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PD-1 and PD-L1 expression in molecularly selected non-small-cell lung cancer patients

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Background: Agents targeting programmed death-1 receptor (PD-1) and its ligand (PD-L1) are showing promising results in non-small-cell lung cancer (NSCLC). It is unknown whether PD-1/PD-L1 are differently expressed in oncogene-addicted NSCLC.

Methods: We analysed a cohort of 125 NSCLC patients, including 56 *EGFR* mutated, 29 *KRAS* mutated, 10 *ALK* translocated and 30 *EGFR/KRAS/ALK* wild type. PD-L1 and PD-1 expression were assessed by immunohistochemistry. All cases with moderate or strong staining (2+/3+) in >5% of tumour cells were considered as positive.

Results: PD-1 positive (+) was significantly associated with current smoking status ($P=0.02$) and with the presence of *KRAS* mutations ($P=0.006$), whereas PD-L1+ was significantly associated to adenocarcinoma histology ($P=0.005$) and with presence of *EGFR* mutations ($P=0.001$). In patients treated with EGFR tyrosine kinase inhibitors ($N=95$), sensitivity to gefitinib or erlotinib was higher in PD-L1+ *vs* PD-L1 negative in terms of the response rate (RR: $P=0.01$) time to progression (TTP: $P<0.0001$) and survival (OS: $P=0.09$), with no difference in PD1+ *vs* PD-1 negative. In the subset of 54 *EGFR* mutated patients, TTP was significantly longer in PD-L1+ than in PD-L1 negative ($P=0.01$).

Conclusions: PD-1 and PD-L1 are differentially expressed in oncogene-addicted NSCLC supporting further investigation of specific checkpoint inhibitors in combination with targeted therapies.

Lung cancer is the leading cause of cancer-related mortality worldwide in men and is second only to breast cancer in women (Siegel *et al*, 2013). Non-small cell lung cancer (NSCLC) accounts for 75% of all lung cancers and includes different subtypes, underlying relevant biological differences. Most patients with NSCLC are diagnosed in advanced stage, with a 5-year survival rate of <5% (Goldstraw *et al*, 2007). During the past years, drugs targeting the epidermal growth factor receptor (EGFR), such as gefitinib, erlotinib or afatinib and drugs targeting anaplastic lymphoma kinase (*ALK*) translocation, such as crizotinib, are offering new hopes, respectively, to the patients with *EGFR* mutations or *ALK* translocations (Mok *et al*, 2009; Maemondo *et al*, 2010; Mitsudomi *et al*, 2010; Zhou *et al*, 2011; Han *et al*, 2012; Rosell *et al*, 2012; Sequist *et al*, 2013; Shaw *et al*, 2013; Wu *et al*, 2014). Nevertheless, even with targeted agents, no patient with metastatic NSCLC can obtain a definitive cure and inevitably disease progresses after a median of 8–12 months, highlighting the urgent need for additional and more effective strategies.

For many years, several investigators evaluated the possibility to modulate the immune system for treating lung cancer, with disappointing results (Topalian *et al*, 2011; Hall *et al*, 2013). Lack of efficacy was probably related to the ability of the tumour to

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activate several distinct pathways to escape immune activity, including endogenous 'immune checkpoints'. Immunotherapy of cancer has entered into a new phase from the discovery of drugs able to interfere with specific immune checkpoints such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and, more recently, programmed death-1 receptor (PD-1) and its ligand (PD-L1).

Programmed cell death protein 1, also known as PD-1, is a 288 amino acid cell surface protein molecule. PD-1 has two ligands, PD-L1 and PD-L2, which are members of the B7 family. Several lines of evidence suggest that PD-1 and its ligands negatively regulate immune responses. Different studies have showed that cancer microenvironment manipulates the PD-L1/PD-1 pathway and that the induction of PD-L1 expression on tumour cells leads to the inhibition of immune responses against cancer, permitting cancer progression and metastasis (Drake *et al*, 2006; Pardoll, 2012). Spranger *et al*, 2013 demonstrated in a murine mechanistic experiments that the induction of PD-L1 in melanoma micro-environment is mediated by IFN-y produced by the T cells that are antigen experienced and therefore express PD-1.

Expression of PD-L1 has been correlated with poor clinical outcomes in a number of human cancers, including melanoma, lung, breast, bladder, ovarian, pancreatic cancers, oesophagus adenocarcinoma, kidney tumours as well as in hematopoietic malignancies (Zou and Chen, 2008). However, other reports indicated a lack of association between PD-L1 expression and outcome (Konishi *et al*, 2004; Mischinger *et al*, 2010) or that PD-L1 expression was associated with an improved survival and increased TILs (Taube *et al*, 2012).

