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Inherited functional variants of the lymphocyte receptor CD5 influence melanoma survival

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Abstract

Despite the recent progress in treatment options, malignant melanoma remains a deadly disease. Besides therapy, inherited factors might modulate clinical outcome, explaining in part widely varying survival rates. T-cell effector function regulators on antitumor immune responses could also influence survival. CD5, a T-cell receptor inhibitory molecule, contributes to the modulation of antimelanoma immune responses as deduced from genetically-modified mouse models. The CD5 SNPs rs2241002 (NM_014207.3:c.671C>T, p.Pro224Leu) and rs2229177 (NM_014207.3:c.1412C>T, p.Ala471Val) constitute an ancestral haplotype (Pro224-Ala471) that confers T-cell hyper-responsiveness and worsens clinical autoimmune outcome. The assessment of these SNPs on survival impact from two melanoma patient cohorts (Barcelona, N=493 and Essen, N=216)

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reveals that p.Ala471 correlates with a better outcome (OR= 0.57, 95% CI=0.33-0.99, Adj. *P*=0.043, in Barcelona OR=0.63, 95% CI=0.40-1.01, Adj. *P*=0.051, in Essen). While, p.Leu224 was associated with increased melanoma-associated mortality in both cohorts (OR=1.87, 95% CI=1.07-3.24, Adj. *P*=0.030 in Barcelona and OR=1.84, 95% CI=1.04-3.26, Adj. *P*=0.037, in Essen). Furthermore survival analyses showed that the Pro224-Ala471 haplotype in homozygosis improved melanoma survival in the entire set of patients (HR=0.27, 95% CI 0.11 to 0.67, Adj. *P*=0.005). These findings highlight the relevance of genetic variability in immune-related genes for clinical outcome in melanoma.

Introduction

Melanoma is the most deadly of the common skin cancers in Caucasians. To date, histological features of the primary melanoma are important hallmarks of early melanoma prognosis and staging.1 However, patients within the same tumor stage exhibit a high variation in survival rates suggesting that other intrinsic factors modulate outcome.2 Melanoma is highly immunogenic, but can evade immune responses via several mechanisms including loss of expression of class I major histocompatibility complex antigens, production of immunosuppressive cytokines, activation of regulatory T-cells and expression of inhibitors for effector T cells (CTLA-4, PD-1/PD-L1).3 Progress in understanding the relationship between immune and tumor cells has led to the development of successful immunotherapies targeting the so called immune checkpoints (e.g. CTLA-4, PD-L1 and PD-1 inhibitors).4 Unfortunately, not all patients respond to these therapies and duration and effectiveness vary among responders.5 Thus, the identification of new therapeutic targets and prognostic biomarkers is essential to further improve patient care.

Accumulating clinical and experimental evidence indicates that the T-cell modulatory properties of CD5 might play a role in the antitumor immune response by acting as a putative immune regulator checkpoint.6, 7 CD5 is a lymphoid-specific 67kDa receptor, mainly expressed by all T cells and the small B1 subset involved in the production of polyreactive natural antibodies.8 Even though the nature of the CD5 ligand is still a controversial matter, it is widely accepted that CD5 is indeed a negative regulator of signaling by the clonotypic antigen-specific receptor present on T an B1 cells9, 10 to which it physically associates and co-localizes at the centre of the immune synapse.11 Noteworthy, CD5 is found to be up-regulated in T and B cells with regulatory/suppressor function as well as in T (either CD4+ or CD8+) and B cells energized by repeated antigen stimulation.7, 8

No CD5 deficiencies have been reported in humans so far. However, several coding nonsynonymous single nucleotide polymorphisms (SNP) have been identified in the *CD5* gene.12 Interestingly, the *CD5* gene has been under recent evolutive selective pressure, probably long after the first colonization of East Asia by anatomically modern humans, the nonsynonymous SNP rs2229177 (NM_014207.3:c.1412C>T), coding for an Ala to Val substitution at the cytoplasmic tail of CD5 (p.Ala471Val), being the most probable target for selection. The rs2229177 variants together with another frequent SNP coding for a Pro to Leu substitution at the extracellular region of CD5 (rs2241002, NM_014207.3:c.671C>T, p.Pro224Leu) constitute different haplotypes, one of which (Pro224-Val471) has been

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positively selected in East Asian populations.12 Functional analyses reveal that homozygous carriers of the ancestral Pro224-Ala471 (CC) haplotype present higher in vitro T cell proliferative responses and a more severe clinical form of Systemic Lupus Erythematosis compared to homozygous individuals for the more recently derived Pro224-Val471 (CT) haplotype.13 This finding suggests a link between differential regulation of T-cell signaling by CD5 variants and distinct autoimmune disease outcome.

