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ORIGINAL ARTICLE

The predictive and prognostic role of single nucleotide gene variants of PD-1 and PD-L1 in patients with advanced melanoma treated with PD-1 inhibitors

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Background: Despite having revolutionized the treatment paradigm for advanced melanoma, not all patients benefit from immune checkpoint inhibitor therapy. To date, there are no predictive biomarkers for response or the occurrence of immune-related adverse events (irAEs) to programmed cell death protein 1 (PD-1) inhibitors. Our aim was to investigate the predictive and prognostic role of single nucleotide variants (SNVs) of genes involved in the PD-1 axis.

Methods: We analysed, in metastatic melanoma patients treated with nivolumab or pembrolizumab, five PD-1 SNVs, namely PD1.3 G>A (rs11568821), PD1.5 C>T (rs2227981), PD1.6 G>A (rs10204525), PD1.7 T>C(rs7421861), PD1.10 C>G (rs5582977) and three programmed death-ligand 1 (PD-L1) SNVs: +8293 C>A (rs2890658), PD-L1 C>T (rs2297136) and PD-L1 G>C (rs4143815). Association of SNV genotypic frequencies with best overall response to PD-1 inhibitors and development of irAEs were estimated through a modified Poisson regression. A Cox regression modelling approach was applied to evaluate the SNV association with OS.

Results: A total of 125 patients with advanced melanoma were included in the analysis. A reduction in irAEs risk was observed in patients carrying the PD-L1 +8293 C/A genotype compared with those carrying the C/C genotype (risk ratio = 0.45; 95% CL 0.22-0.93; $P = 0.031$). A trend for a reduction in irAEs was also observed with the PD1.5 T allele (risk ratio = 0.70, 95% confidence limits 0.48-1.01 versus C allele). None of the SNVs was associated with response to therapy. Finally, a survival benefit was observed in patients harbouring the PD1.7 C/C genotype (hazard ratio = 0.37; 95% confidence limits 0.14-0.96; $P = 0.028$) in the homozygous model.

Conclusions: Our study showed that PD-1.5 and PD-L1 +8293 SNVs may play a role as a predictive biomarker of development of irAEs to PD-1 inhibitors. PD1.7 SNV may also be associated with a reduction of the risk of death, although further translational research is needed to confirm these results.

Key words: melanoma, SNV, single nucleotide gene variant, PD-1, PD-L1

INTRODUCTION

The introduction of immune checkpoint inhibitors (ICIs) has changed the treatment paradigm for advanced melanoma patients, leading to a considerable increase in life expectancy. Before the advent of ICIs, median overall survival (mOS) in these patients was <1 year.[1] With the introduction of cytotoxic T-lymphocyte antigen 4 (CTLA-4) inhibitor ipilimumab, the mOS increased to almost 2 years,[2-4] to ~3 years with the programmed cell death protein 1 (PD-1) inhibitors nivolumab and pembrolizumab,[5,6] and up to ~6 years with the combination of the CTLA-4 inhibitor ipilimumab and the PD-1 inhibitor nivolumab.[2]

This long-term clinical benefit occurs only in about half of patients receiving anti-PD-1, however, with or without anti-CTLA-4 drugs.[2] Another relevant aspect that can be frequently (15%-55%) associated with treatment with ICIs is the occurrence of inflammatory side-effects known as immune-related adverse events (irAEs). These adverse events can be severe, particularly with ICI combinations, in about half of patients.[3,7]

These factors have led to considerable efforts in the search for potential predictive biomarkers of both objective response and the occurrence of severe toxicities upon ICI treatment.

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In this context, we previously investigated the role of CTLA-4 gene single nucleotide variants (SNVs) in patients with advanced melanoma treated with ipilimumab. In particular, both -1577 G>A and CT60 G>A SNVs were found to be associated with best response to therapy and long-term survival,[8] whereas -1661 A>G SNV correlated with the onset of endocrine irAEs.[9] Indeed, these SNVs may affect the transcriptional efficiency of the CTLA-4 gene, leading to alteration of CTLA-4 expression levels and inhibitory function in T cells.

In the present work, we extended the analysis to the SNVs of other immune checkpoint molecules, namely PD-1 and programmed death-ligand 1 (PD-L1), to investigate their implication in clinical response and survival of patients with advanced melanoma treated with anti-PD-1 agents.

The PD-1/PD-L1 axis is an important regulatory mechanism of the immune response that can be exploited by melanoma cells to escape the immune system through inhibition of T lymphocytes' cytotoxic ability to attack and destroy tumour cells. This mechanism of inhibition is known as 'immune tolerance'.[10] Therefore, therapeutic monoclonal antibodies targeting PD-1 and PD-L1 can restore cytotoxic T cell activation and induce tumour cell death by blocking the interaction between PD-1 and PD-L1.[11]

SNVs in the PD-1/PD-L1 axis could modify receptor/ligand interactions and affect the regulation of the antitumour immune response. Indeed, certain functional SNVs can influence PD-1 and PD-L1 transcriptional efficiency, mRNA stability or splicing process[10,12] and, consequently, the expression levels of cell surface PD-1 and PD-L1 proteins.

