


Relevance of PD-L1 Non-Coding Polymorphisms on the Prognosis of a Genetically Admixed NSCLC Cohort


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Purpose: Although non-small cell lung cancer (NSCLC) remains a deadly disease, new predictive biomarkers have emerged to assist in managing the disease, of which one of the most promising is the programmed death-ligand 1 (PD-L1). Each, *PD-L1* variant seem to modulate the function of immune checkpoints differently and affect response to adjuvant treatment and outcome in NSCLC patients. We thus investigated the influence of these PD-L1 genetic variations in genetically admixed NSCLC tissue samples, and correlated these values with clinicopathological characteristics, including prognosis.

Materials and Methods: We evaluated PD-L1 non-coding genetic variants and protein expression in lung adenocarcinomas (ADC), squamous cell carcinomas (SqCC), and large cell carcinomas (LCC) in silico. Microarray paraffin blocks from 70 samples of ADC (N=33), SqCC (N=24), and LCC (N=13) were used to create PD-L1 multiplex immunofluorescence assays with a Cell Signaling E1L3N clone. Fifteen polymorphisms of the *PD-L1* gene were investigated by targeted sequencing and evaluated in silico using dedicated tools.

Results: Although *PD-L1* polymorphisms seemed not to interfere with protein expression, PD-L1 expression varied among different histological subtypes, as did clinical outcomes, with the rs4742098A>G, rs4143815G>C, and rs7041009G>A variants being associated with relapse ($P=0.01$; $P=0.05$; $P=0.02$, respectively). The rs7041009 GG genotype showed a significant correlation with younger and alive patients compared to carriers of the A allele ($P=0.02$ and $P<0.01$, respectively). The Cox regression model showed that the rs7041009 GG genotype may influence OS ($P<0.01$) as a co-dependent factor associated with radiotherapy and recurrence in NSCLC patients. Furthermore, the Kaplan–Meier survival curves showed that rs7041009 and rs4742098 might impact PPS in relapsed patients. In silico approaches identified the variants as benign.

Conclusion: *PD-L1* non-coding variants play an important role in modulating immune checkpoint function and may be explored as immunotherapy biomarkers. We highlight the rs7041009 variant, which impacts OS and PPS in NSCLC patients.

Keywords: *PD-L1*, non-small cell lung cancer, single nucleotide polymorphisms, next-generation sequencing

Introduction

Lung cancer is one of the leading causes of cancer-related death globally.¹ Although there are different types of lung cancer, non-small cell lung cancer (NSCLC) represents 85% of all primary lung tumors. NSCLC is a grim disease that is aggravated by the fact that patients normally either receive their diagnosis at advanced stages or present with recurrent disease after initial locoregional

treatment.² Over the last few decades, conventional chemotherapy, mainly platinum-based chemotherapy, used to be the only therapeutic option for those not eligible for radical intent treatment: a treatment with limited efficacy and very few long-term survivors (5-year overall survival less than 15%). Furthermore, these patients often lacked therapeutic options beyond first-line treatment.³

More recently, though, immune checkpoint molecules involved in tumor immune evasion were identified and immune checkpoint inhibitors (ICIs) were introduced in antitumor immunotherapy. This new therapeutic approach targets an inhibitory receptor, the programmed cell death-1 (PD-1) receptor, to assist the immune system in identifying and neutralizing malignant cells. However, tumor cells may evade the host immunosurveillance by expressing the programmed death-1-ligand 1 (PD-L1) as an adaptive, resistant mechanism to suppress this inhibitory receptor.⁴ Thus, because PD-L1 up-regulation by tumor cells can protect them from antitumor immune response, the blockade of PD-L1/PD-1 interactions has been recently selected for antitumor immune therapy.⁵ Agents targeting the PD-1/PD-L1 signaling pathway have shown promising responses in different types of cancer, including NSCLC. These results point to PD-L1 protein expression as a potential predictive marker for a successful blockade of PD-L1/PD-1 interactions.⁶ However, several challenges remain in producing robust evidence to support the use of this biomarker.

In this context, some studies with NSCLC patients have demonstrated that those with more than 50% PD-L1 positive tumor cells are non-responders to anti-PD-1/PD-L1 treatment. In contrast, others have shown that patients whose tumors do not express PD-L1 are good responders.⁷⁻⁹ To explain the controversies that affect PD-L1 expression, some studies have considered that the heterogeneity between axis expression and response to PD-1/PD-L1 treatment in NSCLC depends on other factors, such as more precise methods to investigate immune evasion mechanisms and the immune microenvironment, as well as greater knowledge on the immune checkpoint genomic profile and the genetic variants of the *PD-L1* gene.¹⁰⁻¹³

Some genetic variants have been shown to affect normal gene activation and transcriptional initiation, and hence influence the amount of mRNA and encoded protein in the cell.¹⁴ Non-coding variants also presumably affect genetic regulatory elements, since a majority of driver variants in cancer genomes occur in non-coding regions.^{15,16}

Thus, several studies have investigated the association between *PD-1* and *PD-L1* genetic variants and the risk of various cancers, but their findings have yet failed to completely elucidate this question.¹⁷ A previous study suggested that *PD-L1* polymorphism may predict chemotherapy response and survival rates in advanced-stage NSCLC patients after first-line paclitaxel-cisplatin.¹⁸ More recently, *PD-L1* copy number variations, point mutations, and 3'-UTR disruptions have been highlighted as genetic mechanisms of PD-L1 deregulation.¹⁹ Furthermore, previous research on Brazilian patients suggested that their ethnic background could account for their distinct cancer's molecular profile, perhaps due to their characteristic genetic admixture, inherited from European, African, and Native American ancestors.²⁰⁻²²

We hypothesize that *PD-L1* non-coding genetic variants modulate the function of this immune checkpoint in NSCLC. To explore this issue, we investigated fifteen *PD-L1* non-coding genetic variants using next-generation sequencing (NGS) in a Brazilian cohort, aiming to uncover the effect of their ethnic admixture on NSCLC. We also combined our analyses with an in-silico approach to predict the impact of these genetic variants on the disease. We evaluated the associations between PD-L1 protein expression level and clinicopathological characteristics, including the prognosis of NSCLC patients undergoing surgical resection, glimpsing the impact of genetic variants on post progression survival (PPS) and overall survival (OS). Herein, in this context, we intend to expand the existing literature on *PD-L1* gene alterations at the genetic level and their impact on NSCLC in patients from different ethnicities, thus increasing the knowledge about the molecular basis of immunotherapy biomarkers.

