PD-L1 SNPs as biomarkers to define benefit in patients with advanced NSCLC treated with immune checkpoint inhibitors

PD-L1 single nucleotide polymorphisms as potential biomarkers to define benefit to immune checkpoint inhibitors: a prospective study of correlations between genotype and outcome in advanced NSCLC patients

ABSTRACT

Immune checkpoint inhibitors (ICIs) have led to improved patient outcome in advanced NSCLC, however, predictive parameters of treatment benefit remain an unsolved issue. Here, we investigate the role of *CTLA-4*, *PD-1* and *PD-L1* single nucleotide polymorphisms (SNPs) in predicting clinical outcome of advanced NSCLC patients treated with ICIs.

We prospectively included 166 consecutive advanced NSCLC patients treated with ICIs in two Italian Institutions. *CTLA-4*, *PD-1* and *PD-L1* SNPs were correlated with outcome measures: clinical benefit (CB: defined as tumor complete, partial response and stable disease lasting at least 6 months), progression-free survival, time to treatment failure and overall survival. Moreover, the incidence of the three polymorphisms in Not-Responder (NR, including patients with disease progression and stable lasting less than 6 months as best response) and Long Clinical Benefit (LCB, defined if response or stable disease with PFS ≥ 12 months) patients were evaluated. Tissue PD-L1 (tPD-L1) expression was immunohistochemically assessed in a patient subset.

Considering the entire cohort, no significant correlations were found between SNPs and clinical outcome. A significant association was observed between *PD-L1* rs4143815 SNP and LCB group ($p = 0.02$). Furthermore, the NR cohort displayed distinctive *PD-L1* haplotype ($p = 0.05$). The potential correlation of *PD-L1* SNPs with tPD-L1 was not documented in our study, probably due to the limited tissue sample availability (48%). The prognostic impact of *PD-L1* polymorphisms deserves further investigations in larger patient cohorts. The design of specific studies dissecting PD-L1 pathway at gene and protein levels is warranted in the near future.

Keywords: NSCLC, Immunotherapy, PD-L1, SNPs, Biomarker.

INTRODUCTION

Lung cancer is the first cause of death among oncologic patients and the second most common cancer worldwide [1]. Non-small cell lung cancer (NSCLC) represents the 85-90% of lung cancers [2]. More than 50% of NSCLC is diagnosed in advanced stage (IIIB-IV), but in the last years, the outcome of these patients has significantly improved thanks to new therapies targeting driver gene alterations or immune-checkpoints [3]. Use of immune-checkpoints inhibitors (ICIs), including nivolumab, pembrolizumab and atezolizumab, is rapidly increasing both in first and subsequent lines of therapy [4]. Based on their notable efficacy, the aforementioned ICIs targeting PD-1 or PD-L1 have been approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA), alone or combined with platinum-based doublet chemotherapy (and bevacizumab), for the treatment of advanced NSCLC [5-13].

The key problem concerning ICIs therapy is the great heterogeneity of responses, in particular when used as single agent: some patients showed clinical benefit whereas others do not respond at all, resulting in an overall nearly 20% response rate (ORR) [14]. PD-L1 expression, as assessed by immunohistochemistry (IHC), is thought to be critical in the clinical benefit from ICIs and represents the safest biomarker of response to anti PD-1/PD-L1 inhibitors [15]. However, PD-L1 dynamic changes during treatment as well as the limited tissue availability in metastatic NSCLC setting represent potential issues for its routine application.

Other putative predictive factors have been investigated [16]. The tumour mutational burden may be related to sensitivity to immunotherapy, since higher non-synonymous mutation burden (HMB) has been correlated with a better outcome in NSCLC patients treated with ICIs; a similar effect was observed with DNA repair pathway mutations [17]. Despite these results, the role of genomic variants as reproducible clinical markers is still far from being established due to lack of validated cut-off, expensiveness of the technique and extensive genomic variability among NSCLC patients worldwide [16].