Although PD-1 and PD-L1 germline SNVs have been extensively investigated in some cancers, with particular reference to their association with cancer susceptibility,[13] a smaller number of studies addressed their correlation with response rate and OS in patients treated with ICI, and such studies are mainly related to non-small-cell lung cancer (NSCLC).[14,15] Thus, there are very few reports in the literature of patients with advanced melanoma treated with ICIs.[12,16-18] In this context, PD1.3 G>A SNV has been associated with better response to therapy and longer progression-free survival (PFS),[12] whereas PD1.5 C>T SNV with a worse OS[16] in patients treated with an anti-PD-1 agent.

Given the association of PD-1 and PD-L1 with susceptibility to autoimmune diseases,[19,20] it is reasonable to also investigate the association of PD-1 and PD-L1 SNVs with the development of irAEs, which seems to have an autoimmune pathogenesis. To the best of our knowledge, there are no data on this topic in melanoma patients treated with anti-PD-1 agents.

In our study, we analysed the possible association of eight functional SNVs of PD-1 and PD-L1 with best overall response (BOR), the occurrence of irAEs and OS, in a cohort of metastatic melanoma patients treated with nivolumab or pembrolizumab in an Italian centre.

METHODS

Patients and controls

Patients with advanced melanoma treated with an anti-PD-1 agent between 1 January 2013 and 31 December 2020 in a single Italian centre, have been included in our study. The following baseline clinical data have been collected: age, sex, primary melanoma subtype, Eastern Oncology Cooperative Group (ECOG) performance status, serum lactate dehydrogenase (LDH) baseline level, TNM American Joint Committee on Cancer (AJCC) 8th Edition staging, number of metastases, presence of brain metastases, previous treatments received, BOR according to the RECIST 1.1 and iRECIST criteria, adverse events according to the Common Terminology Criteria for Adverse Events (CTCAE), and OS. Study protocol has been approved by the Local Ethics Committee (N. Registro CER Liguria: 046REG2017).

DNA extraction and genotyping

In this study, we analysed five PD-1 SNVs, namely PD1.3 G>A (rs11568821), PD1.5 C>T (rs2227981), PD1.6 G>A (rs10204525), PD1.7 T>C (rs7421861), PD1.10 C>G (rs5582977) and three PD-L1 SNVs: +8293 C>A (rs2890658), PD-L1 C>T (rs2297136) and PD-L1 G>C (rs4143815). SNVs were selected on the basis of their association to susceptibility to autoimmune diseases[21] and cancer.[13] Genomic DNA was extracted from peripherical blood samples using a standard proteinase K/salting out method,[22] and genotyping was carried out by pyrosequencing (PSQ) methods,[23] or by real-time polymerase chain reaction (PCR).[24]

To carry out the PSQ method, we utilized 100 ng of genomic DNA in a final volume of 50 µl containing 200 mol/l deoxynucleotide triphosphates, 1× GeneAmp buffer, 1.5 mM $MgCl_2$, 1.25 U of Immolase Hot Start polymerase (Bioline, Milan, Italy) and 0.3 µM of the PCR primer pairs specific for each SNP. Primers design, including PCR primer pair and the related sequencing primer for the PSQ assays, was carried out making use of Pyrosequencing Assay Design software (Biotage, Uppsala, Sweden).

PCR products were sequenced using a PSQ96MA instrument (Qiagen, Milan, Italy) and the sequencing reactions were carried out with the Pyro Gold reagent kit PSQ 96MA following the manufacturer's protocol. Analysis and allele assignment were carried out with the PSQTM 96MA (version 2.02) software.

Primer sequences and PCR conditions are listed in Supplementary Table S1, available at https://doi.org/10.1016/j.iotech.2023.100408.

The analysis of PD1.3 G>A (rs11568821) SNV was carried out by real time PCR based on LightCycler Technology, using the LightSNiP assay developed by TIB Molbiol (Genova, Italy), that can detect SNVs based on melting curve analysis. Samples amplification was carried out in a LightCycler®480II, real-time PCR instrument (Roche, Basel, Switzerland).

Statistical analyses

The distributions of patient and disease characteristics were explored using descriptive statistics. In this context, the distribution of age at diagnosis was summarized using the median value and the interquartile range (IQR), and then

categorized using 60, 70 and 80 years as cut-off points. All categorical variables were expressed in terms of absolute and relative frequencies (percentages).

Preliminarily, departures from the Hardy–Weinberg Equilibrium (HWE) for the eight SNV genotypes were assessed with the Pearson chi-square test by using the de-Finetti software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). A *P* value $\leq$0.05 indicates a lack of HWE.

The correlation between BOR and irAEs with each SNV genotype was evaluated through the modified Poisson regression method, whereas the joint prognostic effect of the same SNV and irAEs on OS was estimated using the Cox regression method. In both regression settings, a relative risk parameter was computed as an index of association: the risk ratio (RR) in the former and the mortality rate (hazard) ratio (HR) in the latter. In addition, five genetic models (i.e. dominant, recessive, allelic, homozygous, heterozygous models) were applied for further explorative analyses.[13]

All regression estimates were adjusted for baseline age at diagnosis, gender, ECOG PS, LDH levels, number of metastatic sites and brain metastases, with 95% confidence limits (95% CL). A two-tailed *P* value $\leq$0.05 was considered as statistically significant.

All statistical analyses were carried out using STATA software (StataCorp. Stata: Release 17. Statistical Software. College Station, TX: StataCorp LP, 2021).

