Effects of the IL6 -174G>C promoter polymorphism and IL-6 serum levels on the progression of cutaneous malignant melanoma

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Abstract. Cutaneous malignant melanoma (CMM) is one of the most immunogenic types of cancer, with a 6-fold higher rate of spontaneous regression than any other malignancy. In addition to responsiveness to different immunotherapies, the immunogenicity of CMM highlights the important role of the host immune system in the response to CMM. The present study aimed to explore the role of two functional promoter polymorphisms [*IL6* -174G>C (rs1800785) and *TNFA* -308G>A (rs1800629)] in the regulation of the genes encoding the pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-α, specifically in patients with CMM. A total of 76 patients with CMM and 200 control subjects were genotyped using PCR-restriction fragment length polymorphism. The genotype frequencies for both single nucleotide polymorphisms (SNPs) did not differ significantly between the patients and controls (P=0.358 and P=0.810 for IL6 and TNFA, respectively). However, compared with carriers of C-allele genotypes (CG+CC), patients with the *IL6* -174GG genotype exhibited more advanced melanoma (Clark scale ≥3; P=0.037) and shorter survival times, particularly those who worked outdoors (in conditions with increased sunlight exposure; P=0.016). Furthermore, the serum IL-6 levels of patients with CMM were significantly higher than those of the control subjects, which were associated with unfavorable blood and serum characteristics and tumor progression (development of new distant metastases; P=0.004), and with a shorter overall survival time (P=0.042). Using a Cox proportional hazard model, the *IL6* -174GG genotype was found to be an independent prognostic factor for reduced survival time (P=0.030), together with sex (being male; P=0.004) and occupations with higher exposure to sunlight (P=0.047). In conclusion, the results of the present study indicated that the promoter polymorphisms *IL6* -174G>C and *TNFA* -308G>A are not predisposing factors for CMM. However, the *IL6* -174G>C SNP and IL-6 serum concentrations are likely to influence the progression of the disease, and the GG genotype and higher IL-6 serum levels may indicate shorter survival.

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Introduction

Cutaneous malignant melanoma (CMM) is the most life-threatening primary skin malignancy, with a high global incidence rate amongst the Caucasian population. In Bulgaria, >470 new cases of melanoma are diagnosed each year (1). Once diagnosed, CMM can remain latent for a long period of time or can rapidly metastasize. Following distant metastasis, patient prognosis is poor, with an average survival time of 6-8 months, with only 11% of patients surviving beyond 2 years (2-4).

Numerous studies have investigated the genetic factors involved in the development of sporadic melanoma, including genes involved in the regulation of skin pigmentation, the cell cycle, DNA repair, the oxidative stress defense system and the production of immune modulatory mediators (5-12). Previous evidence also suggested that patients with CMM mounted an efficient immune response towards the tumor leading in some cases to spontaneous regression, although in most cases these responses did not prevent tumor progression (13,14). The pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) are among the factors involved in this response (13,15). IL-6 is a major pro-inflammatory mediator produced by various cell types, including melanoma cells, which exerts different biological activities towards a variety of target cells (16,17). IL-6 is reportedly involved in the differentiation of myeloid-derived suppressor cells and the reinforcement of their suppressive function; it is also associated with increased production of immunosuppressive cytokines by tumor cells, and increased metastasis in melanoma (18,19). Furthermore, the expression of IL-6 has been shown to promote the progression of CMM. Elevated pre-treatment levels of serum IL-6 have been determined as an independent prognostic biomarker of

reduced overall survival (20). Moreover, the pro-tumorigenic effects of IL-6 have been attributed to its regulatory role in tumor angiogenesis, proliferation, survival and tumor cell motility (15,21,22). The effects of IL-6 are mediated by the stimulation of signal transducer and activator of transcription 3, which profoundly influences melanoma angiogenesis and cellular proliferation by transcriptionally regulating basic fibroblast growth factor, vascular endothelial growth factor, matrix metalloproteinase-2, Wnt family member 5A, Twist and N-cadherin (22,23).

TNF-α also plays a role in the development of CMM. Notably, increased TNF-α expression, stimulated by exposure to UV radiation, has been reported to contribute to antitumor immune escape (13). Together with IL-6, TNF-α has been suggested as one of the key modulators of melanoma cell aggressiveness, which these cells secrete in large volumes to initiate a cascade of effects, such as upregulation of matrix metalloproteinases (24). Conflictingly, TNF-α has been reported to both inhibit and promote tumor growth (25,26).

The genes encoding TNF-α and IL-6 are highly polymorphic due to a variety of single nucleotide polymorphisms (SNPs) in their regulatory and coding sequences. rs1800795, a SNP in the promoter region of the *IL6* gene (-174 G>C) is reportedly associated with constitutive IL-6 expression, which results in higher IL-6 expression in carriers of the GG/GC genotype, compared with those of the CC genotype (27).

A G>A substitution at position -308 in the promoter region of the *TNFA* gene has also been identified, and the -308A allele has been associated with enhanced TNF-α expression *in vivo* and *in vitro,* and increased plasma levels of TNF-α compared with the -308G allele (28-30).

To date, only a limited number of studies have investigated the role of polymorphisms in the *IL6* and *TNFA* genes in melanoma (13,31-34). Thus, the current study aimed to clarify the possible effects of the *IL6* -174G>C and *TNFA* -308G>A SNPs on the susceptibility and prognosis of CMM in a Bulgarian population. The present study is the first, to the best of our knowledge, to describe possible effects of these polymorphisms on the progression of CMM in Bulgarian patients.

Materials and methods

Patients. In total, 76 patients with CMM treated at The Oncology Center of Stara Zagora (Stara Zagora, Bulgaria) were enrolled in the present study. All patients with melanoma diagnosed for the first time between January 2011 and December 2015, regardless of disease stage, were invited to participate in the study. Demographic data and information on working conditions were extracted from patient files. According to their occupation, the patients were divided into two groups: i) Those working in conditions less harmful for the skin (e.g., offices and schools); and ii) those working in more harmful conditions (such as agricultural workers, construction workers and those in open mine shafts). The demographic and clinical data obtained from the patients are presented in Table I. The control group comprised 200 individuals without CMM and included 94 (47%) men and 106 (53%) women aged between 19 and 85 years (median age, 58 years) from the same ethnic group and area of Bulgaria. The recruited controls were volunteers or individuals participating in prophylactic examinations who were reported not to have cancer. The patients were treated and followed-up at the Dermatology Unit of The Oncology Center of Stara Zagora. Informed consent was obtained from all participants, and the protocol was approved by The Ethics Committee of The Medical Faculty of Trakia University (Stara Zagora, Bulgaria). The study was also performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards.