Materials and Methods

Cohort

In this retrospective multi-center study, we obtained archival formalin-fixed paraffin-embedded histologic tumor sections from 70 patients diagnosed with NSCLC (33 adenocarcinomas [ADC], 24 squamous cell carcinoma [SqCC] and 13 large cell carcinoma [LCC]) who underwent surgical resection between January 1, 1995, and December 31, 2015. Patients had been treated at the Hospital das Clínicas of the University of São Paulo Medical School (HC-FMUSP),

at the Heart Institute of the University of São Paulo (INCOR), at the Cancer Institute of São Paulo (ICESP), and at the A.C. Camargo Cancer Center in São Paulo, Brazil.

All samples were histologically reviewed by lung pathologists who selected samples with at least 30% of lung cancer cells before nucleic acid extraction. The samples were classified using the 2017 International Association for the Study of Lung Cancer (IASLC) classification system.²³ The clinicopathological features of patients were obtained from the medical records. The study was approved in accordance with the ethical standards of the responsible committee on human experimentation local (Research Ethics Committee of University of São Paulo Medical School - CAAE: 79769017.1.0000.5440; opinion number: 2.673.320) and with the 1964 Helsinki declaration. A waiver of the requirement for informed consent was obtained from committee, and to identity of the subjects under this retrospective analysis was omitted and anonymized.

Multiplex Immunofluorescence Staining

We performed a Multiplex immunofluorescence (mIF) staining using methods that had been previously described and validated.^{24,25} Four-micrometer-thick consecutive TMA sections were stained using an automated staining system (BOND-RX; Leica Biosystems, Buffalo Grove, IL) to characterize PD-L1 (clone E1L3N, dilution 1:100; Cell Signaling Technology, Danvers, MA). The PD-L1 marker was stained with its respective fluorophore from the Opal 7 kit (catalogue #NEL797001KT; Akoya Biosciences/PerkinElmer, Waltham, MA). A complete validation using immunofluorescence (IF) allowed us to obtain a uniform, specific, and appropriate signal across all the channel; ie, a well-balanced staining pattern for the multiplex staining.^{24,25} We also defined and optimized the correct fluorophore signals between 10 and 30 counts of intensity to maintain good balance and similar thresholds of intensity across all antibodies. In parallel, to detect possible variations in staining and optimize the separation of the signal, positive and negative (autofluorescence) controls were included during the staining process to ensure that all the antibodies performed well together. Autofluorescence controls with an expected spectral resolution of 488nm were able to accurately remove the autofluorescence from all the label signals during the analysis. The stained slides were then scanned using a multispectral microscope, the Vectra Polaris 3.0 imaging system (Akoya

Biosciences/PerkinElmer, Waltham, MA), under fluorescence conditions.

Multiplex Immunofluorescence Quantitation

Multispectral images of tumor sections from each core were analyzed with inForm 2.2.1 (Akoya Biosciences/PerkinElmer, Waltham, MA) software. Individual cells, which were defined by nuclei staining and identified by the InForm cell segmentation tool, were subjected to a phenotyping pattern-recognition learning algorithm to characterize co-localization of the various cell populations using panel labeling.²⁶ The panel labeling was as follows: Malignant cells (MCs), with the AE1/AE3+ marker, including those with and without PD-L1 expression (AE1/AE3+ PD-L1+ and AE1/AE3+ PD-L1-, respectively). The individual cell phenotype report produced by the InForm software was processed using Excel 2010 (Microsoft, Houston, TX), and a final summary of the data, which contained the median of each individual phenotype (given as number of cells/mm²) and the percentage of macrophages and MCs expressing PD-L1, was created for statistical analysis. If the percentage of MCs or macrophages expressing PD-L1 was greater than the median value, the PD-L1 expression was considered positive. If the percentage of macrophages or MCs expressing PD-L1 was lower than or equal to the median, the PD-L1 expression was considered negative.

DNA Extraction

Genomic DNA (gDNA) was extracted from frozen NSCLC tissue using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA concentration was measured using the Qubit[®] 3.0 Fluorometer (Invitrogen, Life Technologies, CA, USA). DNA integrity was assessed using the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Sequencing and Data Analysis

We performed a *PD-L1* (*CD274*) full gene screening by deep targeted sequencing using the TruSeq Custom Amplicon Panel v1.5 kit (TSCAP, Illumina, SanDiego, CA) and the MiSeq platform (Illumina, SanDiego, CA). The DNA libraries were performed according to the manufacturer's instructions and consisted of 150 bp paired-end reads (300 cycles).

We performed an NGS data analysis on the Molecular Genetics and Bioinformatics Laboratory of the Experimental Research Unit (UNIPEX) at the Medical School of São Paulo State University (FMB-UNESP). Sequencing quality was assessed by FastQC. Reads were aligned to the human genome (hg19, GRCh37) with BWA software, and SAM tools converted the alignment results to BAM format.²⁷ Next, the mapped reads underwent variant calling for SNP with GATK command line tools, including HaplotypeCaller, SelectVariants, and VariantFiltration programs with default parameters. After the calling step, the variants were annotated using the VEP²⁸ software. Coverage depth was a priori set at 100×. Variants had to have >10 reads of position depth (PD) and/or >6 reads of allele depth (AD) and/or an AD/PD ratio of >0.05 and/or a population frequency higher than 1% (popfreq_all >0.01) were included in the study. Finally, variants were compared using ABraOM, a web-based public database of Brazilian genomic variants.²⁹

