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ORIGINAL ARTICLE

Polymorphisms in the CD28/CTLA4/ICOS genes: role in malignant melanoma susceptibility and prognosis?

Marna G. Bouwhuis · Andreas Gast · Adina Figl · Alexander M. M. Eggermont · Kari Hemminki · Dirk Schadendorf · Rajiv Kumar

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Abstract The appearance of vitiligo and spontaneous regression of the primary lesion in melanoma patients illustrate a relationship between tumor immunity and autoimmunity. T lymphocytes play a major role both in tumor immunity and autoimmunity. CD28, Cytotoxic T lymphocyte antigen 4 (CTLA4) and inducible costimulator (ICOS) molecules are important secondary signal molecules in the T lymphocyte activation. Single nucleotide polymorphisms (SNPs) in the CD28/CTLA4/ICOS gene region were reported to be associated with several autoimmune diseases including, type-1 diabetes, SLE, autoimmune thyroid diseases and celiac disease. In this study, we investigated the association of SNPs in the CD28, CTLA4 and ICOS genes with the risk of melanoma. We also assessed the prognostic effect of the different polymorphisms in melanoma patients. Twenty-four tagging SNPs across the three genes and four additional SNPs were genotyped in a cohort of 763 German melanoma patients and 734 healthy German controls. Influence on prognosis was determined in 587 melanoma cases belonging to stage I or II of the disease. In general, no differences in genotype or allele frequencies were detected between melanoma patients and controls. However, the variant alleles for two polymorphisms in the CD28 gene were differentially distributed in cases and controls. Similarly no association of any polymorphism with prognosis, except for the rs3181098 polymorphism in the CD28 gene, was observed. In addition, individuals with AA genotype for rs11571323 polymorphism in the ICOS gene showed reduced overall survival. However, keeping in view the correction for multiple hypothesis testing our results suggest that the polymorphisms in the CD28, CTLA4 and ICOS genes at least do not modulate risk of melanoma and nor do those influence the disease prognosis in the investigated population.

Keywords Melanoma · Polymorphism · CD28/CTLA4/ICOS · Susceptibility · Prognosis

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M. G. Bouwhuis · A. M. M. Eggermont
Erasmus Medical Center-Daniel den Hoed Cancer Center, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

A. Gast · A. Figl · K. Hemminki · R. Kumar
Division of Molecular Genetic Epidemiology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany

D. Schadendorf
Department of Dermatology, University Hospital Essen, Hufelandstraße 55, 45147 Essen, Germany

A. Gast
Department of Dermatology, University Hospital, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

M. G. Bouwhuis (✉)
Department of Surgery, Laboratory Experimental Surgical Oncology, Section Surgical Oncology, Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands
e-mail: m.bouwhuis@erasmusmc.nl

Introduction

The association between tumor immunity and autoimmunity is complex [1]. Spontaneous regression is believed to be more common in melanoma than any other cancer types. However, the effect of the phenomenon on prognosis is rather unclear; however, the vitiligo is considered a favorable prognostic factor. Autoimmune conditions like thyroiditis and vitiligo, induced by interleukin 2 and/or

Interferon α (IFN) therapy, have been associated with an improved prognosis in melanoma patients [2, 3]. The appearance of autoantibodies or autoimmune manifestations in IFN-treated patients has been reported to be associated with significantly improved recurrence free and overall survival [4]. However, the findings could not be replicated when serum samples were analyzed of patients that were randomized to IFN treatment or observation in the EORTC 18952 and the Nordic Melanoma Group phase III trials [5].