Table I. Demographic and clinical data of the patients with cutaneous malignant melanoma.

Parameter	N (%)
Sex[a]	76
Male	31 (41)
Female	45 (59)
Localization of the tumor[a]	60
Extremities (legs/arms)	24 (40)
Trunk	30 (50)
Head	6 (10)
pT category[a]	61
pT1-2	32 (52)
pT3-4	29 (48)
pN category[a]	61
pN0	55 (90)
pN1-3	6 (10)
Metastasis[a]	60
No	23 (38)
Yes	37 (62)
pTNM clinical stage[a]	60
I	22 (36)
II	29 (48)
III	3 (5)
IV	6 (10)
Clark's level[a]	50
II	17 (34)
III	20 (40)
IV	9 (18)
V	4 (8)
Outcome after follow-up period[a]	75
Alive	41 (55)
Dead	34 (45)
Breslow's thickness, n=18, mm[b]	2.00 (0.20-4.20)
Survival after diagnosis, n=75, months[b]	81.78 (55.90-301.45)
Overall survival, n=75, months[b]	19.42 (0.49-237.52)
Age at diagnosis, n=76, years[b]	59.74 (15.49-83.76)

[a]Data are presented as n (%); [b]data are presented as median (range). Data for some of the 76 patients regarding localization of the tumor, pT category, pN category, metastasis, pTNM clinical stage, Clark's level and outcome after follow-up period were not available.

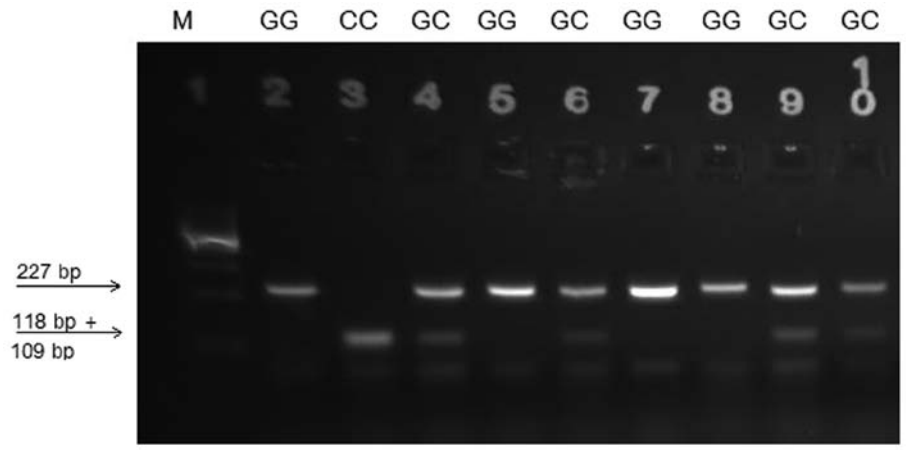

Figure 1. Agarose gel visualization of PCR-restriction fragment length polymorphism products in genotyping for the IL6 -174G>C single nucleotide polymorphism. Only the bigger bands of 227, 118 and 109 bp in length are visible. IL6, interleukin-6; bp, base pair; M, marker.

DNA isolation. Genomic DNA was isolated from 0.2 ml whole blood using the GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich; Merck KGaA). The isolated DNA was stored at -80˚C. DNA concentration was determined using the NanoVue™ Spectrophotometer (GE Healthcare), and purity was assessed by calculating the ratio of the optical density at 260 and 280 nm. The purity and quality of the DNA samples was also confirmed by electrophoresis, using a 1% agarose gel.

Genotyping for TNFA -308G>A (rs1800629) and IL6 -174G>C (rs1800795) SNPs. Genotyping was performed using a PCR-restriction fragment length polymorphism (RFLP)-based method, as previously described (35). The amplification reactions were performed in a final volume of 12 μl using the Mastercycler® instrument (Eppendorf). The amplification mix contained 30-50 ng genomic DNA, 0.8 pmol/μl each primer, 200 μM dNTPs, 1.2 μl 10X buffer with 15 mM $MgCl_2$ (Sigma-Aldrich; Merck KGaA), 0.5 U Taq Polymerase (Sigma-Aldrich; Merck KGaA) and double-distilled H_2O to a final volume of 12 μl. The primers used were as follows: TNF-α, forward 5'-AGGCAATAGGTTTTGAGGGCCAT-3', reverse 5'-TCCTCCCTGCTCCGATTCCG-3'; IL-6, forward 5'-TTGTCAAGACATGCCAAGTGCT-3' and reverse 5'-GCC TGAGAGACATCTCCAGTCC-3'.

Thermocycling conditions for *rs1800629* were: i) Pre-amplification denaturation at 95˚C for 3 min; ii) 5 cycles of denaturation for 30 sec at 94˚C, annealing for 30 sec at 58˚C, and polymerization for 30 sec at 72˚C; iii) 30 cycles of denaturation for 30 sec at 94˚C, annealing for 30 sec at 56˚C and polymerization for 30 sec at 72˚C; and iv) final extension at 72˚C for 7 min.

For *rs1800795*, the thermocycling conditions were: i) Pre-amplification denaturation at 95˚C for 3 min; ii) 35 cycles of denaturation for 30 sec at 95˚C, annealing for 30 sec at 62˚C and polymerization for 30 sec at 72˚C; and iii) final extension at 72˚C for 5 min.

Restriction digestion for the *rs1800629* SNP was performed in a final volume of 16 μl with 12 μl PCR product and 4.8 U *Nco*I in 4 μl 1X ONE Buffer (EUREX Sp. z o.o.) for 16 h at 37˚C. The restriction digestion reaction for the *rs1800795* SNP was performed in a final volume of 17 μl with 12 μl PCR product and 3 U *Hin*I in 5 μl 1X Tango buffer (Thermo Fisher Scientific, Inc.) for 16 h at 37˚C. The obtained restriction fragments were analyzed using electrophoresis with a 3.5% agarose gel stained with ethidium bromide (Sigma-Aldrich; Merck KGaA), and detected using a UV transilluminator (Cleaver Scientific Ltd.). The results were assessed using the Gel documentation system EC3 Imaging system (Ultra-Violet Products Ltd.).