PD-L1 Single Nucleotide Polymorphisms (SNPs) are assumed to have a potential role in conditioning

the clinical benefit from ICIs. Only limited evidence is available about the implication of SNPs of the target molecule in the response to ICIs. In a study conducted in 2017, involving 50 Japanese patients with advanced NSCLC treated with nivolumab, *PD-L1* rs4143815 and *PD-L1* rs2282055 significantly correlated to better ORR and PFS when present in their rare alleles (C and G, respectively). This evidence could be related to a higher PD-L1 expression in patients carrying the rare allele [18].

In this study, we explored the role of *PD-1*, *PD-L1* and *CTLA-4* SNPs in the prediction of clinical outcome in advanced NSCLC patients treated with ICIs.

MATERIALS AND METHODS

Patients and treatment

Advanced NSCLC patients eligible for treatment with single agent anti-PD-1 or anti-PD-L1, regardless of the treatment line, were consecutively enrolled at the Medical Oncology Unit of the University-Hospital of Parma and Bologna (Italy) in an observational prospective study.

Inclusion criteria for the study were as follows: histologically or cytologically proven diagnosis of NSCLC; locally advanced or metastatic (stage IIIB or IV) disease, not susceptible to curative therapy; eligibility for ICIs treatment; availability of blood sample for SNP analysis. ICIs were administered as per clinical practice. Tumour responses were evaluated every 2 months from the beginning of treatment by means of computed tomography (CT) scans; additional evaluations were prescribed by treating physicians according to clinical requirements.

Disease responses were evaluated according to RECIST (Response Evaluation Criteria in Solid Tumours) criteria version 1.1. Patients were categorized, in clinical benefit group (CB), including complete (CR) or partial (PR) response or stable disease (SD) lasting at least 6 months and not-responders (NR), including patients with disease progression (PD) and SD lasting less than 6 months as best response.

Progression-free survival (PFS), time to treatment failure (TTF) and Overall survival (OS) were

calculated from the date of first ICIs administration and the date of death, of disease progression or death (whichever occurred first) and of last ICIs course, respectively.

All patients provided written informed consent and the study was approved by the Ethical Committees of the University-Hospitals of Parma and Bologna.

SNPs genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the Maxwell® 16 Blood DNA Purification Kit (Promega) according to the manufacturer's instructions. Genotyping was performed using the TaqMan genotyping assay (*CTLA-4* gene: C_2415786_20 for rs231775; *PD-1* gene: C_57931321_10 for rs36084323, C_57931291_10 for rs34819629; *PD-L1* gene: C_1409286_1 for rs2282055, C_31941235_10 for rs4143815; Thermofisher, Foster City, CA) and analysed with a Light Cycler 480 Real-Time PCR System (Roche).

The polymerase chain reaction (PCR) solution included 5 µ L of 2X TaqMan Universal PCR Master Mix, 0.25 µ L of primer probe mix, 3.75 µ L of nuclease-free water, and 1 µ L of DNA sample. PCR protocol included a 10 minutes incubation at 95 °C, 40 cycles of denaturing at 92 °C for 15 seconds and annealing and extending at 60 °C for 1 minute. Fluorescence detection was measured at 60°C.

PD-L1 immunohistochemical analysis on tumor tissue

Five µm thick sections from formalin fixed and paraffin embedded NSCLC biopsies were subjected to the immunohistochemical evaluation of tissue PD-L1 (tPD-L1). tPD-L1 levels were measured using anti PD-L1 antibodies (clone SP263, Ventana) revealed by immunoperoxidase and expressed as % of tumor cell surface labelling. Three distinct subgroups were defined according to tPD-L1 score: negative (< 1%), intermediate (1-49%) and high (50-100%).

Statistical analysis

All statistical analyses were performed using SPSS Statistics 22.0 software (IBM Corporation, NY,

USA). Differences in characteristics among the genotypes were assessed using Fisher's exact test for categorical data. Genotypes were analysed separately and as combined allelic groups.