In silico Prediction of Non-Coding Genetic Variants

Several tools were used to predict potential functional effects of SNPs on non-coding binding sites, such as splice sites and binding sites for transcription factors, exonic splicing enhancers (ESEs), and microRNA (miRNA). The impact of each genetic variant was assessed using VarSome³⁰ an integrated search engine that allows access to several databases, forecasting tools, and publications on a single website. Variant pathogenicity was reported using an automatic variant classifier that evaluates each submitted variant according to guideline of the American College of Medical Genetics and Genomics (ACMG) and classifies it as either “pathogenic”, “likely pathogenic”, “likely benign”, “benign” or “uncertain significance”. Varsome predicts the pathogenicity of each variant through a DANN³¹ score, a methodology for scoring deleterious annotations of genetic variants using neural networks that results in a number ranging from 0 to 1. Higher DANN scores represent greater variant deleteriousness.³¹

Next, we applied the Genomic Evolutionary Rate Profiling (GERP)^{32,33} conservation score. This score is used to calculate the reduction of substitutions in a multi-species sequence alignment when compared to a neutral expectation. GERP scores >5.5 are strongly associated with a purifying selection. Mutations that occur at highly

conserved sites in many species are assumed as harmful and therefore contribute to the genetic load within a species.

Finally, we used two tools, SNPinfo (FuncPred)³⁴ and RegulomeDB,³⁵ to track SNPs according to their functions. SNPinfo is a web server that helps researchers investigate SNPs in studies of genetic association and provide different pipelines for SNP selection, whereas RegulomeDB is an online composite database and prediction tool to annotate and prioritize potential regulatory variants from the human genome.³⁵ RegulomeDB divides the variants into six categories: category 1 variants are likely to affect binding and are linked to the expression of a gene target, category 2 variants are likely to affect binding, category 3 variants are less likely to affect binding, and category 4, 5, and 6 variants have minimal binding evidence.³⁵

Statistical Analysis

The allelic and genotypic frequencies of the *PD-L1* polymorphisms found in NSCLC were calculated by Hardy Weinberg equilibrium ($[1 - (hC\ 2H)]/2N$, where “h” stands for a heterozygous genotype, “H” for homozygous genotype and “N” for the number of samples). Associations between polymorphisms, PD-L1 protein expression, and the clinicopathological parameters of NSCLC patients were investigated by Chi-square test. The prognostic value of each polymorphism was assessed by a survival analysis using the Kaplan–Meier method with the Log rank test for statistical significance. In addition, Cox’s proportional hazards regression models were used in a multivariate analysis to test the association between SNPs and PPS and OS. PPS was considered as the period from tumor progression until death or last follow-up. OS was defined as the time from curative surgery to death or last date known to be alive. The statistical software program IBM SPSS (version 22; Armonk, NY, USA) performed all analyses. Differences were considered statistically significant at $P < 0.05$.

Results

Clinicopathologic Characteristics

Of the 70 patients included in the study, 33 presented with ADC (47.1%), 24 with SqCC (34.3%), and 13 with LCC (18.6%). The clinical characteristics by histologic types are summarized in Table 1. While SqCC cases were more frequent in males (81.8%), ADC cases were equally distributed between genders, and LCC cases were close to equal

Table I Demographic and Clinicopathological Characteristics of 70 NSCLC Patients

Characteristics	Histological Subtypes			P-value
	Adenocarcinoma (N=33)	Squamous Cell Carcinoma (N=24)	Large Cell Carcinoma (N=13)	
^a Gender				
Male	10 (50.0%)	9 (81.8%)	5 (55.6%)	0.21
Female	10 (50.0%)	2 (18.2%)	4 (44.4%)	
^a Age (years)				
≤ 63	11 (61.1%)	6 (54.5%)	6 (60.0%)	0.93
> 63	7 (38.9%)	5 (45.5%)	4 (40.0%)	
^a Smoke Status				
Yes	8 (72.7%)	8 (88.9%)	3 (50.0%)	0.25
No	3 (27.3%)	1 (11.1%)	3 (50.0%)	
^a Clinical Stage†				
I–II	8 (47.1%)	4 (40.0%)	3 (42.9%)	0.93
III–IV	9 (52.9%)	6 (60.0%)	4 (57.1%)	
^a Treatment				
Chemotherapy				
Yes	7 (36.8%)	3 (27.3%)	4 (40.0%)	0.80
No	12 (63.2%)	8 (72.7%)	6 (60.0%)	
Radiotherapy				
Yes	1 (5.3%)	1 (9.1%)	0 (0.0%)	0.63
No	18 (94.7%)	10 (90.9%)	10 (100%)	
^a Relapse				
Yes	9 (64.3%)	4 (50.0%)	2 (33.3%)	0.43
No	5 (35.7%)	4 (50.0%)	4 (66.7%)	
^a Mcs+PD-L1 (median cell density)				
≤1.26	13 (54.2%)	10 (52.6%)	3 (30.0%)	0.40
>1.26	11 (45.8%)	9 (47.4%)	7 (70.0%)	
Follow-up (months) 66 (12–144)				
^a Patients censored for survival analysis at last follow-up time	8 (53.3%)	4 (66.7%)	3 (42.9%)	-

Notes: ^aSome cases had missing follow-up information: gender (30); age (31); smoke status (44); clinical stage (36); adjuvant treatment QT/RT (30); relapse (42); Mcs+PD-L1 (17); survival (44). † 8th International Association for the Study of Lung Cancer [23].

Abbreviations: Mcs, malignant cells; PD-L1, programmed death-ligand 1.

distribution (male 55.6%, female 44.4%). All histological types were more frequent in patients aged 63 years or younger. 8 patients reported a history of tobacco smoking in the ADC group (72.7%) and in the SqCC group (88.9%), versus 3 patients in the LCC group (50.0%). All the histological subtypes included advanced stages of disease (9 cases in ADC, 6 cases in SqCC, and 4 cases in LCC). Most of the patients had not received either chemotherapy (12 cases to ADC, 8 cases to SqCC, and 6 cases to LCC) or radiation therapy (18 cases to ADC, 10 cases to SqCC, and LCC) as adjuvant treatment. Malignant cells expressed PD-L1 above the median in 7 LCC cases (70.0%), the most relevant expression compared to the

other two histological subtypes. The median follow-up was 66 (12–144) months. None of the analysis revealed significant differences between histological types ($P>0.05$).