Measurement of IL-6 serum concentration. Serum IL-6 levels of 20 control individuals and 59 patients with CMM were determined using a commercial ELISA kit (cat. no. D6050; R&D Systems, Inc.) according to the manufacturer's protocol. The IL-6 concentrations were recorded in comparison to the standards included in the kit and are presented in pg/ml serum. *Statistical analysis*. Statistical analysis was performed using SPSS v16.0 (SPSS, Inc.). The descriptive data, including the mean, SEM and median, were assessed. Kolmogorov-Smirnov's test and Shapiro-Wilks' W-test were used to analyze the normality of the continuous variables. Continuous variables with normal distribution were compared between ≥2 independent groups using one-way ANOVA followed by a least significant difference post hoc test. Variables with non-normal distribution were compared using a Mann-Whitney U test. The manifestation frequencies of the qualitative (categorical) variables were determined in 2x3 and 2x2 cross-tables and were evaluated using the χ^2 test. Fisher's exact test was used as appropriate (when the expected numbers of ≥1 of the cells of the 2x2 cross-tables were <5). The correlations between the quantitative variables were evaluated using Pearson or Spearman's test according to the distribution (normal or skewed, respectively). The odds ratios and 95% CI values were calculated by binary logistic regression analysis for evaluation of the risk of outcome occurrence (development of melanoma). Hardy-Weinberg equilibrium (HWE) was tested among the controls and patients using the χ^2 test.

Cumulative survival curves were constructed using the Kaplan-Meier method, and the differences in survival were calculated using the log rank test. The prognostic significance of various factors regarding patient survival after surgery was

Table II. Genotype and allele frequencies of the *IL6* -174G>C gene polymorphism in patients with cutaneous melanoma (n=59 and n=118, respectively) and controls (n=173 and n=346, respectively) (binary logistic regression analysis).

A, Genotype frequency (P=0.358; χ^2 test)

Variable	Patients, n (frequency)	Controls, n (frequency)	OR (95% CI)	P-value
GG	30 (0.508)	74 (0.428)	1.0 (reference)	
GC	22 (0.373)	83 (0.480)	0.654 (0.347-1.231)	0.188
CC	7 (0.119)	16 (0.092)	1.079 (0.403-2.888)	0.879
GC+CC	29 (0.492)	99 (0.572)	0.723 (0.399-1.307)	0.283

B, Allele frequency (P=0.878; χ^2 test)

Variable	Patients, n (frequency)	Controls, n (frequency)	OR (95% CI)	P-value
-174 G	82 (0.695)	231 (0.668)	1.0 (reference)	
-174 C	36 (0.305)	115 (0.332)	0.882 (0.563-1.382)	0.649

Frequency was calculated by dividing the number of patients in each group by the total number of patients. OR, odds ratio.

Table III. Biochemical/blood parameters in patients with cutaneous melanoma and different IL6 -174G>C genotypes.

Biochemical/blood parameters	*IL6* -174GG	*IL6* -174GC	*IL6* -174CC	P-value[a]
AsAT, U/l	21.34±2.03	24.77±5.97	18.00±2.51	0.621
AlAT, U/l	22.21±3.38	20.82±7.40	18.40±3.11	0.915
GGT, U/l	51.17±18.03	21.50±4.50	54.00±17.62	0.607
LDH, U/l	215.20±33.26	203.00±25.7	246.33±44.54	0.820
RBC, 10^{12} cells/l	4.82±0.14	4.71±0.11	4.62±0.30	0.777
WBC, 10^9 cells/l	6.94±0.53	7.59±0.46	9.16±2.09	0.239
Lymphocytes, %	36.27±3.01	32.80±3.65	31.70±4.93	0.654
Granulocytes, %	62.88±1.75	62.65±4.45	71.35±1.35	0.080

[a]P-values were calculated by one-way ANOVA followed by a least significant difference post hoc analysis. AsAT, aspartate aminotransferase; AlAT, alanine aminotransaminase; GGT, γ-glutamyl transferase; LDH, lactate dehydrogenase; RBC, red blood cell; WBC, white blood cell; IL6, interleukin-6.

determined by univariate and multivariate Cox regression analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

IL6 -174G>C SNP. Genotyping of the -174G>C polymorphism in the promoter region of *IL6* was performed by PCR-RFLP. The resulting PCR product was 299 bp in length, and the restriction reaction resulted in three fragments for the wild-type G allele (227, 50 and 13 bp). For the variant C allele (CC genotype), the restriction reaction resulted in four fragments of 118, 109, 50 and 13 bp (Fig. 1).

For this SNP, 59 patients with CMM and 173 control individuals were successfully genotyped. The distribution of the genotypes did not deviate from HWE in either group (P=0.997 and P=0.799, respectively; χ^2 test). The genotype distributions in the patient group were 30 (50.8%) GG carriers, 22 (37.3%) GC carriers and 7 (11.9%) CC carriers. The control group comprised 74 (42.8%) carriers of the GG genotype, 83 (48.0%) with the GC genotype and 16 (9.2%) with the CC genotype. Both genotype and allelic distributions did not differ between the patients and controls (P=0.358 and P=0.878; χ^2 test; Table II).

In patients with CMM, there were no associations between different genotypes and biochemical/blood parameters such as total protein, albumin, glucose, bilirubin, creatinine, enzymes [aspartate aminotransferase (AsAT), alanine aminotransferase (AlAT), γ-glutamyl transferase (GGT) and lactate dehydrogenase (LDH)], red blood cell count, white blood cell (WBC) count and the percentages of WBC subpopulations (Table III). According to the Clark scale (36), carriers of the GG genotype predominantly exhibited more advanced melanoma (Clark 3, 4 and 5) than those with C allele genotypes (GC+CC) (P=0.037; χ^2 test; Fig. 2). Similarly, GG carriers more frequently possessed thicker tumors (≥2 mm; 75%) than patients with other genotypes (52.4%; P=0.114; χ^2 test; Fig. 3).