T lymphocytes play an important role both in tumor immunity as well as in autoimmunity. The CD28, cytotoxic T lymphocyte antigen 4 (CTLA4) and inducible co-stimulator (ICOS) molecules are important secondary signaling molecules involved in the T lymphocyte activation. The genes encoding CD28, CTL4 and ICOS are located within a stretch of 300 kb region on chromosome 2q33. Ligation of CD28 molecules with the B7-1 (CD80) or B7-2 (CD86) on antigen presenting cells (APCs), stimulate T cell activation and proliferation. CTLA4 counterbalances this effect by competing with CD28 for B7-1/B7-2 binding and is therefore an important inhibitor of T cell activation [6, 7]. CTLA-4 is also an established negative regulator of T-cell function and proliferation through multiple mechanisms such as reducing interleukin (IL)-2 and IL-2 receptor productions and arresting T-cell at the G1-phase of cell cycle [8]. ICOS is another co-stimulatory molecule which is expressed on activated T cells. It binds to a unique ligand, ICOSL, and does not bind to other ligands such as B7-1/B7-2. Polymorphisms in the CD28/CTLA4/ICOS gene region have been associated with several autoimmune diseases including, type 1 diabetes, SLE, autoimmune thyroid diseases and celiac disease [9, 10]. However, a majority of the studies focused on the known CTLA4 polymorphisms. A high prevalence of AA genotype for the CT60 polymorphism in the gene was observed in patients with renal cell cancer and a positive correlation between the polymorphism and tumor grade was also established [11]. The association between the variants in the promoter region of the CTLA4 gene and breast cancer progression has also been reported [12].

In this study, in order to find an association between polymorphisms in the CD28, CTLA-4 and ICOS genes and risk of cutaneous melanoma we screened patients from Germany and ethnically matched healthy controls. The single nucleotide polymorphisms (SNPs) in the three CD28/CTLA4/ICOS genes were selected by tagging approach in order to cover the entire gene regions. Additionally four SNPs reported to be of interest in literature in the CTLA-4 gene were also included in the study. The association of variants alleles with prognostic outcome was also determined.

Methods

Patients and controls

The study population consisted of 763 melanoma patients from Germany (418 male and 345 female), recruited by the Skin Cancer Unit Mannheim, from 2001 to 2008. Patients with primary cutaneous melanoma with different disease stages that included, 10 cases with in situ melanoma, 615 with stage I/II, 111 stage III and 12 cases with stage IV of the disease. For 15 patients stage was unknown. Disease staging was performed according to the current AJCC criteria from 2001 [13]. Median and mean age of the melanoma cases at diagnosis was 55 and 54 years, respectively. Blood samples from case subjects were taken at their first presentation at the skin cancer unit. DNA was isolated from blood samples using Qiagen mini-preparation kits. Informed consent was obtained from the patients and the study was approved by the institutional ethical review board. Control subjects included 734 healthy German individuals (367 male and 367 female) recruited from blood bank Mannheim, with mean and median age of 60 and 61 years, respectively. They were born in southwest Germany and were matched for ethnicity with cases. The inclusion criteria for controls in the study included cancer free status. The age difference between the cases and controls was statistically significant (T-test; P-value <0.01), whereas, the gender difference was not statistically significant (χ^2-test; P-value >0.05).

Genes and SNPs selection

The selection of polymorphisms in the CD28, CTLA4 and ICOS genes was based on inclusion of known non-synonymous SNPs and those located in regulatory regions as reported in the dbSNP database of the National Center for Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov/SNP/) or reported in published papers. Additionally, tagging SNPs from each gene region were selected from HapMap data using Haploview software 3.32, with pair-wise $r^2 > 0.8$ for each SNP pair and minor allele frequencies >5% (Fig. 1). Ten tagging SNPs in the CD28 gene, five in the CTLA4 gene and 10 in the ICOS gene were selected from HapMap database that covered three genes completely. Four polymorphisms, rs11571319 (CT61), rs11571302 (JO31), rs7665213 (JO30) and rs11571297 (JO27) in the CTLA4 gene, which have been described to correlate with autoimmune disease(s) were also selected. The investigated polymorphisms span a region of 31.0 kb for the CD28 gene region, 6.1 kb for the CTLA4 gene region and 24.7 kb for the ICOS gene region. In total, 29 polymorphisms in three genes (CD28, CTLA4 and ICOS) were identified.