For statistic correlations between SNPs and ICIs response, patients were divided in two cohorts: CB and NR. Moreover, a sub-group analysis was performed, considering NR and Long-CB (LCB, group defined if response or stable disease with PFS $\geq$ 12 months).

SNPs in *PD-L1* gene were analyzed also as a haplotype. Patients were classified as *reference haplotype* when both polymorphisms were homozygous for reference allele and also when only one of them was homozygous for reference allele. Otherwise, they were classified as *alternative haplotype* when both polymorphisms were heterozygous for alternative allele; when both polymorphisms were homozygous for alternative allele, and also when only one of them was homozygous for alternative allele. If one polymorphism was homozygous for reference and the other one for alternative, the patient was classified as *alternative haplotype*.

Clinical benefit among the genotypes were analysed using the Cochrane-Armitage test. Correlations between patient's baseline characteristics and SNPs genotype were analysed using one-way analysis of variance (ANOVA) for continuous variables. The survival curves were estimated using the Kaplan-Meier method. Statistical significance of the relationship between PFS, TTF, OS and SNPs was assessed using the trend log-rank-test. Multivariate Cox proportional hazards models were used to estimate adjusted hazard ratios (HR) with 95% confidence intervals. Multivariable regressions analysis was adjusted for age, gender and histology.

RESULTS

Patient's characteristics

A total of 166 consecutive patients treated with anti-PD-1 or anti-PD-L1 immunotherapy were enrolled in our study from August 2015 to May 2018; 119 were men (71.7%), with a median age at diagnosis of 69.5 [40.8-87.5] years. Ninety-eight patients (59.1%) were histologically diagnosed with

adenocarcinoma, 57 (34.3%) with squamous cell carcinoma while 11 (6.6%) as NSCLC not otherwise specified (NOS). Fifteen patients (9.1%) were treated with ICIs as first-line therapy, 100 (60.2%) as second-line and 51 (30.7%) as third-line or beyond. Anti-PD-1/PD-L1 inhibitors were employed as follow: 123 patients (74.1%) received nivolumab, 22 (13.2%) atezolizumab and 21 (12.7%) pembrolizumab. The main clinical characteristics of the enrolled population of NSCLC patients are shown in Table 1.

Clinical outcome in overall population

Among the 166 patients, ICI treatment resulted in CR or PR in 37 (22.3%), SD in 40 (24.1%) and 89 (53.6%) experienced PD as a best response. According the previous definition, 65 (39.2%) resulted in CB group and 101 (60.8%) in NR group.

The median PFS was 2.6 months (95% confidence interval [CI], 1.2 to 3.9 months), TTF 3.2 (95%CI, 1.9 to 4.4 months) and OS 8.5 months (95%CI, 4.1-12.9 months) (Supplementary Figure S1).

Genotype and association with clinical outcome

Five SNPs were studied in overall NSCLC population: rs231775 in *CTLA-4,* rs36084323 and rs34819629 in *PD-1* and rs4143815 and rs2282055 in *PD-L1* and (Table S1). The two *PD-1* SNPs were excluded from the study because not informative in our population (data not shown). Genotype of investigated SNPs was as not associated with clinico-pathologic variables, except for a correlation between rs2282055 and histology (Table 1).

First, we calculated genotypic and allelic frequencies of all SNPs in NR and CB groups. No differences in SNP distribution of *CTLA-4* rs231775 [p-value for genotype frequencies (p-*gen*)=0.91; p-value for allelic frequencies (p-*all*)=0.84], and *PD-L1* rs2282055 [p-*gen*=0.55; p-*all*=1] and rs4143815 [p-*gen*=0.69; p-*all*=0.42] were observed between NR and CB cohorts. The distribution of genotype and allele frequencies of studied SNPs in the two cohorts is reported in Table S2.