On patient follow-up, the survival time after diagnosis of *IL6* -174GG genotype carriers was shorter, although not

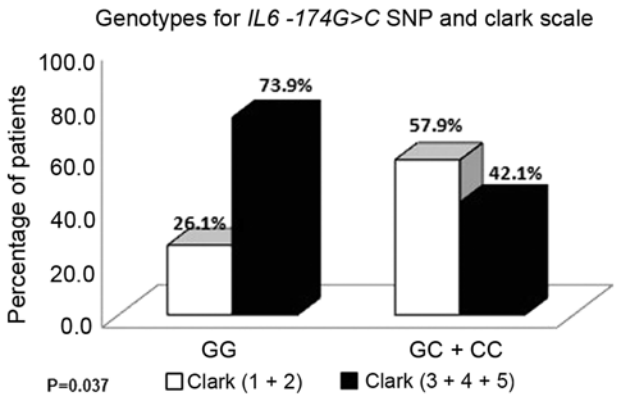

Figure 2. Association between the genotype distribution of the *IL6* -174G>C SNP and the depth of distribution in the skin of patients with cutaneous malignant melanoma (Clark scale). SNP, single nucleotide polymorphism; *IL6*, interleukin-6.

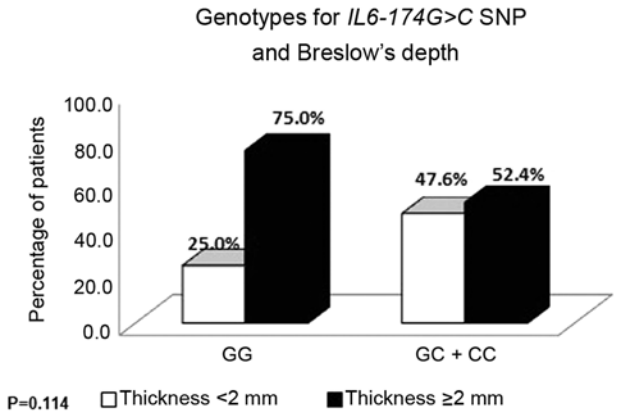

Figure 3. Association between the genotype distribution of the *IL6* -174G>C SNP and Breslow's depth of tumors from the patients with cutaneous malignant melanoma. SNP, single nucleotide polymorphism; *IL6*, interleukin 6.

significantly, than those with a genotype with ≥1 variant *IL6* -174C allele (GC+CC) (mean, 132.58 vs. 166.55 months; P=0.299; log rank test; Fig. 4). When patients were categorized according to their working conditions, those carrying the GG genotype and in occupational conditions with longer periods of sunlight exposure (so called 'high risk conditions') had significantly shorter survival times (24.09 months) compared with carriers of C allele genotype (104.33 months; P=0.016; log rank test; Fig. 5A). Similar results, although not significant, were obtained for the subgroup of patients working indoors, i.e., in conditions with rare sunlight exposure (so called 'low risk conditions'): The mean survival period of patients with the GG genotype who worked indoors was 185.73 months, while that of the patients with C allele genotypes (GC+CC group) was 256.00 months (P=0.121; log rank test; Fig. 5B).

Serum IL-6 levels between patients with CMM (median, 5.68 pg/ml; mean ± SEM), 20.57±5.89 pg/ml) and the control subjects (median, 4.17 pg/ml; mean ± SEM, 5.02±0.74 pg/ml) differed with marginal significance (P=0.033; Fig. 6). No significant difference was observed when comparing the three -174G>C genotypes of both the patient and control groups (P=0.323 and 0.104, respectively; data not shown). However, the IL-6 level was significantly lower in control subjects with

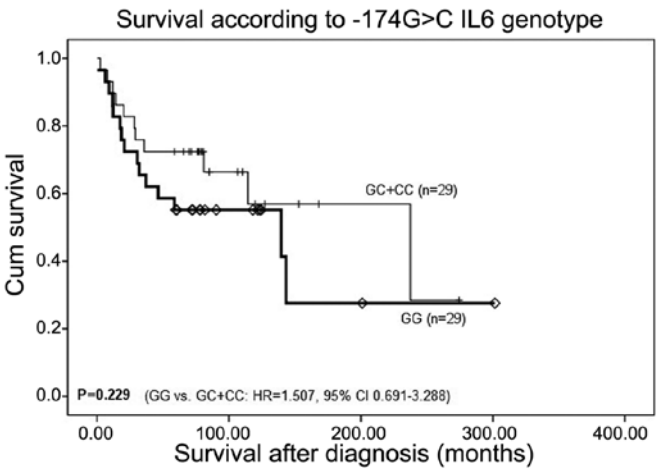

Figure 4. Survival of patients with cutaneous malignant melanoma according to *IL6* -174G> C single nucleotide polymorphism genotype (Log rank test). *IL6*, interleukin-6; HR, hazard ratio; Cum, cumulative.