Considering that the immune system plays an opposite role in autoimmune diseases and cancer, we addressed the putative association between *CD5* allelic variations at SNPs rs2229177 and rs2241002 and clinical outcome of melanoma in two independent cohorts (Barcelona N=493 and Essen N=216).

Materials and Methods

Design and Samples

The retrospective study comprised two independent Hospital Based series of melanoma patients. Recruitment (and therefore blood sampling) took place wherever possible 3–6 months after diagnosis. Patients were included in the study when the following information was available: confirmed alive/death status at last follow-up (melanoma-specific survival), at least on update in follow-up (months) since the date of diagnosis, sentinel lymph node biopsy result (SLN) (positive/negative), gender (male, female), age at diagnosis, and Breslow thickness (mm). Exclusion criteria were lack of germinal DNA sample or lack of signed informed consent.

The first set consisted of a Hospital Based series of 493 melanoma patients from the Melanoma Unit of Hospital Clinic of Barcelona, Spain. Patients were diagnosed with melanoma between 1994 and January 2013 (median time of follow-up: 43 months). Cohort disease status was established through the annual review and review of medical notes, from electronic records of the patients with visits every 3-4 months the first 2 years, every 6 months until 5 years and annual until 10 years.

The second cohort comprised a Hospital Based series of 215 melanoma patients from University Hospital Essen, Germany. Patients were diagnosed with melanoma between 1982 and 2009 (median time of follow-up was 46 months). The cohort disease status was established in the same way as for the Barcelona cohort and included an update of lost to follow-up by phone calls.

In the Barcelona cohort, the patient's stage at diagnosis according to AJCC1 was: 45% stage I (22 IA, 187 IB, 22 I unknown ulceration status), 33% stage II (50 IIA, 53 IIB, 17 IIC, 43 II unknown ulceration status) and 22% stage III (number of positive SLN or presence of micro/macro metastasis was not recorded in our database, thus patients could not be subclassified into IIIA, IIIB or IIIC). In the Essen cohort, the patient's stage at diagnosis according to AJCC was: 28% stage I (61 IB), 45% stage II (40 IIA, 33 IIB, 11 IIC, 16 II unknown ulceration status) and 27% stage III (24 IIIA, 16 IIIB, 5 IIIC, 13 III with unknown number of positive SLN or presence of micro/macro metastasis). The study was approved by the ethical

committee of the Hospital Clinic of Barcelona. The patients gave their written, informed consent.

CD5 Genotyping

Genomic DNA was obtained from peripheral blood lymphocytes. TaqMan Genotyping Assays were used to genotype CD5 SNPs rs2229177 (assay number: C—3237272_10) and rs2241002 (assay number: C—25472293_20) according to the manufacturer's recommendations (TermoFisher). The 7900HT Fast Real-Time PCR System (Applied Biosystems) was used. The results were analysed using the Applied Biosystems TaqMan Genotyper Software (TermoFisher).

In the Barcelona set, SNP genotyping was successful for rs2229177 and rs2241002 in 99.4% (490/493) and 99.8% (492/493) of patients, respectively. In Essen, both SNPs were successfully genotyped in 99.5% (214/215) of patients. In the two patient sets both SNPs were in Hardy-Weinberg equilibrium (Barcelona: rs2229177 p=0.500 and rs2241002 p=1.000, Essen: rs2229177 p=1.000 and rs2241002 p=0.370). The two *CD5* SNPs analyzed were not in linkage disequilibrium in either patient set (D'=0.597, r2=0.100 and D'=0.311, r2=0.018 in Barcelona and Essen, respectively).

Statistical analyses

The main clinical event assessed was melanoma-specific survival. A two-sided Pearson chi-squared test was used for general descriptive analyses for categorical variables. A t-test was used for general descriptive analyses for continuous variables. The Breslow thickness variable did not follow the normal distribution and was transformed using the logarithm function. The melanoma-specific survival according to different haplotypes was assessed using Kaplan-Meier curves and backward multivariate Cox regression analysis. The hazard ratio (HR) and its 95% CI were calculated. SPSS 17.0 was used to perform descriptive statistical analyses and survival analyses. Genotype and haplotype association analyses were performed using the bioinformatics tool SNPStats (http://bioinfo.iconcologia.net/SNPstats). 14 The odds ratio (OR) and its 95% CI were calculated. Gender, SLN, age at diagnosis, and log transformed Breslow were included as covariates in the analyses. Information about Ulceration was not included due to the high number of tumors without this information in the Barcelona set of patients. The tests were considered significant if *P*-value or Adjusted *P*-value as applicable was <0.05.