RESULTS

Patients' characteristics

A total of 125 patients with unresectable or metastatic melanoma treated with an anti-PD-1 agent (nivolumab or pembrolizumab) were included in our study. The median age was 71 years (range: 29-94 years), most patients had an ECOG PS of 0 or 1 (88%), with ~30% of patients having more than three metastatic sites. About 20% had brain metastases at baseline. A total of 29 patients (23%) had the *BRAF* mutation. Out of 125 patients, 66 (52.8%) were treatment-naive, 39 (31.2%) had received one prior line of therapy (including 21 patients treated with ipilimumab, 16 with targeted therapy, and 2 with chemotherapy) and 19 (15.2%) had undergone two or more lines of therapy (including 17 patients who received ipilimumab). In particular, most patients (~70%) were previously untreated with immunotherapy (ipilimumab) or targeted therapy (~75%), and only ~8% had been previously treated with chemotherapy. Response evaluation was available for 97 of 125 patients. At cut-off date, a total 87 patients were dead, and 38 patients were alive. Baseline patients' characteristics are reported in Table 1.

Genotyping and frequencies of PD-1 and PD-L1 SNVs

A total of five PD-1 SNVs, PD1.3 G>A (rs11568821), PD1.5 C>T (rs2227981), PD1.6 G>A (rs10204525), PD1.7 T>C (rs7421861), PD1.10 C>G (rs5582977) and three PD-L1 SNVs, +8293 C>A (rs2890658), PD-L1 C>T (rs2297136) and PD-L1 G>C (rs4143815), were analysed in 125 metastatic melanoma patients treated with nivolumab or pembrolizumab. The distribution of genotype and allele frequencies are reported in Table 2. As shown, no deviation from the HWE was observed for any SNV (*P* value ranging from 0.119 to 0.944).

Association of PD-1 and PD-L1 SNVs with irAEs

Of the included 125 patients, a total of 49 (39%) reported at least one irAE (of any grade and duration), and 76 (61%) did not have any irAE (Table 3).

Correlation of PD-1 and PD-L1 SNVs with irAEs indicated that three of the SNVs, namely PD1.5 C>T, PD1.7 T>C and PD-L1 +8293 C>A, showed differences in codominant genotype frequencies between patients with the presence or absence of irAEs. These differences were further analysed by using different genetic models (dominant, recessive, allelic and homozygous/heterozygous) as shown in Table 3.

When considering PD1.5 C>T SNV, patients carrying the C/T heterozygous (RR = 0.75; 95% CL 0.48-1.18) or the T/T homozygous genotype (RR 0.32; 95% CL 0.08-1.24) tended to have a lower risk of developing irAEs compared with C/C homozygous patients as the reference category (Figure 1, Panel A). This result shows a decreasing trend in the risk of irAEs probably due to the T allele dosage effect as confirmed in the allelic model (RR = 0.70; 95% CL 0.48-1.01), as shown in Table 3.

By contrast, a protective role for the occurrence of irAEs was observed for the C allele of PD1.7 T>C SNV. Indeed, homozygous C/C patients tended to have a 65% risk reduction of developing irAEs compared with T/T homozygous patients (RR = 0.35; 95% CL 0.09-1.31), as shown in Figure 1 (Panel A) and Table 3. This result was also confirmed by the homozygous model (RR = 0.33; 95% CL 0.09-1.15).

Referring to the PD-L1 +8293 C>A SNV, considering C/C homozygous carriers as a reference group, a decrease in irAEs frequency of 55% was estimated for the heterozygous C/A patients (RR = 0.45; 95% CL 0.22-0.93; *P* = 0.079), and an increase of about 40% in A/A homozygous patients (RR = 1.37; 95% CL 0.28-6.69). Both findings were also highlighted by the dominant (RR = 0.50; 95% CL 0.25-0.98; *P* = 0.045) and heterozygous versus homozygous model (RR = 0.45; 95% CL 0.22-0.93; *P* = 0.031) (Table 3).

Patients carrying the PD1.3 G>A SNV, although showing a potential role in increased irAEs risk, were excluded due to the absence of patients without irAEs in the A/A genotype (Table 3).

We attempted to correlate each SNV with a specific irAE, but the limited number of irAE cases for each SNV in our dataset precluded a statistical analysis. Supplementary Table S2, available at https://doi.org/10.1016/j.iotech.2023.100408, provides a comprehensive summary of the irAEs observed in our study. The most frequently observed irAEs were 'general', such as fatigue or fever