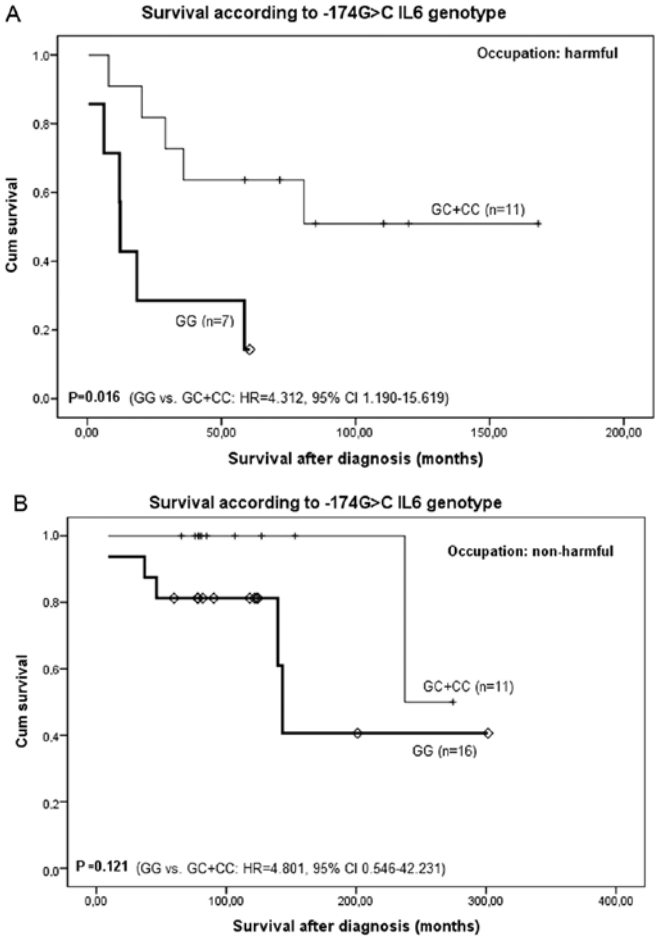

Figure 5. Survival of patients with cutaneous malignant melanoma according to *IL6* -174G> C single nucleotide polymorphism genotype and occupational conditions. (A) Patients with occupational conditions involving longer exposure to sunlight (outdoor work, harmful conditions). (B) Patients with occupational conditions involving rare exposure to sunlight (indoor work, non-harmful conditions). *IL6*, interleukin-6; HR, hazard ratio; Cum, cumulative.

the GG genotype (median, 3.02 pg/ml), compared with those with variant C allele genotypes (GC+CC; 5.01 pg/ml; P=0.039; Fig. 7). Although not statistically significant, a similar trend

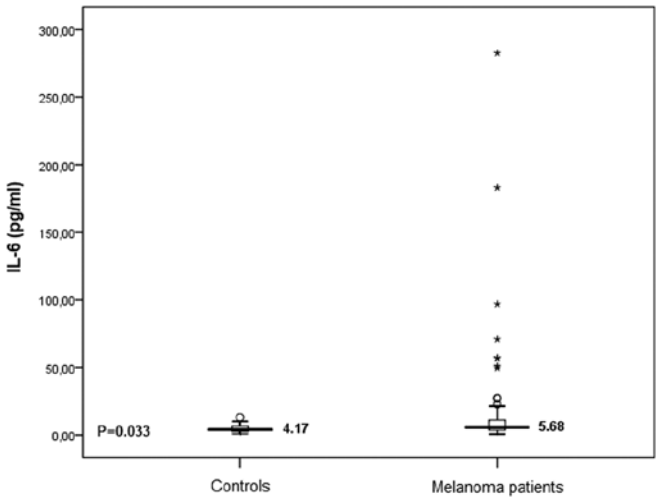

Figure 6. Serum IL-6 levels in patients with cutaneous malignant melanoma and control subjects. Data are presented as box-and-whiskers plots with medians. Statistical analysis was performed using a Mann-Whitney U test (P=0.033). Open circles represent outliers; stars represent extreme cases. IL-6, interleukin-6.

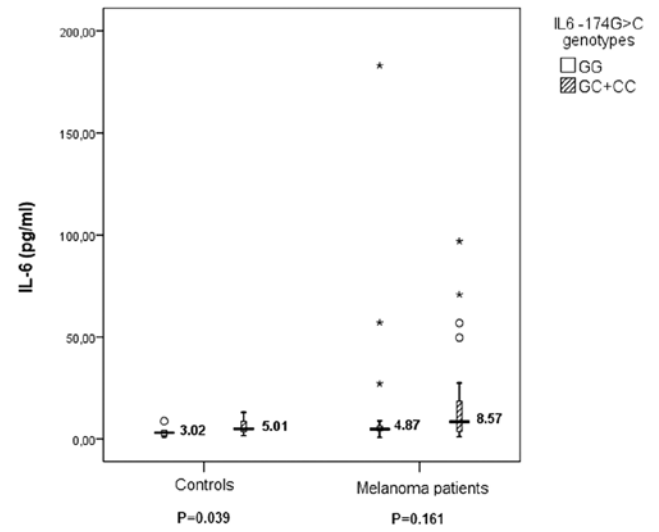

Figure 7. Serum IL-6 levels in patients with cutaneous malignant melanoma and control subjects according to the *IL-6* -174G> C genotypes. Data are presented as box-and-whiskers plots with medians. Statistical analysis was performed using a Mann-Whitney U test (P=0.039 for controls and P=0.161 for melanoma patients). Open circles represent outliers; stars represent extreme cases. IL-6, interleukin-6.

was observed in the patient group (median, 4.87 pg/ml vs. 8.57 pg/ml; P=0.161; Fig. 7).

Serum IL-6 levels were not associated with clinical or histological tumor parameters such as the Clark scale (1+2 vs. 3+4+5; P=0.404), Breslow's thickness (<2 mm vs. ≥2 mm; P=0.808), TNM staging (37) (1 vs. 2 vs. 3 vs. 4; P=0.187) and the presence of distant metastases at the time of diagnosis (P=0.440; data not shown). However, the IL-6 serum levels between the patients with disease progression and development of new distant metastases (median, 22.81 pg/ml), and those without new distant metastases (median, 5.10 pg/ml), were significantly different (P=0.004; Fig. 8A). Moreover, the serum levels differed significantly between patients whose occupation