The contribution of rare alleles (heterozygous + homozygous) vs wild type was investigated in NR

and CB cohorts. No significant differences were found for *CTLA-4* rs231775 (p=1), *PD-L1* rs2282055 (p=0.75) and rs4143815 (p=0.43) (Table 2). Kaplan-Meier survival curves did not show associations between these SNPs and PFS, TTF (Table 2 and Supplementary Figure 2A) and OS (Table 2 and Figure 1A).

Also analyzing the two *PD-L1* SNPs as haplotype, *reference haplotype* vs *alternative* in NR and CB, not statistically significant difference (p = 0.19) was observed (Table 2); however, the *reference haplotype* showed a worst survival outcome, either in PFS/TTF (Table 2 and Supplementary Figure 2B) and in OS (Table 2 and Figure 1B).

After stratification of patients in NR (n = 101) and LCB (n = 39), all SNPs were analyzed (Table 3). In the group with rare allele GC and CC of *PD-L1* rs4143815, we observed a higher percentage of patients with LCB than in the group with the reference allele (35.4 vs 18%, respectively), resulting in a statistically significant difference (p = 0.02). Similar results were obtained analyzing *PD-L1* haplotype (p = 0.05). Corresponding survival curves are shown in Figure 2A and 2B (OS) and in Supplementary Figure 3A and 3B (PFS and TTF); no statistically significant differences in survival outcome were found.

All these analyses were conducted also considering only the pre-treated population (n = 151) and similar results were observed. These data are summarized in Tables S3 and S4; corresponding survival curves are shown in Supplementary Figure 4 and 5.

Correlation of Tissue PD-L1 expression with genotype and clinical outcome

Data on tPD-L1 expression were available in 80 (48.2%) cases. Twenty-three (28.8%) tissue samples displayed ≥ 50% tPD-L1 score, while negative cases represented only 15% (n = 12).

As shown by Kaplan Meier curves (Supplementary Figure 6), a positive prognostic impact of tPD-L1 was documented in terms of PFS, TTF and OS. Specifically, prolonged PFS, TTF and OS were detected in NSCLC displaying high tPD-L1 (≥ 50%) expression with a median of 17.6, 19.6 and 20.9 months, respectively. Within high tPD-L1 cases, the rate of CB was significantly higher (73.9%)

compared to cases displaying intermediate (22.2%) or low (41.7%) tPD-L1 expression (Table S5).

No relevant correlations between tPD-L1 levels and *CTLA-4, PD-L1* SNPs or haplotype were observed either in the overall population (Table S6) or in the subgroup of NR and LCB patients (n=65, data not shown).

DISCUSSION

The first- and second-line treatment of advanced NSCLC patients has been revolutionized in recent years by drugs blocking the interaction of programmed death ligand 1 (PD-L1) with its receptor PD-1 [19]. Although the advent of immunotherapy has demonstrated impressive initial results in improving both PFS and OS of metastatic NSCLC, the *a priori* identification of subsets of patients who can really benefit from immune checkpoint inhibitors is still an unmet need. In advanced NSCLC biopsy-derived tissue is limited and many efforts have been undertaken to exploit alternative sources of bio-humoral parameters able to foresee the clinical benefit from ICIs.

In the last years, several studies have been conducted on the identification of immuno-biological markers that can predict the response to immunotherapeutic drugs. In particular, *CTLA4, PD-1* and *PD-L1* SNPs could play a role in cancer growth and progression [20-23] and in predicting the response and immune-related adverse events (irAEs) of immunotherapy [24, 25]. Nevertheless, current available results still appear limited and conflicting, probably in reason of the exiguous study cohorts which entail a low statistical power.

Here, we reported the analysis of one *CTLA4* (rs231775) and two *PD-L1* (rs2282055 and rs4143815) SNPs as potential biomarkers in 166 Italian advanced NSCLC patients treated with ICIs. To date, this study describes the largest cohort of metastatic NSCLC patients treated with ICIs deeply characterized at least in terms of potential correlations between genotype and clinical outcome. Two SNPs in *PD-1* were also investigated but excluded from the study because not informative in our population.