Allele and Genotype Distributions of PD-L1 Gene Polymorphisms

All NSCLC patients who underwent surgical resection were successfully genotyped for fifteen *PD-L1* SNPs: rs76805387T>C, rs4742098A>G, rs47946526A>G, rs10217310G>T, rs7864231G>A, rs41280725C>T, rs573692330A>G, rs1011769981G>A, rs41280723T>C, rs138135676T>C, rs4143815G>C, rs2297136G>A,

rs148242519G>A, rs41303227C>T, and rs7041009G>A. [Supplementary Table 1](#) shows the SNP identification numbers, allele and genotype frequencies, and *P*-value for HWE. Of the 15 SNPs studied, 11 were found to be monomorphic, whereas 4 SNPs, namely rs4742098, rs4143815, rs2297136, and rs7041009, were polymorphic in NSCLC. Monomorphic SNPs were excluded from further analysis. All the polymorphic SNPs were found to be in equilibrium ($P>0.05$) for HWE. The allele frequency of our cohort was compared to different populations in the 1000 Genomes Project ([Supplementary Table 2](#)).

Correlation Between PD-L1 Gene Polymorphisms and Clinicopathological Characteristics in NSCLC

We performed stratified analyses on the associations between clinical characteristics and the four *PD-L1* polymorphisms with different genotypic distributions. [Table 2](#) shows each SNP genotype frequency and their associated clinicopathological characteristics. Three of the four *PD-L1* gene polymorphisms (rs4742098, rs4143815, and rs7041009) were significantly associated with relapse

Table 2 Clinicopathological Characteristics of 70 NSCLC Patients Stratified by the *PD-L1* Polymorphisms rs4742098, rs4143815, rs2297136 and rs7041009

Characteristics	rs4742098 AA vs AG/GG			rs4143815 GG vs CG/CC			rs2297136 GG vs AG/AA			rs7041009 GG vs AG/AA		
	No. of Patients (N=70) (%)			No. of Patients (N=70) (%)			No. of Patients (N=70) (%)			No. of Patients (N=70) (%)		
	A/A	A/G+G/G	P	G/G	C/G+C/C	P	G/G	AG+A/A	P	G/G	A/G+A/A	P
Age (years), median												
≤ 63	13 (56.5%)	10 (62.5%)	0.75	11 (57.9%)	12 (60.0%)	1.00	4 (57.1%)	19 (59.4%)	1.00	16 (76.2%)	7 (38.9%)	0.02
>63	10 (43.5%)	6 (37.5%)		8 (42.1%)	8 (40.0%)		3 (42.9%)	13 (40.6%)		5 (23.8%)	11 (61.1%)	
Gender, n (%)												
Male	13 (56.5%)	11 (64.7%)	0.74	12 (63.2%)	12 (57.1%)	0.75	4 (57.1%)	20 (60.6%)	1.00	12 (57.1%)	12 (63.2%)	0.75
Female	10 (43.5%)	6 (35.3%)		7 (36.8%)	9 (42.9%)		3 (42.9%)	13 (39.4%)		9 (42.9%)	7 (36.8%)	
Smoke status												
Yes	10 (71.4%)	9 (75.0%)	1.00	9 (75.0%)	10 (71.4%)	1.00	4 (66.7%)	15 (75.0%)	1.00	9 (60.0%)	10 (90.9%)	0.17
No	4 (28.6%)	3 (25.0%)		3 (25.0%)	4 (28.6%)		2 (33.3%)	5 (25.0%)		6 (40.0%)	1 (9.1%)	
Histology												
ADC	18 (43.9%)	15 (51.7%)	0.61	15 (41.7%)	18 (52.9%)	0.40	7 (53.8%)	26 (45.6%)	0.86	15 (41.7%)	18 (55.9%)	0.34
SqCC	16 (39.0%)	8 (27.6%)		15 (41.7%)	9 (26.5%)		4 (30.8%)	20 (35.1%)		12 (33.3%)	12 (35.3%)	
LCC	7 (17.1%)	6 (20.7%)		6 (16.7%)	7 (20.6%)		2 (15.4%)	11 (16.3%)		9 (25.0%)	4 (11.8%)	
Clinical stage†												
I–II	8 (40.0%)	7 (50.0%)	0.72	7 (41.2%)	8 (47.1%)	1.00	1 (14.3%)	14 (51.9%)	0.10	8 (44.4%)	7 (43.8%)	1.00
III–IV	12 (60.0%)	7 (50.0%)		10 (58.8%)	9 (52.9%)		6 (85.7%)	13 (48.1%)		10 (55.6%)	9 (56.3%)	
Mcs+PD-L1 (median)												
≤1.26	15 (57.7%)	11 (42.3%)	0.78	14 (53.8%)	12 (46.2%)	1.00	6 (23.1%)	20 (76.9%)	0.74	11 (42.3%)	15 (57.7%)	0.27
>1.26	14 (51.9%)	13 (48.1%)		14 (51.9%)	13 (48.1%)		5 (18.5%)	22 (81.5%)		16 (59.3%)	11 (40.7%)	
Chemotherapy												
Yes	7 (30.4%)	7 (41.2%)	0.52	5 (26.3%)	9 (42.9%)	0.33	3 (42.9%)	11 (33.3%)	0.67	10 (47.6%)	4 (21.1%)	0.10
No	16 (69.6%)	10 (58.8%)		14 (73.7%)	12 (57.1%)		4 (57.1%)	22 (66.7%)		11 (52.4%)	15 (78.9%)	
Radiotherapy												
Yes	1 (4.3%)	1 (5.9%)	1.00	1 (5.3%)	1 (4.8%)	1.00	1 (14.3%)	1 (3.00%)	0.32	0 (0.0%)	2 (10.5%)	0.21
No	22 (95.7%)	16 (94.1%)		18 (94.7%)	20 (95.2%)		6 (85.7%)	32 (97.0%)		21 (100.0%)	17 (89.5%)	
Relapse												
Yes	11 (78.6%)	4 (28.6%)	0.01	9 (75.0%)	6 (37.5%)	0.05	5 (71.4%)	10 (47.6%)	0.39	5 (33.3%)	10 (76.9%)	0.02
No	3 (21.4%)	10 (71.4%)		3 (25.0%)	10 (62.5%)		2 (28.6%)	11 (52.4%)		10 (66.7%)	3 (23.1%)	
Status												
Live	5 (38.5%)	8 (53.3%)	0.47	5 (45.5%)	8 (47.1%)	1.00	3 (50.0%)	10 (45.5%)	1.00	11 (78.6%)	2 (14.3%)	0.00
Dead	8 (61.5%)	7 (46.7%)		6 (54.5%)	9 (52.9%)		3 (50.0%)	12 (54.5%)		3 (21.4%)	12 (85.7%)	