Table 1. Baseline patients' characteristics

Characteristics	No.	%
Age at diagnosis (median, IQR)	71 (56-78)	—
29-60	38	30.4
61-70	23	18.4
71-80	44	35.2
81-94	19	15.2
Unknown	1	0.8
Gender		
Female	61	48.8
Male	64	51.2
Primary melanoma subtype		
Acral	3	2.4
Cutaneous	96	76.8
Mucosal	8	6.4
Unknown	18	14.4
ECOG performance status		
0	84	67.2
1	26	20.8
2	3	2.4
Unknown	12	9.6
Serum LDH[a]		
Normal level for female	28	22.4
≥Upper limit of normal for female	26	20.8
Normal level for male	25	20.0
≥Upper limit of normal for male	34	27.2
Unknown	12	9.6
BRAF mutation		
Present	29	23.2
Absent	79	63.2
Unknown	17	13.6
Number of metastases sites		
1	54	43.2
2	34	27.2
≥3	36	28.8
Unknown	1	0.8
Brain metastases		
Present	23	18.4
Absent	101	80.8
Unknown	1	0.8
Immunotherapy before to anti-PD-1[b]		
Yes	38	30.4
No	86	68.8
Unknown	1	0.8
Targeted therapy before anti-PD-1[c]		
Yes	29	23.2
No	95	76.0
Unknown	1	0.8
Chemotherapy before anti-PD-1		
Yes	11	8.8
No	113	90.4
Unknown	1	0.8
Radiotherapy		0.0
Yes	59	47.2
No	65	52.0
Unknown	1	0.8
Stage		
III	9	7.2
IV	115	92.0
Unknown	1	0.8
Treatment regimen		
Pembrolizumab	73	58.4
Nivolumab	52	41.6
TNM stage (AJCC 8th Ed.)		
III	9	7.2
M1a	28	22.4
M1b	16	12.8
M1c	48	38.4
M1d	23	18.4
Unknown	1	0.8
Number of immune related adverse events (irAE)		
0	76	60.8
1	32	25.6
2	12	9.6
≥3	5	4.0
Vital status		
Deceased	87	69.6
Alive	38	30.4
Total	125	100.0

ECOG, Eastern Oncology Cooperative Group; IQR, interquartile range; LDH, lactate dehydrogenase; No./%, number and percentage of patients; PD-1, programmed cell death protein 1.
[a]Normal LDH levels: 135-214 U/l for females, 135-225 U/l for males.
[b]A total of 21 patients received first-line ipilimumab, and 17 received second-line ipilimumab.
[c]BRAF/MEK inhibitor ($n = 16$; 12.8%); BRAF inhibitor ($n = 4$; 3.2%); MEK inhibitor ($n = 6$; 4.8%); imatinib ($n = 3$; 2.4%).

($n = 14$; 28%), cutaneous ($n = 15$; 30%) and endocrine ($n = 10$; 20%).

Association of PD-1 and PD-L1 SNVs with response to anti-PD-1 therapy

A total of 33 out of 97 patients (34%) included in the analysis had progressive disease (PD), and 64 (66%) had complete response (CR), partial response (PR) or stable disease (SD) as BOR, according to iRECIST criteria.

The comparison of SNV genotypic frequencies in patients with PD versus NPD (non-progressive disease, i.e. CR, PR or SD) is reported in Figure 1 (Panel B) and Supplementary Table S3, available at https://doi.org/10.1016/j.iotech.2023.100408. Using the wild-type genotype as reference category (RR = 1.00), none of the evaluated SNVs were associated with a reduced risk of having PD as best response.

Association of PD-1 and PD-L1 SNVs with OS

We analysed the association between the included SNVs and OS (Supplementary Tables S4-S7, available at https://doi.org/10.1016/j.iotech.2023.100408). A T-allele dose-dependent positive trend in OS was observed for PD1.7 T>C (Figure 2). In particular, patients carrying the T/C and C/C genotypes had a reduction in the risk of death of ∼25% (HR = 0.74; 95% CL 0.43-1.25) and 60% (HR = 0.41; 95% CL 0.16-1.00), respectively, when compared with patients with homozygous T/T genotype (Supplementary Table S6, available at https://doi.org/10.1016/j.iotech.2023.100408). This survival benefit was confirmed in patients harbouring the PD1.7 C/C genotype (HR = 0.37; 95% CL 0.14-0.96; $P = 0.028$) in the homozygous model.

DISCUSSION

Our study provides descriptive and exploratory analyses on the possible role of PD-1 and PD-L1 germline variants in the prediction of tumour response and development of irAEs in patients with advanced melanoma treated with anti-PD-1 agents.

Table 2. Distribution of genotypic and allelic frequencies of five PD-1 and three PD-L1 gene variants in 125 patients with advanced melanoma

Single nucleotide variant	Genotype	Patients (n = 125)		Allele	Alleles ($2n$ = 250)	
		No.	%		No.	%
PD1.3 G>A (rs11568821)	G/G	92	73.6	G	216	86.4
	G/A	62	25.6	A	34	13.6
	A/A	1	10.4			
	(*P* value)	(0.318)				
PD1.5 C>T (rs2227981)	C/C	51	40.8	C	163	65.2
	C/T	61	48.8	T	87	34.8
	T/T	13	10.4			
	(*P* value)	(0.399)				
PD1.6 G>A (rs10204525)	G/G	109	87.2	G	234	93.6
	G/A	16	12.8	A	16	6.4
	A/A	0	0.0			
	(*P* value)	(0.444)				
PD1.7 T>C (rs7421861)	T/T	59	47.2	T	166	66.4
	T/C	48	38.4	C	84	33.6
	C/C	18	14.4			
	(*P* value)	(0.119)				
PD1.10 C>G (rs5582977)	C/C	117	93.6	C	242	96.8
	C/G	8	6.4	G	8	3.2
	G/G	0	0.0			
	(*P* value)	(0.712)				
PD-L1 +8293 C>A (rs2890658)	C/C	87	69.6	C	210	84.0
	C/T	36	28.8	A	40	16.0
	T/T	2	1.6			
	(*P* value)	(0.416)				
PD-L1 C>T (rs2297136)	C/C	13	10.4	C	91	36.4
	C/T	65	52.0	T	159	63.6
	T/T	47	37.6			
	(*P* value)	(0.169)				
PD-L1 G>C (rs4143815)	G/G	62	49.6	G	176	70.4
	G/C	52	41.6	C	74	29.6
	C/C	11	8.8			
	(*P* value)	(0.944)				

P value: probability level associated with the chi-square test for departures from the Hardy—Weinberg equilibrium.
PD-L1, programmed death-ligand 1.