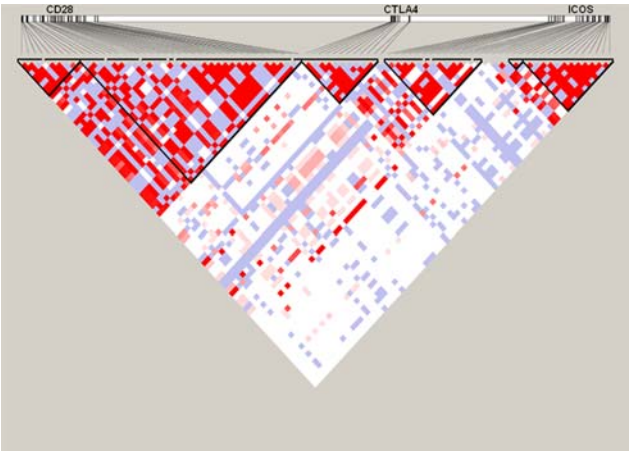

Fig. 1 Haplotype blocks in the genomic region with CTLA-4, CD28 and ICOS genes based on HapMap data

Validation of the SNPs by DNA sequencing

The validation of the 29 selected polymorphisms was carried out by sequencing a set of 32 DNA samples from control subjects. Sequencing reactions were performed using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Ca, USA) and the following conditions were used; initial 94°C for 1 min followed by 27 cycles at 96°C for 16 s, 56°C for 5 s and 60°C for 4 min. Reaction products were run on ABI prism 3100 Genetic analyzer (Applied Biosystems). Primers used in PCR amplification and sequencing reaction are listed in Supplementary Table.

Genotyping

Genotyping of the validated SNPs was performed by allelic discrimination technique (TaqMan assays, 'by demand or design' Applied Biosystems, Supplementary Table). Genotyping for one polymorphism in the ICOS gene (rs4355090) failed and was, thus, excluded from the study. Genotype failure rate was 0.14%, calculated from samples that could not be genotyped after two repeated assays and by direct DNA sequencing. Genotyping data were confirmed by random direct DNA sequencing of 5% of all samples, which showed 100% concordance.

Statistical analysis

The association between malignant melanoma and different genotypes was estimated as odds ratios (OR), 95% confidence intervals (CI) and *P*-values using SAS version 9.1. Estimates were adjusted for gender and age. Haplotype procedure of SAS/Genetics Software was used to calculate haplotype frequencies in cases and controls. Linkage disequilibrium (LD) was calculated with Haploview software (www.broad.mit.edu/mpg/haploview/documentation.php). The association between genotypes and different survival parameters, adjusted for age, gender and Breslow thickness, was carried out using proportional hazard regression (Cox) model. Metastases free survival (MFS) was the time from date of diagnosis until the first metastasis (either lymph node or distant metastasis) and overall survival (OS) was time from diagnosis to death. The follow-up of patients without metastases or who did not decease has been censored at the latest visit/last contact.

Results

Case-control study

The allelic distribution of polymorphisms in the CD28, CTLA4 and ICOS genes was assessed in 763 German melanoma patients and compared with 734 healthy German controls. A total of 28 SNPs were studied and genotype and allele distributions of all the polymorphisms are summarized in Table 1. Genotype frequency in controls for all the polymorphisms was in accordance with the Hardy–Weinberg equilibrium. Minor allele frequency (MAF) for the rs3181098 polymorphism was higher in cases than in controls (OR: 1.18, 95% CI: 1.00–1.38; $P = 0.05$). And for the rs3181100 (C > G) polymorphisms the MAF was lower in cases than controls (OR: 0.83, 95% CI: 0.71–0.97; $P = 0.02$). None of the inferred haplotypes in three genes showed differential distribution between cases and controls (data not shown).