Results and Discussion

Descriptive analyses of the clinical and genetic variables showed that the characteristics known to be associated with worse melanoma prognosis (male gender, melanoma-death, and positive sentinel lymph node) and rs2241002 genotype frequencies were significantly different between the two cohorts (Tables S1-S2). The association analysis of SNP rs2229177 with melanoma-specific survival (Table 1) showed that the ancestral C allele (p.Ala471) had a statistically significant effect on melanoma outcome in the Barcelona-cohort (OR= 0.57, 95% CI=0.33-0.99, Adj. *P*=0.043) and a trend close to statistical significance in the Essen-cohort (OR=0.63, 95% CI=0.40-1.01, Adj. *P*=0.051). Additionally,

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the minor T allele of rs2241002 (p.Leu224) was associated with increased melanoma-associated mortality in both the Barcelona-cohort (OR=1.87, 95% CI=1.07-3.24, Adj. *P*=0.030) and the Essen-cohort (OR=1.84, 95% CI=1.04-3.26, Adj. *P*=0.037).

Haplotype analyses with SNPStats showed that the presence of T alleles for both SNPs (Leu224-Val471 haplotype) was associated with increased risk of melanoma-associated death in the Barcelona-cohort (OR=2.52, 95% CI=1.22-5.22, Adj. *P*= 0.013), while in Essen, the presence of the C allele in both SNPs (Pro224-Ala471 haplotype) had a protective role on melanoma survival (OR=0.49, 95% CI=0.27-0.90, Adj. *P*=0.022). Thus, the ancestral *CD5* Pro224-Ala471 haplotype associates with increased melanoma-specific survival, while the more recently derived Leu224-Val471 associates with reduced melanoma survival. As functional analyses have revealed that the ancestral *CD5* Pro224-Ala471 haplotype increases the immune activity compared with the Pro224-Val471 haplotype,13 we assessed the melanoma-specific survival using Kaplan-Meier and Cox regression analyses, comparing individuals homozygous for each haplotype in the entire patient set (Figure 1a). We identified that carriers of the Pro224-Ala471 haplotype in homozygosis have a better survival compared with the carriers of the Pro224-Val471 haplotype in homozygosis (HR=0.21, 95% CI 0.07 to 0.58, Adj. *P*=0.003). These individuals also have a better survival compared with other haplotype combinations (HR=0.27, 95% CI 0.11 to 0.67, Adj. *P*=0.005) and also compared with homozygotes for the Leu224-Val471 haplotype (HR=0.20, 95% CI 0.05 to 0.80, Adj. *P*=0.022), the most extreme haplotype combination (Figure 1b). When AJCC staging was included in the model instead of log transformed Breslow and SLN status, the results were similarly significant (data not shown).

As patients from different stages at diagnosis (I, II and III) were included in the study, we performed survival analyses according to the functional Pro224-Ala471 vs. Pro224-Val471 haplotypes, grouping patients by staging (Figure S1). Although, we have not enough power to reach significance, a protective effect on melanoma survival of the Pro224-Ala471 haplotype in homozygosis could be observed in the three stages (Stage I: HR=0.18, 95% CI 0.21 to 1.53, Adj *P*=0.117; Stage II: HR=0.15, 95% CI 0.02 to 1.22, Adj. *P*=0.077, and Stage III: HR=0.39, 95% CI 0.11 to 1.43, Adj. *P*=0.154). Thus, the effect of CD5 variants on the modulation of melanoma outcome is independent of the stage at diagnosis.

The results represent, as far as we know, the first report on the impact of functional germline variants from an immune-regulatory receptor in melanoma outcome. Previous studies have identified SNPs from several non-immune related genes that impact melanoma prognosis. This is the case for the GC gene (rs2282679), linking lower serum levels of vitamin D with increased melanoma-specific deaths,15 and for the MC1R gene,16 for which loss of function variants up-regulate oxidative stress-related pathways and DNA damage,17 favoring the apoptosis of damaged cells. SNPs in Nucleotide Excision Repair (NER) and Poly [ADP-Ribose] Polymerase 1 (PARP1) genes have also been implicated in melanoma prognosis, showing the relevance of the DNA damage and repair system for tumor survival.18, 19 A similar situation applies to genes from the Hippo pathway, which control cell migration, development, and organ sizes in diverse species,,20 as well as genes from the Notch21 and Fanconi anemia22 pathways. Related with immunity, SNPs in the interleukin locus and angiogenesis have also been associated with melanoma progression.23, 24