To date, very few studies investigated these issues.[12,16,18] To the best of our knowledge, our study is the first one reporting data on both PD-1 and PD-L1 genomic variants in the same cohort of melanoma patients.

The rationale for investigating both PD-1 and PD-L1 SNVs was based on the key role of both the encoded proteins in the regulation of the immune response, immune tolerance and immune escape. These mechanisms are widely involved in the genesis of autoimmune diseases and antitumour immunity.[25]

Our results showed that PD1.5 C>T, PD1.7 T>C and PD-L1 8293 C>A SNVs were not associated with clinical response to nivolumab or pembrolizumab, but had a noticeable impact on the development of irAEs.

In particular, patients carrying the PD1.5 homozygous T/T genotype had ~68% reduced risk of developing irAEs compared with patients with homozygous C/C genotype. These results were observed in both the recessive model (i.e. in the absence of the C allele) and the dominant model (i.e. in the presence of the T allele), suggesting a possible protective role of the PD1.5 T-positive genotype in respect to irAEs development.

Indeed, in a previous study, the PD1.5 C/T or T/T genotypes have been associated with increased PD-1 expression on the surface of CD4 T lymphocytes,[18] most likely resulting in increased PD-1/PD-L1 axis activity. Moreover, other studies have associated the presence of PD1.5 SNVs with a lower risk of developing irAEs.[14,16,21]

Our results showed that patients harbouring the C/A genotype of the PD-L1 +8293 SNV had a lower risk for having irAEs compared with patients harbouring the C/C genotype. This effect was also suggested by previous studies showing increased susceptibility of the C/C genotype in the development of autoimmune diseases, particularly Graves' disease.[19,26] The pathophysiological mechanism is unclear, and is probably related to reduced protein function secondary to the presence of the C allele.[19] This may be based on the fact that PD-L1 +8293 C>A is an SNV located near the binding site of transcription factors, resulting in the production of altered or non-functional proteins.[15,16]

The clinical implications of the of PD1.7 T>C variant are less known. This SNV is placed in intron 1 of the PD-1 gene and may have a role in the normal splicing process disruption and mRNA secondary structure alteration, leading to altered gene expression and potential translation inhibition.[27,28] Our results showed that the presence of the C allele may have both a trend for a protective role in the onset of irAEs and, in the homozygous C/C genotype, a

Table 3. Codominant, dominant, recessive, allelic and other genetic models for PD1.3 G>A, PD1.5 C>T, PD1.7 G>A and PD-L1 +8293 C>A estimated through the modified Poisson regression analysis

Genotypic model	irAE		Non-irAE		Total	Relative risk of irAEs		
	No.	%	No.	%		RR	95% CL	*P* value
PD1.3 G>A (rs11568821)								
Codominant								0.001
G/G	34	37.0	58	63.0	92	1.00	(Ref.)	
G/A	14	43.8	18	56.3	32	1.35	0.76-2.41	
A/A	1	100.0	0	0.0	1	3.94	1.90-8.18	
Dominant								0.207
G/G	34	37.0	58	63.0	92	1.00	(Ref.)	
G/A + A/A	15	45.5	18	54.5	33	1.43	0.81-2.50	
Recessive								<0.001
G/A+G/G	48	38.7	76	61.3	124	1.00	(Ref.)	
A/A	1	100.0	0	0.0	1	3.61	1.82-7.16	
Allelic								0.157
G	82	38.0	134	62.0	216	1.00	(Ref.)	
A	16	47.1	18	52.9	34	1.41	0.88-2.27	
Heterozygous versus homozygous								0.308
G/G	34	37.0	58	63.0	92	1.00	(Ref.)	
G/A	14	43.8	18	56.3	32	1.35	0.76-2.41	
PD1.5 C>T (rs2227981)								
Codominant								0.170
C/C	23	45.1	28	54.9	51	1.00	(Ref.)	
C/T	24	39.3	37	60.7	61	0.75	0.48-1.18	
T/T	2	15.4	11	84.6	13	0.32	0.08-1.24	
Dominant								0.088
C/C	23	45.1	28	54.9	51	1.00	(Ref.)	
C/T + T/T	26	35.1	48	64.9	74	0.67	0.43-1.06	
Recessive								0.144
C/C+C/T	47	42.0	65	58.0	112	1.00	(Ref.)	
T/T	2	15.4	11	84.6	13	0.37	0.10-1.39	
Allelic								0.058
C	70	42.9	93	57.1	163	1.00	(Ref.)	
T	28	32.2	59	67.8	87	0.70	0.48-1.01	
Homozygous								0.253
C/C	23	45.1	28	54.9	51	1.00	(Ref.)	
T/T	2	15.4	11	84.6	13	0.44	0.11-1.79	
Heterozygous versus homozygous								0.166
C/C	23	45.1	28	54.9	51	1.00	(Ref.)	
C/T	24	39.3	37	60.7	61	0.73	0.47-1.14	
PD1.7 T>C (rs7421861)								
Codominant								0.283
T/T	26	44.1	33	55.9	59	1.00	(Ref.)	
T/C	20	41.7	28	58.3	48	1.04	0.64-1.69	
C/C	3	16.7	15	83.3	18	0.35	0.09-1.31	
Dominant								0.610
T/T	26	44.1	33	55.9	59	1.00	(Ref.)	
T/C + C/C	23	34.8	43	65.2	66	0.88	0.54-1.43	
Recessive								0.112
T/C+T/T	46	43.0	61	57.0	107	1.00	(Ref.)	
C/C	3	16.7	15	83.3	18	0.35	0.09-1.28	
Allelic								0.206
T	72	43.4	94	56.6	166	1.00	(Ref.)	
C	26	31.0	58	69.0	84	0.77	0.51-1.15	
Homozygous								0.082
T/T	26	44.1	33	55.9	59	1.00	(Ref.)	
C/C	3	16.7	15	83.3	18	0.33	0.09-1.15	
PD-L1 +8293 C>A (rs2890658)								
Codominant								0.079
C/C	40	46.0	47	54.0	87	1.00	(Ref.)	
C/A	8	22.2	28	77.8	36	0.45	0.22-0.93	
A/A	1	50.0	1	50.0	2	1.37	0.28-6.69	
Dominant								0.045
C/C	40	46.0	47	54.0	87	1.00	(Ref.)	
C/A + A/A	9	23.7	29	76.3	38	0.50	0.25-0.98	
Recessive								0.563
C/C + C/A	48	39.0	75	61.0	123	1.00	(Ref.)	
A/A	1	50.0	1	50.0	2	1.60	0.38-7.80	
Allelic								0.100
C	88	41.9	122	58.1	210	1.00	(Ref.)	