Association between polymorphisms and prognosis

The association between polymorphisms and survival parameters was evaluated for melanoma patients in stage I and II. Information regarding metastases free survival (MFS), overall survival (OS) and Breslow thickness was available for 587 patients (321 male and 266 female). Mean age was 54 years (median 55 years), the mean and median Breslow thickness was 1.84 and 1.50 mm, respectively. Ulceration status of the primary tumor was not systematically recorded in the past and is therefore lacking. Age, gender and Breslow thickness were included as covariates in the Cox regression analysis. Overall, on comparing carriers versus non-carriers, no significant differences in OS were observed (Tables 2, 3, 4). A single SNP in the CD28 gene (rs3181098) showed an association with reduced metastases free survival (HR 1.34 95% CI: 1.02–1.77). In addition to the carrier versus non-carrier approach, effect of the different genotypes on prognosis was analyzed. According to this analysis, one SNP (AA) in the ICOS gene

Table 1 Case control

SNP	Genotype	Cases $N = 763$	(%)	Controls $N = 734$	(%)	OR	95% CI	*P*-value
CD28								
rs3181098	GG	315	41	331	45			
	AG	331	43	325	44	1.05	0.84–1.31	
	AA	117	15	78	11	1.50	1.07–2.11	0.06
	G-allele	961	63	987	67			
	A-allele	565	37	481	33	1.18	1.00–1.38	0.05
rs3181100	CC	279	37	229	31			
	CG	368	48	357	49	0.87	0.69–1.10	
	GG	116	15	145	20	0.68	0.50–0.93	0.05
	C-allele	926	61	815	56			
	G-allele	600	39	647	44	0.83	0.71–0.97	0.02
rs3181101	CC	570	75	559	77			
	CG	175	23	165	23	0.98	0.76–1.26	
	GG	17	2	7	10	1.97	0.78–4.96	0.35
	C-allele	1,315	86	1,283	88			
	G-allele	209	14	179	12	1.06	0.85–1.33	0.61
rs1181390	GG	474	62	467	64			
	GT	257	34	233	32	1.14	0.91–1.43	
	TT	32	4	33	5	0.92	0.55–1.55	0.47
	G-allele	1,205	79	1,167	80			
	T-allele	321	21	299	20	1.06	0.88–1.28	0.53
rs1181388	GG	575	76	545	74			
	AG	169	22	170	23	0.98	0.76–1.26	
	AA	17	2	18	3	0.90	0.45–1.81	0.95
	G-allele	1,319	87	1,260	86			
	A-allele	203	13	206	14	0.97	0.78–1.20	0.76
rs17533594	AA	483	63	474	65			
	AG	257	34	232	32	1.11	0.89–1.40	
	GG	23	3	24	3	0.94	0.52–1.73	0.63
	A-allele	1,223	80	1,180	81			
	G-allele	303	20	280	19	1.06	0.88–1.28	0.55
rs3116494	AA	414	54	393	54			
	AG	307	40	299	41	1.01	0.81–1.26	
	GG	42	6	39	5	1.09	0.67–1.75	0.94
	A-allele	1,135	74	1,085	74			
	G-allele	391	26	377	26	1.02	0.86–1.21	0.79
rs3181107	AA	659	86	620	85			
	AG	100	13	106	15	0.92	0.67–1.25	
	GG	4	1	6	1	0.74	0.20–2.79	0.78
	A-allele	1,418	93	1,346	92			
	G-allele	108	7	118	8	0.91	0.68–1.20	0.49
rs3116496 (IVS3 + 17)	TT	487	64	475	65			
	CT	254	33	231	32	1.10	0.88–1.38	
	CC	22	3	24	3	0.89	0.48–1.64	0.63
	T-allele	1,228	81	1,181	81			
	C-allele	298	20	279	20	1.04	0.86–1.26	0.66