The presence of immune cells in the tumor microenvironment is known to influence melanoma prognosis.25 Currently available melanoma immunotherapies target lymphocyte receptors involved in down-regulating T-cell effector functions (e.g. CTLA-4, PD-1 and PD-1 ligand), collectively known as “immune check-points”. In accordance with previously published studies,7 the present association study supports a role for CD5 as a new immune modulatory receptor, which paves the way for improvement of current therapies against melanoma. Indeed, available evidence supports the involvement of CD5 in the regulation of antitumour immune responses. Early mouse studies showed the efficacy of a non-depleting anti-CD5 monoclonal against lymphoid and nonlymphoid tumors.26 Later reports found that *in situ* sensory adaptation of TILs from patients undergoing lung carcinoma involves down-regulation in CD5 surface expression.6 More recently, studies involving CD5-deficient mice6 and transgenic mice expressing a soluble form of human CD5,27 showed improved anti-tumor responses using non-orthotopic mouse melanoma models (B16 cells).

In conclusion, the present study illustrates an unprecedented, although predictable, fact: the genetic variability of the host’s immune response influences melanoma survival. The results are also in line with a recent observation from our group showing that rs2229177 variants impact the survival response of chronic lymphocytic leukemia patients to conventional chemotherapy regimens.28 Thus, the identification of new inherited variants in immune-related genes may also be useful to identify patients that are going to respond better to available treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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CD5 functional variants influence melanoma outcome, illustrating the contribution of the genetic variability of the host's immune response on prospects for survival in melanoma patients. The results of this study add new evidence that proposes the CD5 immune checkpoint as a new target for the improvement and development of new cancer immunotherapies.