Notes: Bolded values are statically significant. †8th International Association for the Study of Lung Cancer [23].

Abbreviations: ADC, adenocarcinoma; SqCC, squamous cell carcinoma; LCC, large cell carcinoma; Mcs, malignant cells; PD-L1, programmed death-ligand 1.

($P=0.01$; $P=0.05$; $P=0.02$, respectively). For the rs4742098 variant, carriers of the G allele (AG or GG genotypes) were less likely to relapse ($P=0.01$). Similarly, for rs4143815, carriers of the alternative C allele (CG or CC genotypes) were also less likely to relapse ($P=0.05$). In rs7041009, however, carriers of the alternative allele A (AG or GG genotypes) were more likely to relapse ($P=0.02$). Moreover, GG genotype (reference) of rs7041009 showed a significant correlation with age, being more prevalent among younger patients (16 patients, or 69.6%), and status, being more prevalent among patients who were alive (11 patients, or 84.6%), compared to carriers of the A allele ($P=0.02$ and $P<0.01$, respectively). No statistical significance was observed in the association between rs2297136 genotypes and clinico-pathological variants.

Correlation Between PD-L1 Gene Polymorphisms and PD-L1 Protein Expression

The correlation between PD-L1 protein expression and *PD-L1* gene polymorphisms are shown in Table 2. There were no statistically significant associations between PD-L1 protein expression in malignant cells and *PD-L1* gene polymorphisms. In our cohort, the four *PD-L1* gene polymorphisms were in non-coding regions and, apparently, cause no interference in PD-L1 protein expression in NSCLC malignant cells. However, when we correlated PD-L1 protein expression with histological subtype, we observed that the expression in malignant cells was above the median in 70% of patients with LLC, in contrast with 45.8% and 47.4% of patients with ADC and SqCC, respectively.

Associations Between PD-L1 Gene Polymorphisms and Survival Outcomes

Our first statistical test examined the individual effect of patients' characteristics to estimate statistical differences in survival using the Kaplan–Meier method (Table 3). Patients younger than 63 years showed increased OS, 111.62 vs 66.54 months in older patients ($P=0.05$). Choice of treatment was also an independent factor in diagnosis, with patients who did not receive radiotherapy presenting a better survival rate when compared with those who were treated with radiotherapy, 94.43 vs 12.00 months, respectively ($P<0.01$). Patients who presented disease recurrence had lower survival rates and poorer

Table 3 A Survival Analysis Conducted by the Kaplan–Meier Method Showing the Difference in the Means of the Log Rank Test According to the Optimal Upper and Lower Binary Cut-off Limits of Different Variables

Variables	Overall Survival (Months)		Chi-Square (Log Rank)	P-value
	Mean	Standard Error		
Age				
≤63	111.62	13.92	3.88	0.05
>63	66.54	15.99		
Treatment				
Radiotherapy				
Yes	12.00	0.00	11.46	<0.01
No	94.43	11.31		
Relapse				
Yes	48.23	9.84	11.01	<0.01
No	123.10	13.48		
PD-L1 polymorphisms				
rs7041009 (GG vs AG/AA genotype)				
GG	116.93	13.81	7.49	<0.01
AG/AA	59.00	13.67		

Note: Bolded values are statically significant.

Abbreviation: PD-L1, programmed death-ligand 1.

prognostic when compared with those who did not relapse, 48.23 vs 123.10 months, respectively ($P<0.01$).

Moreover, differences in the genotypes of *PD-L1* polymorphisms seemed to also impact the prognosis of NSCLC patients. *PD-L1* rs7041009, for instance, led to a statistically significant difference in OS (Figure 1), with carriers of the A allele of rs7041009 having lower OS than carriers of the GG genotype (reference), 59.00 vs 116.93 months, respectively ($P<0.01$).

Next, using a univariate Cox Regression analysis, we were able to associate the following variables with a lower risk of death: the absence of radiotherapy treatment, relapse, and GG genotype of *PD-L1* rs7041009 (Table 4). However, after feeding these variables into a multivariate analysis, only the absence of radiotherapy treatment and relapse were considered to be independent factors for OS (HR 9.82, $P=0.02$; HR 6.15, $P=0.04$, respectively).

Then, we introduced the *PD-L1* polymorphisms into the Cox model, controlling for radiotherapy treatment and tumor relapse. Of the four SNPs, only rs7041009 was identified as a co-dependent factor associated with radiotherapy and relapse. We thus inferred that patients with NSCLC who