Table 1 continued

SNP	Genotype	Cases $N = 763$	(%)	Controls $N = 734$	(%)	OR	95% CI	P-value
CTLA4								
rs16840252	CC	521	68	489	67	1.00		
	CT	218	29	222	30	0.91	0.72–1.15	
	TT	23	3	21	3	1.02	0.54–1.91	0.74
	C-allele	1,260	83	1,200	82			
	T-allele	264	17	264	18	0.95	0.78–1.15	0.57
rs5742909 (CT44)	CC	619	81	596	81	1.00		
	CT	136	18	130	18	0.96	0.73–1.27	
	TT	8	1	8	1	0.89	0.32–2.49	0.95
	C-allele	1,374	90	1,322	90			
	T-allele	152	10	146	10	0.96	0.75–1.23	0.74
rs231775 (CT42)	AA	289	38	283	39	1.00		
	AG	369	48	345	47	1.08	0.86–1.36	
	GG	104	14	106	14	0.97	0.70–1.36	0.71
	A-allele	947	62	911	62			
	G-allele	577	38	557	38	1.01	0.87–1.18	0.90
rs231777	CC	539	71	514	70	1.00		
	CT	208	27	203	28	0.97	0.76–1.23	
	TT	15	2	16	2	0.83	0.39–1.77	0.87
	C-allele	1,286	84	1,231	84			
	T-allele	238	16	235	16	0.95	0.78–1.17	0.64
rs3087243 (CT60)	GG	246	32	223	30	1.00		
	AG	355	47	388	53	0.81	0.63–1.03	
	AA	162	21	122	17	1.22	0.89–1.65	0.01
	G-allele	847	56	834	57			
	A-allele	679	45	632	43	1.06	0.91–1.23	0.45
rs11571319 (CT61)	GG	518	68	488	67	1.00		
	AG	222	29	223	31	0.93	0.73–1.17	
	AA	23	3	21	3	1.02	0.54–1.92	0.81
	G-allele	1,258	82	1,199	82			
	A-allele	268	18	265	18	0.96	0.79–1.16	0.64
rs11571302 (JO31)	GG	225	30	210	29	1.00		
	GT	370	49	383	52	0.87	0.68–1.12	
	TT	168	22	140	19	1.14	0.84–1.55	0.15
	G-allele	820	54	803	55			
	T-allele	706	46	663	45	1.05	0.90–1.22	0.53
rs7665213 (JO30)	GG	228	30	211	29	1.00		
	AG	370	49	383	52	0.87	0.68–1.12	
	AA	165	22	137	19	1.13	0.83–1.53	0.17
	G-allele	826	54	805	55			
	A-allele	700	46	657	45	1.04	0.90–1.21	0.59
rs11571297 (JO27)	TT	214	28	193	26	1.00		
	CT	376	49	393	54	0.84	0.65–1.08	
	CC	173	23	148	20	1.07	0.79–1.45	0.15
	T-allele	804	53	779	53			
	C-allele	722	47	689	47	1.02	0.88–1.18	0.81

Table 1 continued

SNP	Genotype	Cases $N = 763$	(%)	Controls $N = 734$	(%)	OR	95% CI	*P*-value
ICOS								
rs10932029 (+173)	TT	538	71	488	67	1.00		
	CT	204	27	228	31	0.82	0.65–1.03	
	CC	21	3	15	2	1.14	0.57–2.27	0.20
	T-allele	1,280	84	1,204	82			
	C-allele	246	16	258	18	0.89	0.73–1.08	0.23
rs4335928	TT	579	76	559	76	1.00		
	CT	170	22	162	22	0.99	0.77–1.28	
	CC	14	2	12	2	1.11	0.50–2.47	0.96
	T-allele	1,328	87	1,280	87			
	C-allele	198	13	186	13	1.01	0.80–1.26	0.96
rs4675374	CC	457	60	436	60	1.00		
	CT	272	36	258	35	0.98	0.78–1.23	
	TT	34	5	38	5	0.85	0.52–1.40	0.82
	C-allele	1,186	78	1,130	77			
	T-allele	340	22	334	23	0.96	0.80–1.14	0.62
rs7602383	AA	550	72	533	73	1.00		
	AG	197	26	183	25	1.01	0.79–1.29	
	GG	16	2	17	2	0.93	0.46–1.90	0.98
	A-allele	1,297	85	1,249	85			
	G-allele	229	15	217	15	0.99	0.81–1.23	0.95
rs4521021	TT	450	59	451	61	1.00		
	CT	276	36	260	35	1.00	0.80–1.25	
	CC	37	5	23	3	1.47	0.83–2.60	0.41
	T-allele	1,176	77	1,162	80			
	C-allele	350	23	306	21	1.07	0.89–1.28	0.45
rs11571323	GG	587	77	534	73	1.00		
	AG	161	21	184	25	0.81	0.63–1.04	
	AA	15	2	13	2	1.30	0.60–2.84	0.18
	G-allele	1,335	88	1,252	86			
	A-allele	191	13	210	14	0.89	0.71–1.10	0.29
rs12466129	TT	448	59	451	62	1.00		
	AT	273	36	239	33	1.14	0.91–1.43	
	AA	42	6	42	6	0,94	0.59–1.51	0.47
	T-allele	1,169	77	1,141	78			
	A-allele	357	23	323	22	1.06	0.89–1.27	0.54
rs10172036	GG	301	40	283	39	1.00		
	GT	353	46	352	48	0.92	0.73–1.15	
	TT	109	14	97	13	1.04	0.75–1.45	0.63
	G-allele	955	63	918	63			
	T-allele	571	37	546	37	0.99	0.85–1.16	0.93
rs10183087	AA	461	61	418	57	1.00		
	AC	263	35	277	38	0.85	0.68–1.06	
	CC	38	5	37	5	1.04	0.63–1.70	0.32
	A-allele	1,185	78	1,113	76			
	C-allele	339	22	351	24	0.92	0.77–1.10	0.36