Recent studies showed that, in tumour cells, PD-L1 expression may range from 45 to 50% in NSCLC biopsies, irrespective of histology (Grosso *et al*, 2013). The evidence that PD-L1 is commonly up-regulated in NSCLC and that PD-1 is expressed on the majority of tumour infiltrating lymphocytes, represented the rationale for the development of monoclonal antibodies against PD-L1 or PD-1, and several agents are currently under investigation. Preliminary results suggested that PD-L1 positivity may correlate with response to treatment with PD-1 pathway inhibitors (Topalian *et al*, 2012; Grosso *et al*, 2013). Moreover, recent data suggested that checkpoint inhibitors could be more effective in smokers (Soria *et al*, 2013), where somatic gene mutations are frequent, suggesting a potential different expression of PD-1/PD-L1 in presence of specific molecular events including *KRAS* mutations. In addition, a recent study demonstrated that expression of mutant EGFR in bronchial epithelial cells induced PD-L1, and PD-L1 expression was reduced by EGFR inhibitors in NSCLC cell lines with activated *EGFR* (Akbay *et al*, 2013).

Based on these premises we supposed that PD-1/PD-L1 expression could differ according to the molecular phenotype of the tumour. The aim of the present study was to assess whether PD-1/PD-L1 expression was differently expressed in NSCLC patients according to presence or absence of *EGFR* mutations, *ALK* translocations or *KRAS* mutations.

MATERIALS AND METHODS

Patient selection. This retrospective study was conducted in a cohort of 125 metastatic NSCLC patients followed in three Italian centres. We selected two cohorts of patients (*EGFR* mutated and *EGFR* wild type) with availability of additional tumour tissue from the same tumour sample previously used for *EGFR* assessment. In addition, we included onto the study only cases evaluated for *KRAS* and *ALK* status, with full clinical data including previous therapies and survival. *EGFR* mutations and *KRAS* mutations were evaluated using Polymerase Chain Reaction and direct sequencing, while presence of *ALK* translocations were detected using fluorescence *in situ* hybridisation. All tests were performed locally as a part of clinical practice. The study was approved by the local Ethics Committee and was conducted in accordance with the ethical principles stated in the most recent version of the Declaration of Helsinki or the applicable guidelines on good clinical practice, whichever represented the greater protection of the individuals.

Immunohistochemistry. Four-micron sections of 125 primary or metastatic NSCLC samples were used throughout this study. Standard indirect immunoperoxidase procedures were used for immunohistochemistry (IHC; ABC-Elite, Vector Laboratories, Burlingame, CA, USA). Briefly, slides were dewaxed and rehydrated in distilled water. Endogenous peroxidase activity was blocked using 0.5% H_2O_2. The sections were treated with 10% normal goat serum (DakoCytomation; Dako, Carpinteria, CA, USA) for 20 min and incubated with primary antibodies PD-L1 (CD274) ab58810 (Abcam, Cambridge, UK) (Bloch *et al*, 2013; Shi *et al*, 2013) and PD-1 760–4448 (Ventana, Tucson, AZ, USA) at room temperature. Sections were further incubated with peroxidase-labelled secondary antibody (DakoCytomation) for 30 min at room temperature. For visualisation of the antigen, the sections were immersed in 3-amino-9-ethylcarbazole plus substrate-chromogen (DakoCytomation) for 30 min, and counterstained with Gill's hematoxylin.

Two well-experienced pathologists (MA and LT) examined the immunohistochemical slides without any prior information on the clinicopathological features of the patient samples.

Percentages of PD-L1 and PD-1 positive tumour cells and staining intensity were evaluated for each sample. Staining intensity was scored considering 0 as negative or trace, 1 as weak, 2 as moderate and 3 as high. In absence of any standardised scoring system, all cases with staining intensity $\geqslant 2$ in more than 5% of tumour cells were considered as positive, similarly to previous studies (Antonia *et al*, 2013; Grosso *et al*, 2013; Soria *et al*, 2013). Moreover, a semi-quantitative approach was used to generate a score for each tissue core. The percentage of stained cells (0–100%) was multiplied by the dominant intensity pattern of staining ranging from 0 to 3. Therefore, the overall semiquantitative score ranged from 0 to 300.