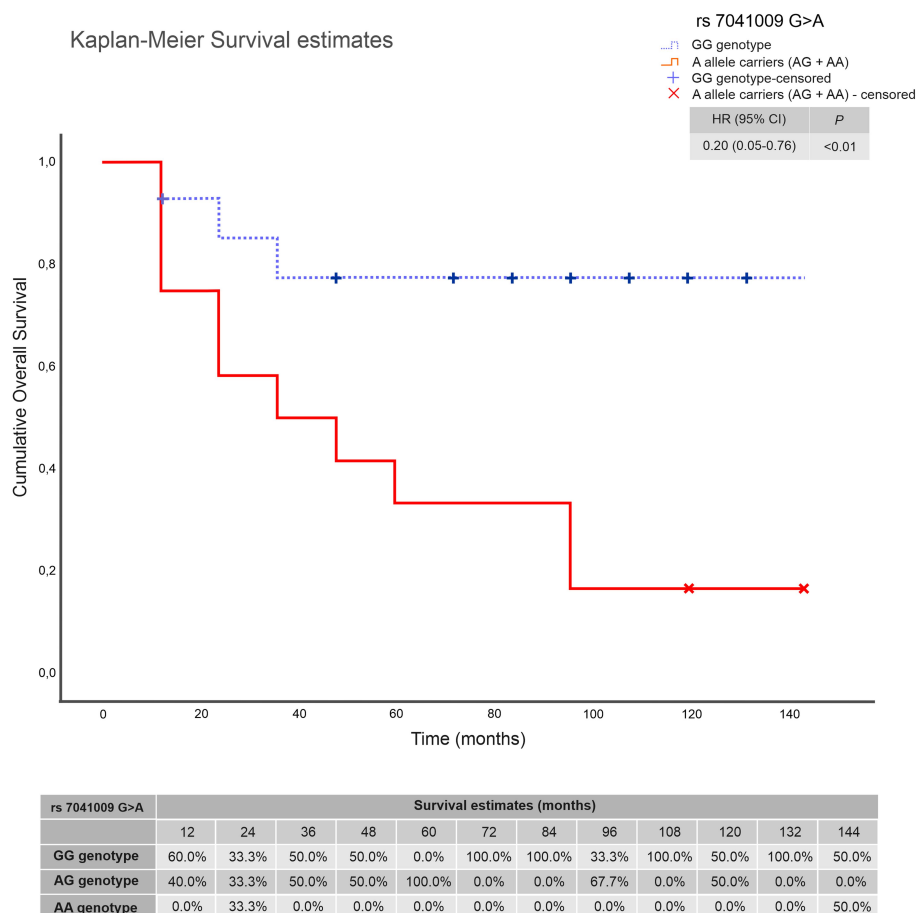


Figure 1 Kaplan–Meier survival curve for *PD-L1* rs7041009 G>A. A allele carriers (AG+AA) presented worse prognosis and a lower survival rate when compared to GG genotyped patients ($P<0.01$).

carried the A allele (AG/AA) presented a higher risk of relapse in the presence of radiotherapy, resulting in a poorer prognosis and decreased survival rates than patients who carried the rs7041009 GG genotype. In relapsed patients, we observed that the *PD-L1* polymorphisms rs7041009 and rs4742098 might have an impact on PPS (Figure 2). Patients with the rs7041009 GG genotype had a higher PPS than those with the alternative A allele of rs7041009 (AG/AA), 110.98 vs 56.18 months, respectively ($P<0.01$); whereas, patients who carried the reference rs4742098 AA genotype had lower PPS than those who carried the alternative G allele of rs4742098 (AG/GG), 56.00 vs 115.71 months, respectively ($P=0.02$).

In silico Prediction of PD-L1 Gene Polymorphisms

The in silico analysis predicted the *PD-L1* variants rs4742098 (c.*2635A>G), rs4143815 (c.*395G>C), rs2297136 (c.*93G>A), and rs7041009 (c.682+122G>A) to be benign (Table 5). Not only was the DANN score low for all four variants (0.8226, 0.6475, 0.7056, and 0.5428, respectively),

but their GERP score was also lower than 5.5 (−1.74, 2.38, 4.4, and 1.81, respectively), indicating that these variants are found in non-conserved positions and are unlikely to be harmful.

SNPinfo predicted miRNA-binding function to be affected by two of these variants, namely rs2297136 and rs4143815. Rs2297136 was predicted to affect the binding function of hsa-miR-324-5p and hsa-miR-632, whereas rs4143815 was found to correlate with hsa-miR-1252, hsa-miR-1253, hsa-miR-539, hsa-miR-548, and hsa-miR-570 (Table 6). RegulomeDB was then used to complement the SNP analysis. Three of the four SNPs, rs4742098, rs4143815, and rs2297136, had a RegulomeDB score of 5, whereas rs7041009 had a score of 6, meaning that all four variants show minimal binding evidence (Table 5).

Discussion

Lung cancer has a high mortality rate and lacks suitable markers for early diagnosis and prognosis. Thus, it is essential to detect the best potential biomarker out of the several genetic and protein markers. Fortunately for

Table 4 Variables Associated with Overall Survival (OS) in 70 Patients Diagnosed with NSCLC. Univariate and Multivariate Analyses Employing a Cox Proportional Hazards Model

Clinicopathological Characteristics	Univariate Analysis			Multivariate Analysis	
	HR (95% CI)	HR	P value	HR ^c (95% CI)	P-value
Age (yrs): ≤63 vs >63	0.33 (0.10–1.10)	–1.10	0.07		
Gender Male	1.83 (0.54–6.14)	0.60	0.32		
Smoke status Yes	1.33 (0.33–5.36)	0.28	0.68		
Histology ADC SqCC LCC (reference)	1.25 (0.32–4.86) 1.67 (0.33–8.37) -	0.22 0.51 -	0.74 0.53 0.82		
Clinical stage † I–II III–IV (reference)	0.41 (0.11–1.52) -	–0.87 -	0.18 -		
Therapy Chemotherapy Yes	0.36 (0.10–1.34)	–1.00	0.13		
Relapse Yes	8.47 (1.81–39.62)	2.13	0.00	6.15 (1.03–36.44)	0.04
Immune checkpoint PD-L1+/mm ²	1.00 (0.95–1.05)	0.00	0.94		
polymorphisms in PD-L1					
rs4742098 AA genotype G allele carrier (reference)	2.02 (0.65–6.30) -	0.70 -	0.22 -		
rs4143815 GG genotype C allele carrier (reference)	1.58 (0.52–4.81) -	0.46 -	0.41 -		
rs2297136 GG genotype A allele carrier (reference)	1.24 (0.34–4.55) -	0.22 -	0.73 -		
rs7041009 GG genotype A allele carrier (reference)	0.20 (0.05–0.76) -	–1.56 -	0.01 -	0.57 (0.12–2.70)	0.48

Notes: Bolded values are statically significant. †8th International Association for the Study of Lung Cancer [23].