Table 1 continued

SNP	Genotype	Cases $N = 763$	(%)	Controls $N = 734$	(%)	OR	95% CI	*P*-value
rs10932036	AA	611	80	594	81	1.00		
	AT	144	19	129	18	1.09	0.83–1.44	
	TT	7	1	7	1	1.12	0.37–3.35	0.81
	A-allele	1,366	90	1,317	90			
	T-allele	158	10	143	10	1.08	0.85–1.39	0.53

Table 2 CD28

SNP	Genotype	Cases (%) $N = 587$	Metastases free survival			Overall survival		
			OR	95% CI	*P*-value	OR	95% CI	*P*-value
rs3181098	GG	247 (42)						
	AG/AA	340 (58)	1.34	1.02–1.77	0.04	1.18	0.81–1.72	0.38
rs3181100	CC	212 (36)						
	CG/GG	375 (64)	0.82	0.62–1.08	0.16	0.82	0.56–1.20	0.31
rs3181101	CC	440 (75)						
	CG/GG	146 (25)	1.28	0.94–1.74	0.12	1.21	0.80–1.83	0.38
rs1181390	GG	360 (61)						
	GT/TT	227 (39)	0.92	0.70–1.21	0.56	1.16	0.80–1.67	0.45
rs1181388	GG	440 (75)						
	AG/AA	146 (25)	0.86	0.63–1.17	0.32	0.82	0.53–1.25	0.35
rs17533594	AA	372 (63)						
	AG/GG	215 (37)	1.03	0.78–1.35	0.84	1.23	0.85–1.79	0.28
rs3116494	AA	319 (54)						
	AG/GG	268 (46)	0.99	0.76–1.30	0.96	1.13	0.79–1.62	0.52
rs3181107	AA	506 (86)						
	AG/GG	81 (14)	0.81	0.54–1.21	0.30	0.73	0.41–1.29	0.28
rs3116496 (IVS3 +17)	TT	378 (64)						
	CT/CC	209 (36)	1.16	0.88–1.53	0.29	1.39	0.96–2.02	0.08

(rs11571323) was associated with reduced overall survival, $P = 0.04$, HR 3.60; 95% CI, 1.31–9.91, however, only 13 (2.2%) melanoma patients carried AA genotype.

Discussion

The immunogenic nature of malignant melanoma is clinically manifested by spontaneous regression and appearance of vitiligo. The phenomenon of autoimmunity observed during various forms of immunotherapy, IL-2, IFN and anti-CTLA4 therapy, have been linked to the treatment response [2–4]. To understand the link between tumor immunity and autoimmunity in melanoma and to explore its implication on disease susceptibility and prognosis remains a challenge [14]. The results from studies evaluating polymorphisms in various autoimmune diseases suggest the existence of a common autoimmune disease locus in the CTLA4 gene [9].

