancer cells. Accordingly, *in vitro*, macrophages from C− subjects produce higher IL-6 levels that those from C+ subjects (27). This observation is in line with that made by Piancatelli *et al.* (28), who studied the expression of different cytokines in tumor tissue, in normal mucosa, and in the peripheral blood mononuclear cells of CRC patients, and found specific *IL-6* gene expression in the tumor microenvironment. As CRC cell lines did not express *IL-6* transcripts, the authors suggested that tumoral IL-6 originates in tumor infiltrating mononuclear cells (28). However, in depth studies are required to clarify this aspect, because other authors have observed different IL-6 production patterns in CRC cell lines (29, 30).

In the present study, we found a close correlation between high levels of circulating IL-6 and the presence of hepatic metastasis. This is in line with the findings of other clinical studies: in 46 patients with metastatic breast cancer, Zhang and Adachi (5) reported that patients with hepatic metastasis had higher serum IL-6 levels than those without liver metastasis; similarly, in their study on 24 patients with CRC, Ueda *et al.* (31) found that serum IL-6 levels were significantly higher in patients with than in those without hepatic metastasis.

The association between IL-6 serum level and CRC hepatic metastasis may depend on IL-6 being a potent stimulator of metastasis, up-regulating the expression on endothelial cells of adhesion receptors such as intercellular adhesion molecule-1 and leukocyte adhesion molecule-1, and inducing the production of growth factors, such as hepatocyte growth factor and vascular endothelial growth factor, both of which may stimulate tumor progression (2–4). Moreover, the high levels of circulating IL-6 in patients with metastasis could also be caused by the transcription activation of *IL-6* by NFκB. This transcription factor, known to regulate *IL-6* transcription, is reportedly overexpressed in highly metastatic cell lines as well as in metastatic tissue (32, 33). *IL-6* promoter activation involves synergism between the transcription factors NF-IL-6 and NFκB (34), and this may explain why we found that IL-6 serum levels varied significantly in the subset of patients with hepatic metastasis, depending on the *−174 G>C* polymorphism status.

In conclusion, our findings indicate that *−174 G>C* polymorphism influences circulating levels of IL-6 in patients with CRC. This observation may in fact be of clinical relevance, because high serum IL-6 level has been shown to be a negative prognostic factor in patients with several tumor types, including CRC (5, 9–11). As this genetic variant may be a prognostic molecular marker, it should be additionally investigated in prospective studies.

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