Continued

Table 3. Continued

Genotypic model	irAE		Non-irAE		Total	Relative risk of irAEs		
	No.	%	No.	%		RR	95% CL	*P* value
A	10	25.0	30	75.0	40	0.59	0.32-1.10	
Heterozygous versus homozygous								0.031
C/C	40	46.0	47	54.0	87	1.00	(Ref.)	
C/A	8	22.2	28	77.8	36	0.45	0.22-0.93	
Total	49	39	76	61	125	—	—	—

P value: probability level associated with the likelihood ratio test result.

95% CL, 95% confidence limits for RR; irAEs, immune-related adverse events; Ref., reference category; RR, (risk ratio) irAE relative frequency in each SNV genotype in comparison to the irAE relative frequency in the reference genotype, adjusted for baseline age, gender, Eastern Oncology Cooperative Group performance status (ECOG PS), lactate dehydrogenase (LDH) levels, number of metastatic sites at first and brain metastases; SNV, single nucleotide variant.

significant role in reducing the risk of death in advanced melanoma patients. This might be explained considering the PD1.7 SNV effect in reducing expression of PD-1 as directly associated with a more efficient antitumour T-cell immunity.

In our study, we strived to comprehensively document the observed irAEs and their potential associations with specific SNVs. It is imperative, however, to acknowledge the limitations of our analysis. The relatively small number of irAE cases for each toxicity type presented challenges in conducting robust correlation analyses between SNVs and specific toxicities. This limitation highlights the need for larger datasets to explore potential biological links between SNVs and distinct irAEs, as such investigations could yield valuable insights into the underlying mechanisms of