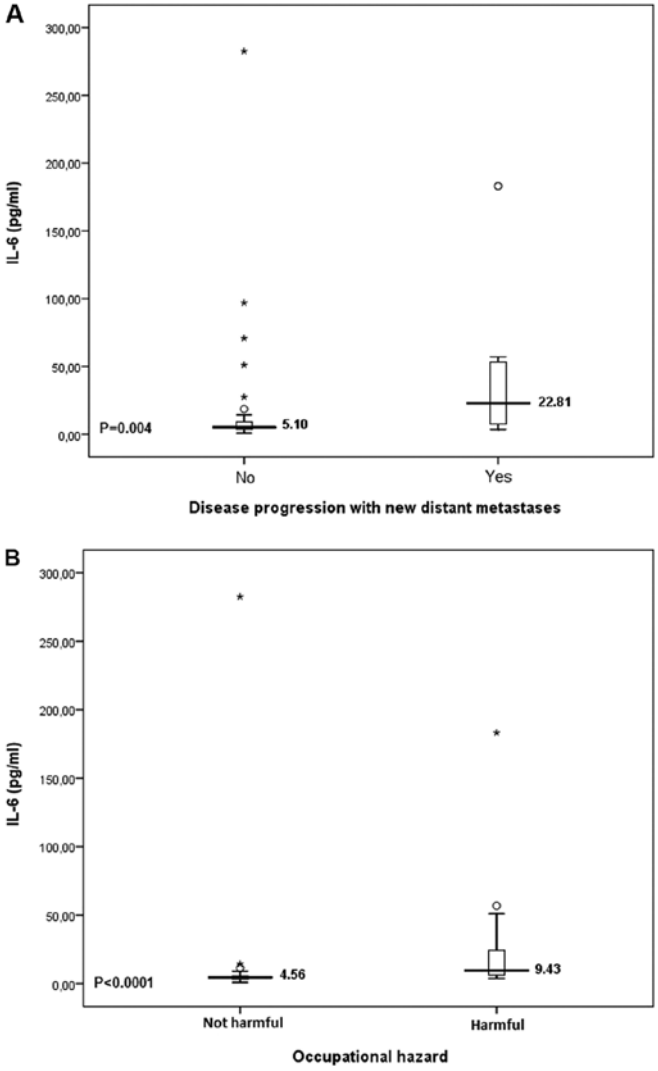

Figure 8. Serum IL-6 levels in patients with cutaneous malignant melanoma (A) according to disease progression and development of new distant metastases (P=0.004) and (B) according to occupational hazard (P<0.0001, vs. patients who worked indoors). Data are presented as box-and-whiskers plots with medians. Statistical analysis was performed using a Mann-Whitney U test. Open circles represent outliers; stars represent extreme cases. IL-6, interleukin-6.

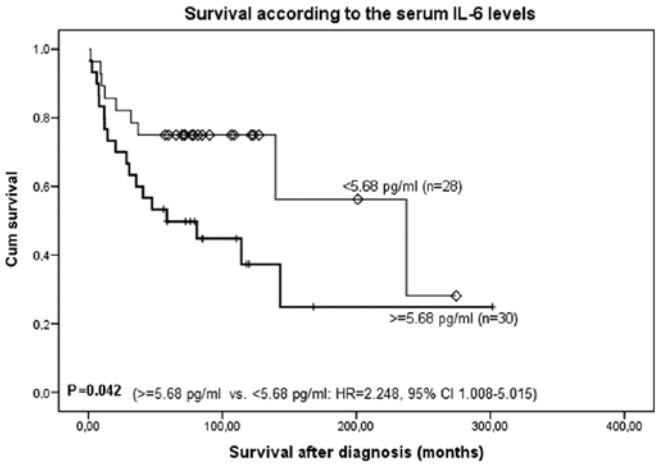

Figure 9. Survival of patients with cutaneous malignant melanoma according to IL-6 serum levels. Cut-off, 5.68 ng/ml (median serum IL-6 level in patients). IL-6, interleukin-6.

Table IV. Correlation between serum IL-6 and serum biochemical and blood parameters in patients with cutaneous melanoma.

Biochemical/ blood parameters	AsAT	AlAT	GGT	Serum glucose	Serum creatinine	WBC
ϱ	0.408	0.328	0.758	0.621	0.434	0.384
P-value	0.015	0.050	0.007	<0.0001	0.015	0.019

AsAT, aspartate aminotransferase; AlAT, alanine aminotransaminase; GGT, γ-glutamyl transferase; WBC, white blood cell.

Table V. Univariate and multivariate Cox proportional analysis of the survival of patients with cutaneous melanoma. (pTNM staging system is not included in multivariate analysis because it depends on another variable in the analysis, the presence of lymph node metastases, LN metastases)

	Univariate analysis		Multivariate analysis model 1, n=30		Multivariate analysis model 2, n=32	
Variable	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)
Sex, category (n)						
Male (32) vs. female (44)	<0.0001	4.51 (2.19-9.28)	0.015	44.29 (2.08-942.27)	0.004	14.09 (2.34-84.74)
Age	0.010	1.03	0.217	1.06 (0.97-1.15)	0.105	1.06 (0.99 -1.15)
Clark score, category (n)						
3+4+5 (34) vs. 1+2 (18)	0.024	4.41 (1.21-16.04)	0.263	3.52 (0.39-31.90)	0.719	1.41 (0.22-9.21)
LN metastasis, category (n)						
Yes (6) vs. no (55)	<0.0001	9.54 (3.48-26.26)	0.098	42.27 (0.50-358.20)	0.211	16.43 (0.20-132.40)
pTNM staging, category (n)						
III-IV (10) vs. I-II (54)	0.002	4.04 (1.67-9.76)				
Occupational hazard, category (n)						
Present (36) vs. absent (30)	0.022	3.03 (1.18-7.81)	0.188	0.11 (0.01-2.93)	0.047	6.83 (1.03-45.45)
Hemoglobin, g/l (n)						
<120 (10) vs. ≥120 (39)	0.001	5.09 (2.01-12.88)	0.588	2.29 (0.11-46.29)	0.248	13.60 (0.16-113.70)
Serum IL-6, pg/ml (n)						
≥5.68 (30) vs. <5.68 (28) vs.	0.048	2.25 (1.01-5.02)	0.299	5.67 (0.21-149.70)		
-174G>C IL6, SNP (n)						
GG (29) vs. GC+CC (29)	0.303	1.51 (0.69-3.29)			0.030	9.61 (1.24-74.28)

Multivariate analysis model 1 used serum IL-6 levels as a co-variable, while multivariate analysis model 2 used *-174G>C IL6* genotypes as a co-variable. pTNM staging system was not included in the multivariate analyses since this was not an independent variable but depended on another variable in the analysis (the presence of LN metastases). LN, lymph node; IL-6/IL6, interleukin-6; SNP, single nucleotide polymorphism.

was associated with increased exposure to sunlight (presence of occupational hazard; median, 9.43 pg/ml) and those who worked indoors (median, 4.56 pg/ml; P<0.0001; Fig. 8B).

IL-6 serum levels were positively correlated with liver-specific enzyme levels, including AsAT (ϱ=0.408; P=0.015) AlAT (ϱ=0.328; P=0.050) and GGT (ϱ=0.758; P=0.007). IL-6 was also positively correlated with blood glucose (Rho=0.621; P<0.0001) and creatinine levels (ϱ=0.434; P=0.015), as well as WBC count (ϱ=0.384; P=0.019; Table IV).