Unfortunately, rs231775 *CTLA-4* SNP did not provide significant results. In literature, it was

described as an useful genetic prognostic marker in advanced NSCLC Chinese patients treated with chemotherapy and/or radiotherapy [23]. However, due to the employment of anti-PD-1/PD-L1 agents and the absence of significant results in our population, *CTLA-4* SNP will not be discussed further.

In 2017, Nomizo and colleagues reported the association of rs2282055 and rs4143815 *PD-L1* SNPs with improved ORR and PFS in a study including 50 Japanese advanced NSCLC patients [18]. In particular, the alternative G allele of rs2282055 and C allele of rs4143815 were associated with improved response to nivolumab. In addition, rs2282055 GG/GT and rs4143815 GC/CC genotypes were associated with better PFS compared with TT and GG genotypes, respectively. In our population, no significant correlations were found between *PD-L1* SNPs and clinical outcome. A possible explanation of these contrasting results may reside in opposite allele frequencies of rs2282055 and rs4143815 *PD-L1* SNPs between the European and Japanese populations. Indeed, the rs2282055 minor allele results G (minor allele frequency [MAF]=0,268) in European population, while in Asian population is T (MAF=0,351). Similarly, rs4143815 minor allele results C (MAF=0,330) in Europeans while in Japanese population is G (MAF=0,462) [26]. These population-specific differences in allele frequencies of SNPs between cohorts could justify different results in association studies.

However, following a clinically oriented stratification of cases in NR and LCB, our analysis highlighted a statistically significant correlation between alternative allele C of *PD-L1* rs4143815, either in homozygous or heterozygous, and LCB. The same results were observed also when only pre-treated population was analysed. Previous studies have shown that the alternative C allele of rs4143815 significantly increases the surface PD-L1 expression via down regulation of miRNA 570 which inhibits PD-L1 expression [27]. Therefore, a major benefit observed in patients with C allele of rs4143815 is in line with the known correlation between PD-L1 expression and better outcome [28-31]. Unfortunately, results of tissue PD-L1 expression in our NR/LCB subgroups were not statistically significant, likely due to a small number of testable cases (65 out of 135 patients), resulting in an unbalanced distribution. The assessment of miR-570 levels may provide further

insights on the involvement of this pathway in predicting immunotherapy outcomes in NSCLC patients. However, the role of *PD-L1* polymorphisms in regulating PD-L1 expression remains largely unclear.

In addition, we observed that NR patients commonly carried a peculiar haplotype based on homozygous reference alleles (TT and GG of *PD-L1* rs2282055 and rs4143815, respectively), confirming the hypothesis that the alternative C allele of rs4143815 could be a favourable genetic marker of ICIs benefit. The association between disease progression and role of germinal polymorphism was recently investigated also by Refae et al. [32]. Contrary to our findings, alternative G allele of rs2282055 *PD-L1*, either in homozygous or heterozygous, were found to be significantly associated to higher risk of developing hyper-progressive disease. The discrepancy with our results should be likely sought in the analysed heterogeneous population. Refae et al. enrolled patients affected by different malignancies (NSCLC, HNSCC, RCC and melanoma) while we focused only on advanced NSCLC patients. Importantly, in line with our haplotype, Yeo et al. speculated that rs4143815 genotype GG might be a suitable predictor of poor prognosis in lung adenocarcinoma cases [28].

Main limitations of the present study involve the inclusion of patients under different line of immunotherapy and the restricted availability of tissue PD-L1 expression. Future studies in larger and better stratified cohorts are required to ascertain the role of *PD-L1* SNPs in predicting extreme response to ICIs. The combination of genotypic and phenotypic assessment of PD-L1 pathway might implement the actual definition of predictive biomarkers and expand the population of patients who can benefit from immunotherapy.