We genotyped 28 polymorphisms located in the CD28, CTLA4 and ICOS genes in melanoma patients and healthy controls. Use of tagging approach covered the entire loci for all three genes. To the best of our knowledge, the screen for SNPS in the CTLA4 gene was the largest ever performed in melanoma patients (and controls) and the first one for the ICOS and CD28 genes. Our results showed that the variant alleles for two polymorphisms in the CD28 gene (rs3181098 and rs3181100) were differentially distributed in cases and controls. No differences in genotype or allele frequencies were detected between melanoma patients and controls for any other polymorphism. Similarly, carriers of the variant allele for the polymorphism rs3181098 in the CD28 gene showed reduced metastasis free survival and for the polymorphism rs11571323 the individuals with variant allele homozygous genotype were associated with reduced overall survival. However, keeping in view the number of tests carried out in the present study, the observed significant

Table 3 CTLA4

SNP	Genotype	Cases (%) $N = 587$	Metastases free survival			Overall survival		
			OR	95% CI	*P*-value	OR	95% CI	*P*-value
rs16840252	CC	399 (68)						
	CT/TT	187 (32)	1.11	0.84–1.48	0.46	1.25	0.85–1.82	0.26
rs5742909 (CT44)	CC	476 (81)						
	CT/TT	111 (19)	1.24	0.88–1.72	0.22	1.40	0.90–2.18	0.14
rs231775 (CT42)	AA	219 (37)						
	AG/GG	367 (63)	0.99	0.75–1.30	0.93	0.92	0.63–1.33	0.64
rs231777	CC	414 (71)						
	CT/TT	172 (29)	1.04	0.78–1.39	0.79	1.23	0.83–1.80	0.30
rs3087243 (CT60)	GG	195 (33)						
	AG/AA	392 (67)	0.99	0.74–1.31	0.92	0.93	0.63–1.36	0.69
rs11571319 (CT61)	GG	397 (68)						
	AG/AA	190 (32)	1.10	0.83–1.45	0.51	1.19	0.81–1.73	0.38
rs11571302 (JO31)	GG	173 (30)						
	GT/TT	414 (71)	0.95	0.71–1.27	0.72	0.80	0.55–1.17	0.26
rs7665213 (JO30)	GG	176 (30)						
	AG/AA	411 (70)	0.95	0.72–1.27	0.74	0.83	0.57–1.21	0.32
rs11571297 (JO27)	TT	163 (28)						
	CT/CC	424 (72)	0.87	0.65–1.16	0.33	0.76	0.52–1.11	0.16

Table 4 ICOS

SNP	Genotype	Cases (%) $N = 587$	Metastases free survival			Overall survival		
			OR	95% CI	*P*-value	OR	95% CI	*P*-value
rs10932029 (+173)	TT	412 (70)						
	CT/CC	175 (30)	0.87	0.65–1.17	0.36	0.79	0.53–1.18	0.25
rs4335928	TT	446 (76)						
	CT/CC	141 (24)	1.17	0.86–1.60	0.32	1.26	0.82–1.92	0.29
rs4675374	CC	352 (60)						
	CT/TT	235 (40)	1.08	0.82–1.42	0.58	1.31	0.91–1.88	0.15
rs7602383	AA	421 (72)						
	AG/GG	166 (28)	1.02	0.76–1.37	0.88	1.12	0.75–1.66	0.59
rs4521021	TT	345 (59)						
	CT/CC	242 (41)	0.96	0.73–1.26	0.77	1.19	0.83–1.71	0.35
rs11571323	GG	452 (77)						
	AG/AA	135 (23)	1.07	0.78–1.46	0.70	1.27	0.83–1.94	0.28
rs12466129	TT	340 (58)						
	AT/AA	247 (42)	0.86	0.66–1.13	0.28	1.03	0.72–1.48	0.88
rs10172036	GG	232 (40)						
	GT/TT	355 (61)	1.12	0.85–1.50	0.42	0.86	0.58–1.26	0.43
rs10183087	AA	352 (60)						
	AC/CC	234 (40)	0.87	0.66–1.15	0.33	0.93	0.64–1.35	0.70
rs10932036	AA	467 (80)						
	AT/TT	119 (20)	0.76	0.54–1.06	0.11	0.76	0.48–1.19	0.23

associations would be lost upon multiple hypothesis correction. Moreover, the detected association would also require confirmatory testing in an independent population.