Subsequently, the median patient IL-6 serum level (5.68 pg/ml) was selected as the cut-off value for survival analysis. Patients with higher serum IL-6 levels exhibited significantly shorter survival times after diagnosis, compared with those with IL-6 levels below the cut-off (median, 58.63 vs. 237.52 months; P=0.042; Fig. 9).

Cox univariate analysis demonstrated that several demographic, clinical and blood parameters had significant adverse effects on the survival of patients with CMM. These included male sex (P<0.0001), advanced age (P=0.010), occupational conditions with increased exposure to sunlight (P=0.022, low hemoglobin levels (P=0.001), greater Clark scale depth (3+4+5; P=0.024) and the presence of lymph node metastases (P<0.0001); higher serum IL-6 levels were also adversely associated with survival (P=0.048; Table V).

Factors identified as significant using univariate analysis were then assessed using the multivariate Cox's proportional hazard model (Model 1). IL-6 serum level was no longer a significant factor (P=0.299), and only the male sex remained an independent risk factor for shorter survival time (P=0.015; Table V). A second Cox's proportional hazard model (Model 2)

Table VI. Genotype and allele frequencies of the *TNFA -308G>A* gene polymorphism in patients with cutaneous malignant melanoma (n=76 and n=152, respectively) and controls (n=198 and n=396, respectively).

A, Genotype frequency (P=0.810; χ^2 test)

Variable	Patients, n (frequency)	Controls, n (frequency)	OR (95% CI)	P-value
GG	63 (0.829)	160 (0.808)	1.0 (reference)	
GA	12 (0.158)	33 (0.167)	0.924 (0.449-1.901)	0.829
AA	1 (0.013)	5 (0.025)	0.508 (0.058-4.434)	0.540
GA+AA	13 (0.171)	38 (0.192)	0.869 (0.434-1.739)	0.691

B, Allele frequency (P=0.878, χ^2 test)

Variable	Patients, n (frequency)	Controls, n (frequency)	OR (95% CI)	P-value
-308 G	138 (0.908)	353 (0.891)	1.0 (reference)	
-308 A	14 (0.092)	43 (0.109)	0.833 (0.445-1.558)	0.641

Frequency was calculated by dividing the number of patients in each group by the total number of patients. OR, odds ratio; TNFA, tumor necrosis factor-α.

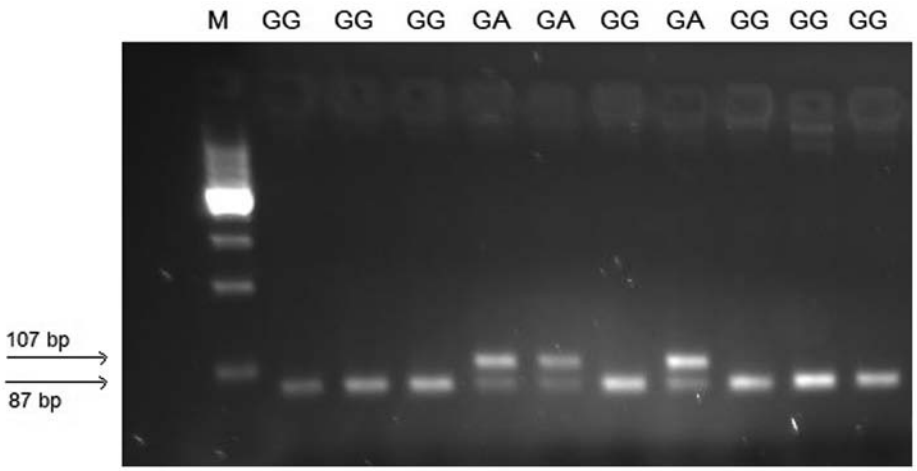

Figure 10. Agarose gel visualization of PCR-restriction fragment length polymorphism products when genotyping for the *TNFA -308G>A* single nucleotide polymorphism. Only the bigger bands of 107 and 87 bp in length are visible. TNFA, tumor necrosis factor-α; bp, base pair; M, marker.

was also used, which included genotypes with the *IL6* -174G>C SNP together with all significant factors identified during univariate analysis of routine demographic, clinical and blood parameters. Male sex (P=0.004), occupational conditions with increased exposure to sunlight (P=0.047 and the GG genotype (P=0.030) remained independent prognostic factors for shorter survival time (Table V).

TNFA -308G>A SNP. Genotyping for the -308G>A polymorphism in the *TNFA* promoter was performed by PCR-RFLP. The resulting PCR product was 107 bp in length, and the subsequent restriction reaction for the G allele (GG genotype) resulted in 2 fragments of 87 and 20 bp. The variant A allele remained unchanged (107 bp; Fig. 10).

Genotyping of the *TNFA* -308G>A SNP was successfully performed in 76 patients with CMM and 198 control individuals. The genotype distribution in both the control and patient group did not deviate from HWE (P=0.148 and P=0.889, respectively; χ^2 test). In the patient group, 63 (82.9%) carried the GG genotype, 12 (15.8%) were GA genotype-positive, and only one patient (1.3%) carried the AA genotype. The genotype distribution among the controls was 160 (80.8%) with the GG genotype, 33 (16.7%) with GA genotype and 5 (2.5%) with the AA genotype. No significant differences in genotype and allelic distribution between the patients and controls were observed (P=0.810 and P=0.982; χ^2 test; Table VI). Furthermore, no associations were found between genotypes with the *TNFA -308G>A* SNP and biochemical or clinical parameters in patients with CMM (data not shown).

Discussion

Melanoma cells are derived from normal melanocytes transformed due to various extrinsic and intrinsic factors. The immune response strongly influences the development and

progression of melanoma (14,34). Previous studies identified IL-6 as one of the most important regulatory cytokines in tumor biology, which is involved in key stages of tumor development, such as proliferation, apoptosis, angiogenesis and differentiation (38,39). However, IL-6 has pleiotropic effects in carcinogenesis, including strong growth-stimulating, as well as antitumor effects (39). During tumor progression, IL-6 changes from a paracrine inhibitor of normal melanocytes in the early stages, to an autocrine mitogen in the advanced stages of disease (15). Additionally, it exerts a paracrine effect on tumor angiogenesis and cells of the immune system (15). Thus, IL-6 acts as an inhibitor of tumor growth in early melanoma, but appears to be an important growth factor in advances stages of the disease (33,40).