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Figure Legends

Figure 1. Overall survival (OS) curves and polymorphism in the overall population (166 patients).

A) Kaplan Meier curves illustrating the impact of *CTLA4* rs231775, *PD-L1* rs2282055 and rs4143815 SNPs on OS in the overall population. 0=homozygous wild-type; 1=heterozygous and homozygous for rare allele.

B) Kaplan Meier curves illustrating the impact of *PD-L1* haplotype on OS in the overall population.

Figure 2. Overall survival (OS) curves and polymorphism in Not-Responder (NR) vs Long Clinical Benefit (LCB) subgroups (140 patients).

A) Kaplan Meier curves illustrating the impact of *CTLA4* rs231775, *PD-L1* rs2282055 and rs4143815 SNPs on OS in NR/LCB subgroups. 0=homozygous wild-type; 1=heterozygous and homozygous for rare allele.

B) Kaplan Meier curves illustrating the impact of *PD-L1* haplotype on OS in NR/LCB subgroups.

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Supplementary Figure Legends

Supplementary Figure S1. Overall population survival curves. Kaplan Meier curves of progression-free survival (PFS), time-to-treatment failure (TTF) and overall survival (OS) in the overall population.

Supplementary Figure S2. Progression Free Survival (PFS) and Time to treatment failure (TTF) curves and polymorphism in the overall population (166 patients).

A) Kaplan Meier curves illustrating the impact of *CTLA4* and *PD-L1* SNPs on PFS and TTF in the overall population. 0=homozygous wild-type; 1=heterozygous and homozygous for rare allele.

B) Kaplan Meier curves illustrating the impact of *PD-L1* haplotype on PFS and TTF in the overall population.

Supplementary Figure S3. Progression Free Survival (PFS) and Time to Treatment Failure (TTF) curves and polymorphism in Not-Responder (NR) vs Long Clinical Benefit (LCB) subgroups (140 patients).

A) Kaplan Meier curves illustrating the impact of *CTLA4* and *PD-L1* SNPs on PFS and TTF in NR/LCB subgroups. 0=homozygous wild-type; 1=heterozygous and homozygous for rare allele.

B) Kaplan Meier curves illustrating the impact of *PD-L1* haplotype on PFS and TTF in NR/LCB subgroups.

Supplementary Figure S4. Progression Free Survival (PFS), Time to Treatment Failure (TTF) curves and Overall Survival (OS) and polymorphism in pre-treated population (151 patients).

A) Kaplan Meier curves illustrating the impact of *CTLA4* and *PD-L1* SNPs on PFS, TTF and OS in pre-treated population. 0=homozygous wild-type; 1=heterozygous and homozygous for rare allele.

B) Kaplan Meier curves illustrating the impact of *PD-L1* haplotype on PFS, TTF and OS in pre-

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treated population.

Supplementary Figure S5. Progression Free Survival (PFS), Time to Treatment Failure (TTF) curves and Overall Survival (OS) and polymorphism in Not-Responder (NR) vs Long Clinical Benefit (LCB) subgroups of pre-treated population (133 patients).

A) Kaplan Meier curves illustrating the impact of *CTLA4* and *PD-L1* SNPs on PFS, TTF and OS in NR/LCB subgroups of pre-treated population. 0=homozygous wild-type; 1=heterozygous and homozygous for rare allele.

B) Kaplan Meier curves illustrating the impact of *PD-L1* haplotype on PFS, TTF and OS in NR/LCB subgroups of pre-treated population.

Supplementary Figure S6. Tissue PD-L1 and survival outcome.

Kaplan Meier curves illustrating the impact of tissue PD-L1 on progression-free survival (PFS), time-to-treatment failure (TTF) and overall survival (OS) in the overall population. PD-L1 expression was stratified in 3 classes: negative; 1-49%; ≥ 50%.

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