One of the limitations of the present study included lack of pigmentation data, history of sunburns and the existence of statistical significant difference in mean age between

cases and controls. Keeping in view the fact that ethnicity and not the age is major determinant of variant allele frequency, in our study design we ensured complete match between cases and controls for the latter parameter.

Our results are in accordance with a previous study that reported no difference in frequencies of six polymorphisms in the CTLA4 gene in 203 melanoma patients (stage IIb, IIc and III), compared to 288 healthy controls. Also no polymorphism correlated with improved recurrence free or overall survival [15]. However, several studies have reported association of the CTLA4 polymorphisms with other malignancies [16]. In humans cell CTLA4 exists in two isoforms, a full-length and a soluble isoform that lacks exon 3 due to alternative splicing [17]. The CT60 (A/G) polymorphism in the CTLA4 gene is a key susceptibility locus for autoimmune diseases, and the G-allele was shown to be correlated with decreased levels of the soluble isoform [9]. The frequency of the AA genotype for CT60 polymorphism was reported to be higher in renal cell carcinoma (RCC) patients than in controls. In addition, a positive correlation between the AA genotype and tumor grade was also observed, suggesting a role in tumor development [11]. The CT42 polymorphism (49A/G) in exon 1 is the only amino acid (Thr > Ala) altering polymorphism in the CTLA4 gene; and the individuals homozygous for the Ala allele were associated with decreased CTLA4 expression on the T cell surfaces [18]. The AA genotype was correlated with increased frequencies in RCC patients and the A allele, in association with the 3′-untranslated region $(AT)_{82}$ alleles, correlated with non-Hodgkin's lymphoma (NHL) [11, 19]. Interestingly, the GG variant was linked to an increased risk of gastric mucosa-associated lymphoid tissue (MALT) lymphoma [20]. In a study on multiple cancer types, 49A/G polymorphism has been risk of lung, breast and esophageal cancers as well as gastric cardia [16]. CTLA-4 with variant Thr allele has been shown to be associated with stronger inhibitory effect on T-cell activation than that with common allele. Polymorphisms in the promoter region of the CTLA4 gene were described to modulate expression of the gene [21]. This region contains the CT44 polymorphism (−318 C/T) variant. The CC genotype of the CT44 polymorphism was shown to be correlated with significantly reduced lymph node involvement in breast cancer patients [12]. The T allele was linked to an increased risk of B-CLL but to a decreased risk of MALT lymphoma [20, 22]. No correlation was found between the CT44 polymorphism and the risk of colon cancer [23]. The chromosomal region 2q33 containing the CTLA-4 and CD28 genes has been linked with asthma, however, the association with polymorphisms in the genes was not detected [24].

Melanoma patients with thick primary tumors and/or nodal involvement are at high risk for relapse or death [13]. However, adjuvant treatment is only beneficial in a small group of these patients. Genetic variability possibly predicts treatment outcome and could be a predictive marker to select the group benefiting from a certain treatment. In this study, only stage I and II melanoma patients were evaluated for a possible association between SNPs and prognosis. Since these patients do not frequently receive systemic treatment, we could not assess the predictive value of any of the polymorphisms. Nevertheless, recently it was shown that polymorphisms in the CTLA4 gene were correlated with response in melanoma patients (stage IV) receiving anti-CTLA4 treatment [25].

In conclusion, from the results of this large study we did not find convincing evidence for association between polymorphisms in the CD28, CTLA4 and ICOS genes and the risk of melanoma, nor with an effect on prognosis. Even two individual polymorphisms showed differential distribution of variant alleles between cases and controls, the effect nevertheless was marginal and a chance factor could no be ruled. The study was confined to German population, therefore, a strong association of polymorphisms investigated with melanoma susceptibility or disease outcome, in other populations cannot be entirely precluded.

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