The expression of IL-6 depends on a variety of factors, including polymorphisms in the *IL6* gene. The *IL6* -174G>C (rs1800795) polymorphism is localized at the promoter region of the gene, and is associated with altered promoter activity and resulting protein expression levels (41). A previous study reported 2-fold lower expression in HeLa cells transfected with a vector containing the C allele construct, compared with cells transfected with the G allele construct (41). Another *in vitro* study also demonstrated that the G allele of the *IL6* -174G>C SNP was associated with an increased transcriptional response to various stimuli (42). However, the results of studies investigating the levels of circulating IL-6 are conflicting. In healthy subjects, the G allele genotypes (GG and GC) were associated with higher IL-6 plasma levels compared with the CC genotype (41), while Jones *et al* (43) detected high plasma IL-6 levels in individuals with the C allele and CC genotypes. The results of the present study are in a line with the latter study (43), although carriers of C allele genotypes (CG+CC) exhibited significantly higher serum IL-6 levels in the control group only, not in patients with CCM.

The effects of the *IL6* -174G>C SNP on the progression of different cancer types have been widely explored, with quite controversial results (27). Zhai *et al* (27) performed a comprehensive meta-analysis of 17 studies including 4,304 patients with various cancer types, including breast, colorectal, lung, ovarian cancer and lymphoma. This previous study assessed the association between the *IL6* -174 polymorphism and cancer prognosis. No association with overall survival was observed from pooled analysis of the three genotypes with this SNP. However, the GG genotype affected patient survival compared with the C allele genotypes (GC+CC) in bladder, ovarian, gastric and peritoneal cancer, as well as in neuroblastoma and osteosarcoma.

To date, there are only a limited number of studies concerning the possible role of the *IL6* -174G>C SNP in CMM (31,44). The present study is the first, to the best of our knowledge, describing the possible effect of this polymorphism on Bulgarian patients with CMM. The results of the current study concur with those aforementioned (27), which also demonstrated no significant differences in genotype or allelic frequency of the *IL6* -174G>C SNP between patients with CMM and controls. Thus, these results suggested that *IL6* -174G>C is unlikely to be heavily involved in patient susceptibility to CMM (31,44).

Investigating the associations between *IL6* -174G>C SNP genotype frequencies and different clinical characteristics of patients with CMM, the present results suggested that the *IL6* -174 GG genotype was associated with a more advanced stage, thicker tumors and reduced overall survival. Similarly, a previous study on the role of the *IL6* -174G>C SNP demonstrated that the C allele decreased the risk of developing urinary bladder cancer, and that the GG genotype was associated with more advanced disease stages (45). By contrast, Leibovici *et al* (46) reported that the CC genotype was more frequently observed in the advanced stages of bladder cancer.

The association between *IL6* -174G>C and disease progression may be due to the potential effects on IL-6 expression. The results of the present study indicated that higher serum IL-6 levels were associated with unfavorable blood/serum characteristics (poor WBC count, liver enzyme levels, blood glucose and creatinine), tumor progression (development of new distant metastases) and shorter survival time. These results concur with those of Tobin *et al* (19), which suggested an association between increased plasma IL-6 levels in patients with stage IV melanoma and tumor burden, as well as shorter survival.

A notable finding of the present study was the relationship between serum IL-6 and patient working conditions. Those working outdoors with supposedly increased exposure to sunlight had significantly higher IL-6 levels compared with patients working indoors. This finding suggested that sunlight may stimulate IL-6 expression, which is in line with previous observations of increased serum levels of the melanoma tumor markers (IL-1α, IL-4, IL-6, IL-10, TNF-α and interferon-γ), so-called 'melanoma inhibitory activity', following phototherapy with UV light (47).

TNF-α is one of the most important pro-inflammatory cytokines involved in cellular proliferation, differentiation and apoptosis, and has been reported to play a critical role in carcinogenesis (48). A previous study demonstrated that protein expression and transcriptional levels of TNF-α were related to several promoter polymorphisms in cytokine-encoding genes, including the *TNFA* -308G>A SNP (49). In previous studies, the A allele of this SNP was associated with elevated TNF-α transcription *in vitro* (50-52), and with increased serum TNF-α in patients with acute myocardial infarction (53). By contrast, Sharma *et al* (54) reported that the A allele of the *TNFA* -863C>A SNP was associated with reduced serum TNF-α levels in patients with asthma (54). Similarly, the wild-type G-allele of *TNFA* -308G>A was associated with significantly higher TNFA mRNA expression in human blood leucocytes, compared with the A allele (55).

In the current study, no differences were found in the genotype or allelic distributions between patients with CMM and the controls. This result confirms the reported lack of association between the *TNFA* -863C>A SNP and increased risk of melanoma (13,33,56). By contrast, previous meta-analyses suggested that the *TNFA* -308G>A polymorphism was a risk factor for a range of other malignancies, such as gastric, breast and hepatocellular cancer (57-59), whilst other studies did not demonstrate any significant association between *TNFA* -308G>A and the risk of cancer (60).

In conclusion, the results of the present study suggested that the *IL6* -174G>C and the *TNFA* -308G>A promoter polymorphisms were not predisposing factors for CMM. However, the *IL6* -174G>C SNP and IL-6 serum concentrations are likely to influence the progression of CMM. The GG geno-

type and higher serum levels may be associated with tumor progression and shorter survival. Although the GG genotype was associated with lower IL-6 levels, higher IL-6 levels may be influenced by other factors, including sun light, and not only by the genotype.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TV, MK, MD and DV conceived and designed the study. TV, DD, DV and NO wrote and revised the manuscript. TV, AA and MK performed the genotyping. TV, TT, DD, DV and AM collected the biological material and clinical data, and analyzed the data. DD and AM acquired the informed consent and approval by the Ethics Committee, and were involved in drafting the manuscript. All authors contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all participants included in the study. The study protocol was approved by The Ethics Committee at The Medical Faculty of Trakia University. The present study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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