Abbreviations: ADC, adenocarcinoma; SqCC, squamous cell carcinoma; LCC, large cell carcinoma; HR, hazard ratio (β coefficient); CI, confidence interval; PD-L1, programmed death-ligand 1.

patients, the translational impact of such findings is rapidly increasing, and the stimulation of immune response by ICI has emerged as a dramatic paradigm shift in the treatment of advanced tumors, mainly NSCLC.^{19,36} PD-1/PD-L1 monoclonal antibodies have shown potential efficacy in advanced squamous-cell and non-squamous

NSCLC.^{37,38} However, despite the remarkable success achieved by immunotherapy so far, its effectiveness still seems to vary among cancer patients.¹⁹ The expression of PD-L1 on tumor cells remains the only recognized predictive factor for immunotherapy response in NSCLC patients; however, patients without PD-L1 expression on

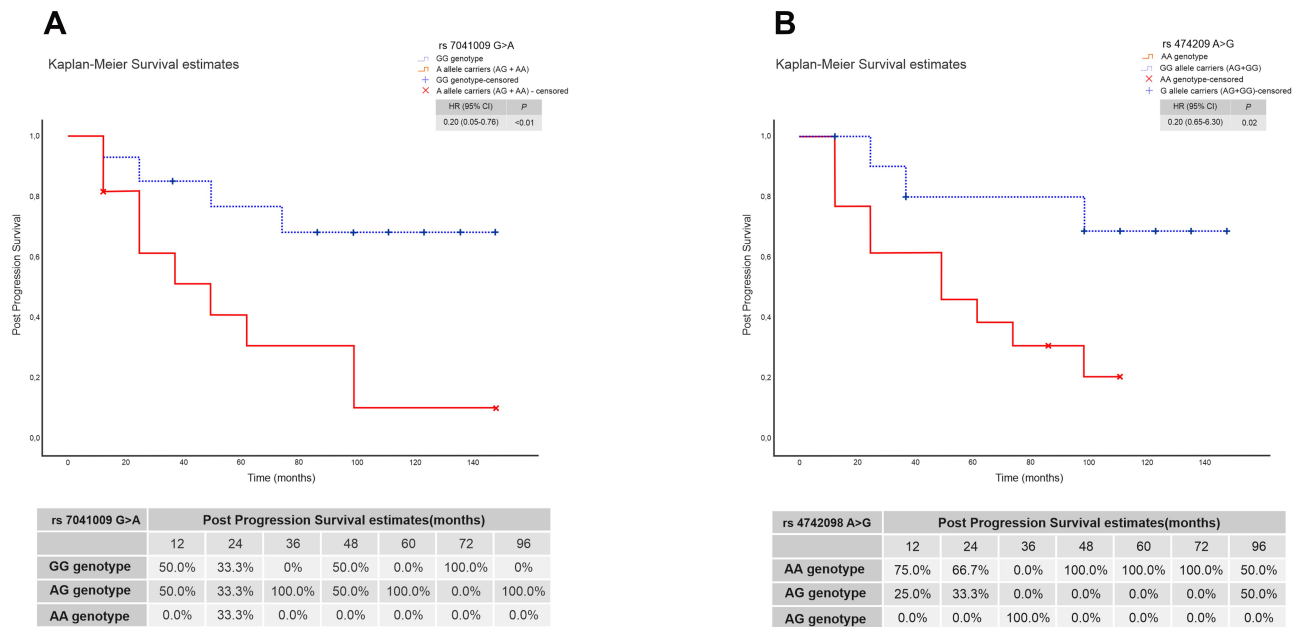


Figure 2 Kaplan–Meier survival curves estimating post-progression survival (PPS) in NSCLC patients according to *PD-L1* polymorphisms. **(A)** Kaplan–Meier survival curve for rs7041009 G>A. A allele carriers (AG+AA) presented worse prognosis and a lower PPS rate when compared GG genotyped patients ($P<0.01$); **(B)** Kaplan–Meier survival curve for rs4742098 A>G. G allele carriers (AG+GG) had a higher PPS rate and better prognosis when compared AA genotyped patients ($P=0.02$).

tumor cells may also respond to immunotherapy.^{39–43} Based on these findings, the present study inferred that *PD-L1* non-coding genetic variants could help predict the prognosis of patients with NSCLC and impact disease recurrence and OS.

In our cohort of 70 NSCLC specimens, we evaluated *PD-L1* protein expression in malignant cells by *PD-L1* multiplex immunofluorescence (mIF) assays, using the Cell Signaling E1L3N clone and image analysis, and investigated *PD-L1* polymorphisms by NGS sequencing. This method resulted in the detection of high *PD-L1* expression in LCC malignant cells when compared to other histological subtypes, suggesting that LCC patients may benefit from ICIs. As described by Shimoji et al,⁴⁴ *PD-L1* expression using the Cell Signaling E1L3N clone was significantly correlated with a consistent vimentin expression, increased Ki-67 labeling index and poor

prognosis in ADC but not in SqCC. Other studies have reported that *PD-L1* was detected at significantly higher frequencies in SqCC than in ADC of the lung.^{44,45} Cha et al⁴⁶ found that *PD-L1* expression using the SP142 clone was significantly associated with an ADC solid subtype histology, p53 aberrant expression, and poor prognosis.

We also assessed *PD-L1* polymorphisms. Of the fifteen genetic variants genotyped, eleven were monomorphic. The four potential variants (rs4742098, rs4143815, rs2297136, and rs7041009) present in the non-coding region were correlated with the clinicopathological characteristics of the NSCLC patients and were submitted to an in silico analysis investigating their functional role. The MAF of the rs4742098, rs4143815, and rs7041009 polymorphisms was consistent with the genotype frequency among European and Mixed Americans populations present in The 1000 Genomes Project, whereas the G allele in

Table 5 List of the Selected Non-Coding SNP and the Tools Used to Study Them

dbSNP ID	Region	FuncPred	RegulomeDB score	VarSome (DANN Score)
rs4742098	3'UTR	–	5	Benign (0.8226)
rs4143815	3'UTR	miRNA-binding function	5	Benign (0.6475)
rs2297136	3'UTR	miRNA-binding function	5	Benign (0.7056)
rs7041009	Intron	–	6	Benign (0.5428)

Note: SNPs that do not affect function.