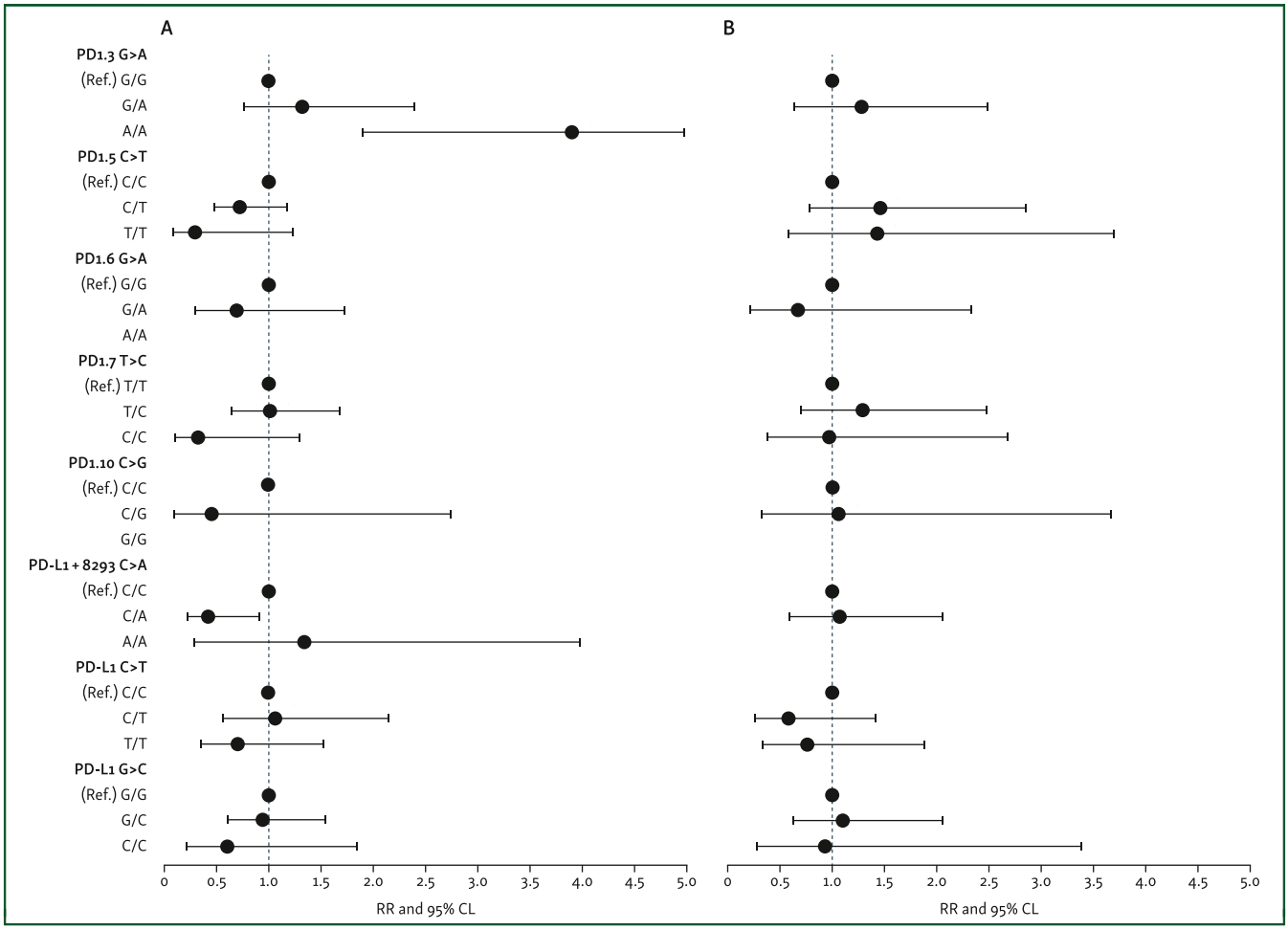

Figure 1. Caterpillar plots of the associations between (A) single nucleotide variants (SNVs) and immune-related adverse events (irAEs) to nivolumab or pembrolizumab and (B) SNVs and best overall response (BOR) evaluated as relative frequency of progressive disease (PD) compared with non-progressive disease (NPD: complete/partial response and stable disease); BOR in patients with advanced melanoma, estimated through multivariable modified Poisson regression analyses. Ref., reference category; RR, (black points) relative frequency of (A) irAEs or (B) PD in each SNV genotype category adjusted for baseline age, gender, Eastern Oncology Cooperative Group performance status (ECOG PS), lactate dehydrogenase levels, number of metastatic sites and brain metastases; 95% CL, (grey whiskers) 95% confidence limits for RR; RR = 1, (vertical dashed line) relative frequency of irAE/ PD in an SNV category equal to that of the reference; RR > 1/RR < 1, frequency in a SNV category greater/lower than that of the reference.