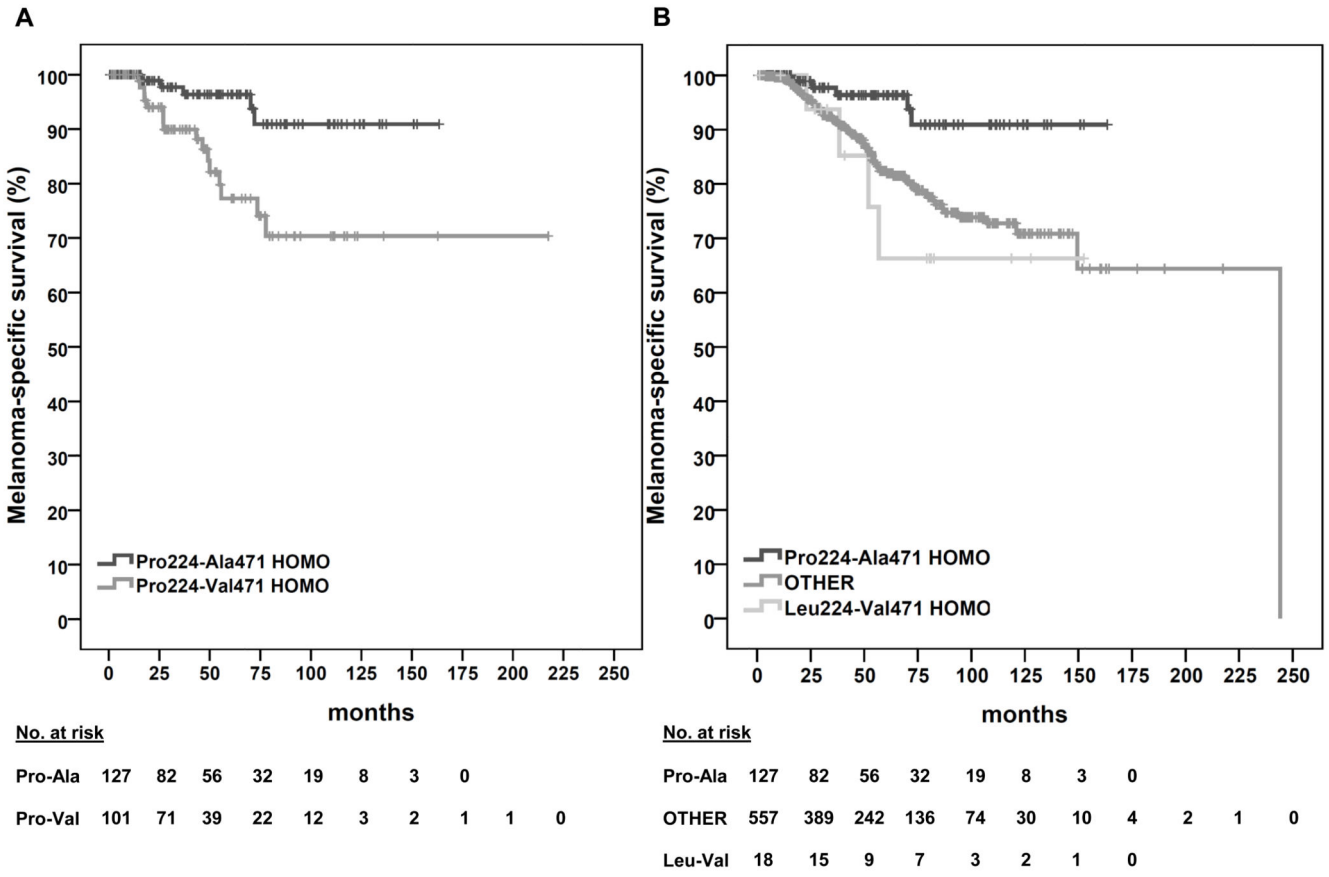

Figure 1. Melanoma-specific survival curve according to the genetic status of *CD5*

a) Melanoma-free survival curve in patients homozygous for the Pro224-Ala471 haplotype (dark gray) vs. Pro224-Val471 (light gray). HR comparing homozygous Pro224-Ala471 with other was 0.21 (95% CI 0.07 to 0.58), Adj. *P*=0.003. The 5-year survival rate was 0.96 for Pro224-Ala471 homozygous and 0.77 for Pro224-Val471 homozygous. HR and *P*-values were adjusted by age at diagnosis, gender, log transformed Breslow and SLN biopsy result (positive/negative).

b) Melanoma-free survival curve in patients homozygous for the Pro224-Ala471 haplotype (dark gray) vs. Leu224-Val471 homozygous (light gray) vs. other haplotype combination (gray) (Log-rank test *p*=0.010). HR comparing homozygous Pro224-Ala471 with other was 0.27 (95% CI 0.11 to 0.67), Adj. *P*=0.005. HR comparing homozygous Leu224-Val471 with other was 1.22 (95% CI 0.44 to 3.36), Adj. *P*=0.703. HR comparing homozygous Pro224-Ala471 with homozygous Leu224-Val471 was 0.20 (95% CI 0.05 to 0.80), Adj. *P*=0.022. The 5-year survival rate was 0.96 for Pro224-Ala471 homozygous, 0.82 for other haplotype combination and 0.66 for Leu224-Val471 homozygous. HR and *P*-values were adjusted by age at diagnosis, gender, log transformed Breslow and SLN biopsy result (positive/negative).

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Table 1

Genetic association of the *CD5* SNPs rs2229177 and rs2241002 with melanoma-specific survival.

	rs2229177 (p.Ala471Val)										
	MAF (C allele)		Genotype Frequency								
			Alive			Death					
	Alive	Death	CC	CT	TT	CC	CT	TT	OR	95%CI	Adj. P^1
Barcelona (N=490)	0.48	0.36	0.24	0.48	0.28	0.12	0.47	0.41	**0.57**	**0.33, 0.99**	**0.043**
Essen (N=214)	0.48	0.41	0.24	0.49	0.27	0.14	0.53	0.33	0.63	0.40, 1.01	0.051
	rs2241002 (p.Pro224Leu)										
	MAF (T allele)		Genotype Frequency								
			Alive			Death					
	Alive	Death	CC	CT	TT	CC	CT	TT	OR	95%CI	Adj. P^1
Barcelona (N=492)	0.23	0.34	0.59	0.36	0.05	0.47	0.37	0.16	**1.87**	**1.07, 3.24**	**0.030**
Essen (N=214)	0.16	0.25	0.71	0.27	0.02	0.54	0.42	0.04	**1.84**	**1.04, 3.26**	**0.037**

The median (range) time of follow-up in months for Barcelona alive and death patients was 42 (1-217) and 44 (3-124), respectively, and for Essen alive and death patients was 48 (1-177) and 38 (8-244), respectively.

MAF: minor allele frequency; OR: Odds Ratio

Minor allele of rs2229177 SNP was C, while minor allele of rs2241002 was T.

The genetic model used was the log-additive.

The statistically significant results are highlighted in bold.

[1] P-values were adjusted by age at diagnosis, gender, log transformed Breslow and sentinel lymph node biopsy result (positive/negative).