Abbreviations: SNPs, single-nucleotide polymorphisms; dbSNP, SNP database; FuncPred, SNP function prediction; RegulomeDB score, RegulomeDB variant classification; VarSome, The human genomics community; DANN score, annotation of genetic variants using neural networks.

Table 6 List of the 3' UTR SNPs Analyzed in FuncPred and Their miRNA Motif

dbSNP ID	Allele	miRNA Motif
rs4143815	C	hsa-miR-1252
	C	hsa-miR-1253
	C	hsa-miR-539
	C	hsa-miR-548
	G	hsa-miR-570
rs2297136	A/G	hsa-miR-324-5p
	A/G	hsa-miR-632

Abbreviations: FuncPred, SNP function prediction; dbSNP ID, SNP database identification.

the rs2297136 polymorphism was the main allele in our cohort to show racial differences.

When assessing patient prognosis, three of the four *PD-L1* variants rs4742098, rs4143815, and rs7041009 were significantly associated with disease recurrence. Carriers of the G allele (individuals with the AG or GG genotypes) of rs4742098 were less likely to relapse compared to carriers of the homozygous AA genotype. Similar findings were published by Du and colleagues,⁴⁷ who reported that the AG genotype differed from the AA genotype in terms of risk of NSCLC recurrence. In the case of rs4143815G, patients with the alternative C allele were less likely to relapse in our study, in agreement with Nomizo et al's report.⁴⁸ In their study, the authors even suggested that this polymorphism might be a biomarker for nivolumab efficacy.⁴⁸ Finally, in our study, for rs7041009G>A, carriers of the alternative G allele were more likely to exhibit relapse. The rs7041009 GG genotype also showed a significant correlation with age, being more present in younger patients, and with status, being more present in patients who are alive, compared to carriers of the A allele. Rs7041009 (c.682+122G>A) is located at position 2377 in intron 4 of the *PD-L1* gene. However, little is known about the exact function of this genetic variation, except that it is located near the transcription factor binding site.

Our cohort showed a significant association between these *PD-L1* polymorphisms and OS in NSCLC. 18 Patients presenting the GG genotype of rs7041009 benefited from longer OS. In addition, our findings indicated that both the rs7041009G and rs4742098G polymorphisms were significantly related to a longer PPS. The clinical impacts of *PD-L1* variants had also been investigated by previous studies. Zhao et al⁴⁹ suggested that patients with the GG

genotype of another *PD-L1* polymorphism (rs822336) had worse disease-free survival and OS in a Chinese patient population.

Further contribution was provided in that respect by Lee et al⁵⁰ who demonstrated that rs4143815 and rs2297136 were significantly associated with clinical outcomes after chemotherapy. Of 379 patients with NSCLC treated with first-line paclitaxel–cisplatin chemotherapy, those carrying the rs4143815 G allele responded better to chemotherapy and had gains in overall survival. In our study, however, the polymorphism rs2297136 showed no significant association with clinical outcome, a finding that was corroborated by Zhao et al.⁴⁹ This difference might be explained by the heterogeneity of patients enrolled in each study, and further research is needed to settle this question.

In our study, we were unable to find any statistical significance between the rs4742098, rs4143815, rs2297136, and rs7041009 genotypes and *PD-L1* protein expression in malignant cells. However, recent data have helped to shed light on the impact of *PD-L1* genetics on *PD-L1* expression, though the existing results remain controversial. Recently, Tao and colleagues⁵¹ showed that rs4143815 and rs10815225 in the *PD-L1* gene contributed to *PD-L1* overexpression in gastric cancer. So far, Lee et al¹⁸ have conducted the largest study on *PD-L1* polymorphisms and *PD-L1* expression in NSCLC. The authors showed that rs822336C, rs822337A and rs4143815G were associated with worse OS in NSCLC patients but found no significant correlation between *PD-L1* expression and the genotypes of these polymorphisms. Krawczyk et al⁵² demonstrated that carriers of the rs822335 CC genotype were predisposed to higher expression of the *PD-L1* protein in NSCLC tumor cells, whereas rs822336 had no effect on *PD-L1* expression in these cells. Their results are consistent with our findings, but future research is needed to clarify remaining confounders.

In our study, using in silico approaches, we report that rs4742098, rs4143815, rs2297136, and rs7041009 can be considered benign variants. However, little is known about the effect that mutations in conserved non-coding regions might have on fitness and how many of them are present in the human genome as deleterious polymorphisms. Moreover, rs2297136 was predicted to affect the miRNA-binding function of hsa-miR-324-5p and hsa-miR-632, whereas the variant rs4143815 was found to correlate with hsa-miR-1252, hsa-miR-1253, hsa-miR-539, hsa-miR-548, and hsa-miR-570. In this context, others have

reported that variants in the 3'UTR region of the *PD-L1* gene can affect the interaction of miRNAs, possibly resulting in *PD-L1* underexpression.⁵³ Therefore, additional studies are necessary to validate our findings. However, there are limitations to our analysis. We did not perform a case-control approach and our cohort comprises a relatively small sample size. Nonetheless, to our knowledge, our research on the effect of the rs7041009 polymorphism of *PD-L1* gene on NSCLC patients is unique.

We believe this study is also the first to evaluate variants in the non-coding region of the *PD-L1* in Brazilian patients with NSCLC, since most studies of *PD-L1* polymorphisms have been conducted in Asian patients. Thus, we consider this exploratory study as a pioneer in the understanding of *PD-L1* polymorphisms in a genetic admixed population.

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Disclosure

The authors report no conflicts of interest related to this work.

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