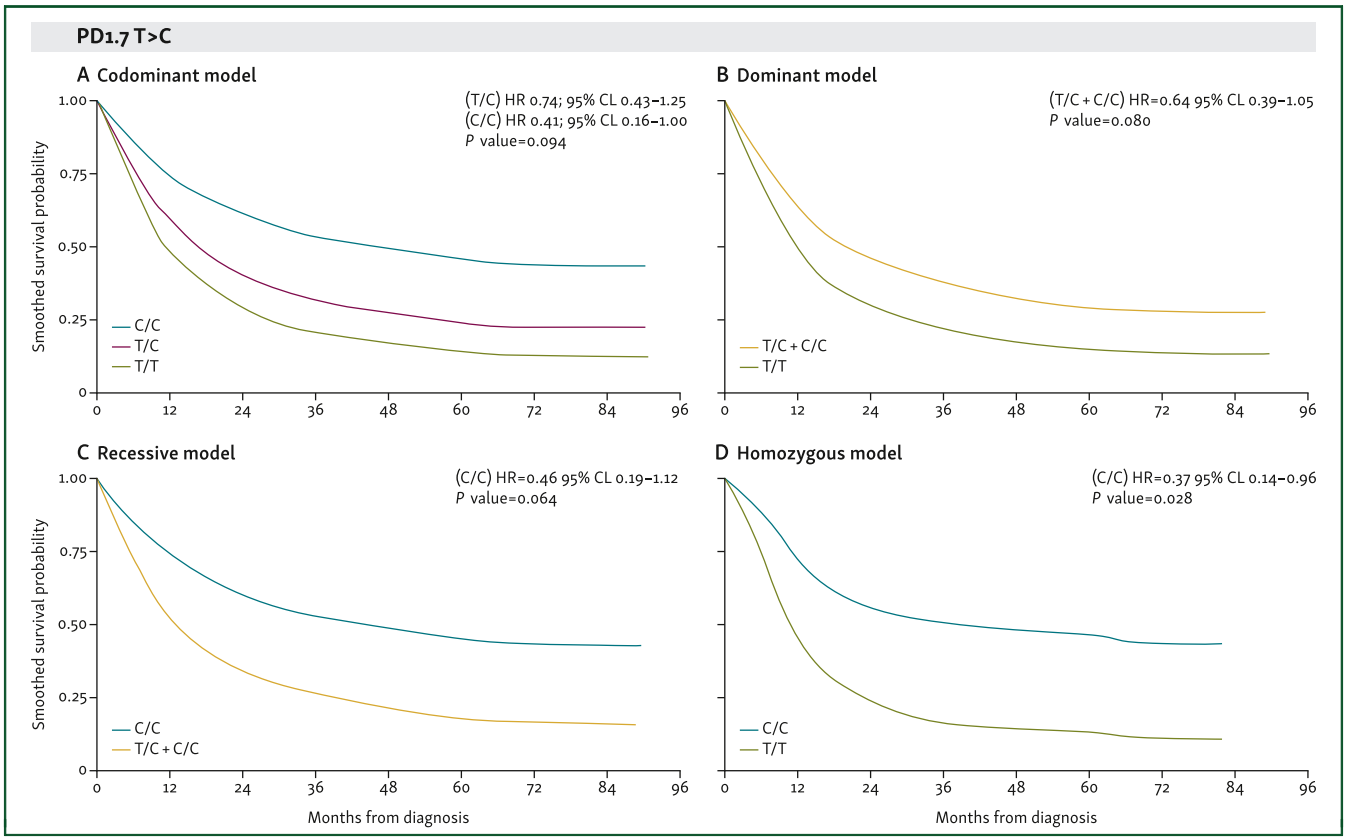

Figure 2. Overall survival probabilities according to PD1.7 T>C genotypic model: (A) codominant, (B) dominant, (C) recessive, (D) homozygous, estimated using the multivariable Cox regression analysis.

HR, mortality rate (hazard) ratio adjusted for baseline age, gender, Eastern Oncology Cooperative Group performance status (ECOG PS), lactate dehydrogenase levels, number of metastases sites, brain metastases and immune-related adverse events; 95% CL, 95% confidence limits for HR; *P* value, probability level associated with the likelihood ratio test.

immune-related toxicity. Future studies with larger cohorts may shed further light on the intricate relationships between genetic factors and irAEs, facilitating more personalized treatment approaches.

Our results showed no association of the analysed SNVs with a lower risk of having disease progression as BOR, unlike, for example, what was observed in a study by Parakh et al.,[12] where patients with the G/G genotype of PD1.3 had more CRs than patients with A/G genotype (16.5% versus 2.6%, respectively) and the G allele was associated with a longer PFS than with the A/G genotype.

This discrepancy could be due to the different disease response assessment we carried out, by using the iRECIST criteria instead of the RECIST 1.1 criteria, which may have resulted in an underestimation of the rate of disease progression. Moreover, shorter median follow-up duration among non-responders may have limited the observation of final events in this subgroup. Furthermore, stringent selection criteria based on follow-up duration were not employed due to potential reductions in sample size and increased data sparseness, which could introduce imprecision in parameter estimates.

Notably, 30% of patients had previous exposure to immunotherapy, predominantly ipilimumab. Additionally, a subset of patients had received BRAF/MEK inhibitors (Table 1). These prior treatments are known to impact both efficacy and the potential for treatment-related toxicity to anti-PD-1 agents. In particular, both nivolumab and pembrolizumab have demonstrated lower overall response rate (ORR) in patients who had progressed to ipilimumab (~30%) compared with the ORR observed in the first-line setting (~40%).[7,29-31] Moreover, another study showed that in patients who experienced irAEs during ipilimumab treatment, a flare of toxicity was observed in ~40% of cases following anti-PD-1 therapy.[32] In patients with prior ipilimumab irAEs requiring immunosuppression, the response rate was 40%.[32] Finally, it should be noted that 19 out of 125 patients had received two or more prior lines of therapy before anti-PD-1 treatment, thus constituting a subgroup of patients with a poorer prognosis.

Despite the limitations of the current study, including the limited sample size, the lack of an external validation cohort, our results may provide a preliminary indication that genotyping of PD-1 and PD-L1 SNVs might represent a useful tool for clinicians to predict or to select patients at higher risk of developing irAEs or having worse outcomes.

Moreover, our results extend similar exploratory studies previously carried out to identify germline variants associated with the risk of irAEs in patients with advanced melanoma receiving ICIs.[33]

This aspect may be clinically relevant since the combination of anti-CTLA-4 and anti-PD-1 is one of the current

standards of care for the first-line treatment of advanced melanoma.[34] Only about half of the patients obtain a long-term clinical benefit, however, which is also burdened by a significant rate of irAEs grade $\geq$3 (in about half of patients).[2,7] These factors make the validation of predictive biomarkers of both response to treatments and the development of irAEs (and in particular of the most severe), increasingly necessary.

In conclusion, our study indicates that PD1.5 (rs2227981), PD1.7 T>C (rs7421861) and PD-L1 +8293 (rs2890658) gene variants may have some predictive role on the onset of irAEs in patients with advanced melanoma treated with anti-PD-1 agents. In addition, in the same patients, the PD1.7 SNV may also have a prognostic role. Functional studies are required to better understand the underlying molecular and immunological mechanisms of both PD-1 and PD-L1 gene variants on the immune system and their interaction with ICIs.

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