Statistical analyses. Sample size was computed assuming a difference of 25% in PD-1 or PD-L1 expression in *EGFR* mutated *vs EGFR* wild type. With a power of 80% and a significance level of 0.05 (1-tailed test), a sample size of at least 49 patients was required for each group. Statistical analyses were performed to compare differences between patients with and without PD-1 and PD-L1 expression according to presence or absence of a specific biomarker. Clinical characteristics and associations with biomarkers were examined comparing the differences by χ^2-test or Fisher's exact test as appropriate. Comparison between the two groups with or without PD-1 or PD-L1 expression was performed by log rank test. A multivariable analysis was performed using a logistic regression model in order to explore the association of PD-1/PD-L1 expression with patient characteristics (sex, smoke, histology, *EGFR, KRAS* and *ALK*). A step-down procedure method was selected. The criterion for variable removal was the likelihood ratio statistic based on the maximum partial likelihood estimates (default *P*-value of 0.10 for removal from the model). Differences between median score was performed by U-Mann–Witney test. Correlative analyses of PD-1 or PD-L1 expression and outcome of patients according to a specific therapy were evaluated. Time to progression (TTP) was calculated from date of therapy start to progression or last follow-up date; overall survival (OS) was calculated from the date of therapy start to death or last follow-up date, with 95% confidence intervals calculated using the Kaplan–Meier method (Kaplan and Meier, 1985). The significance level for all analyses was set at $P<0.05$ and all *P*-values were two-sided. Statistical analysis was performed by using IBM-SPSS Statistics version 20 (Property of IBM Corp., Armonk, NY, USA).

RESULTS

Patient characteristics. A total of 125 metastatic NSCLC patients were included in the study. A total of 98 (78.4%) samples were obtained from the primary tumour and 17 (13.6%) from the metastatic sites. This study included 99 cases treated with EGFR-TKIs (gefitinib: $N=30$, 30.3%; erlotinib: $N=69$, 69.7%) in first ($N=29$, 29.3%) or subsequent lines of treatment ($N=70$, 70.7%). The median age was 64 years (range: 41–84 years). The majority of patients were male ($N=67$, 53.6%), former ($N=58$, 46.4%) or current smokers ($N=17$, 13.6%) and mostly presented two metastatic sites ($N=43$, 34.4%). The most frequent metastatic sites were lung ($N=72$, 57.6%), followed by lymph-nodes ($N=55$, 44.0%), bone ($N=37$, 29.6%), brain ($N=18$, 14.4%), liver ($N=13$, 10.4%) and adrenal glands ($N=8$, 6.4%). Adenocarcinoma was the most frequent histology ($N=83$, 66.4%). All patients were analysed for presence of *EGFR* and *KRAS* mutation and for *ALK* translocation: this analysis included 56 (44.8%) *EGFR* mutated, 29 (23.2%) *KRAS* mutated, 10 (8.0%) *ALK* translocated and 30 (24.0%) *EGFR/KRAS/ALK* wild type, defined as triple negative. Exon 19 deletion ($N=30$, 24.0%) and codon 12 mutation ($N=26$, 20.8%), were the most frequent *EGFR* and *KRAS* alterations, respectively (Table 1). In this study, because of the criteria for patient selection, incidence of *EGFR* mutations, *KRAS* mutations and *ALK* translocations was not representative of a standard Caucasian population.

PD-1/PD-L1 expression and patient characteristics. PD-1 was successfully evaluated in 122 specimens. Median PD-1 expression was 30. As illustrated in Figure 1A–F, median PD-1 expression resulted high in male, in current smokers, in adenocarcinoma histology, in *EGFR* wild type, in *ALK* negative and in patients harbouring *KRAS* mutations. A total of 43 cases (35.2%) had moderate (2+) or strong (3+) staining in at least 5% of cells and were considered as PD1+ as illustrated in Figure 2 and in Supplementary Figure S1. As reported in Table 2, PD-1 positive (+) patients were more frequently male with adenocarcinoma histology, even if the association was not statistically significant. PD-1 positivity was significantly associated with current smoking status ($P=0.02$) and with presence of *KRAS* mutations ($P=0.006$), while no significant association was observed with presence of *EGFR* mutations or *ALK* translocations. A multivariable analysis confirmed the significant association between PD-1 and *KRAS* mutations ($P=0.05$) (Supplementary Table S1).

PD-L1 was successfully evaluated in 123 specimens, with a median expression level of 75. As illustrated in Figure 3A–F, median PD-L1 expression was high in female, in never/former smokers, in adenocarcinoma histology, in patients harbouring *EGFR* mutations, and in patients with *ALK* translocations. A score of 2+ or 3+ in >5% of tumour cells (PD-L1+) was observed in 68 cases (55.3%), as illustrated in Figure 2. As reported in Table 2, PD-L1+ status was significantly associated to adenocarcinoma histology ($P=0.005$) and more frequently observed in female and in never/former smokers, even if the association was not statistically significant. Importantly, presence of *EGFR* mutations was significantly associated with PD-L1 positive status ($P=0.001$). Multivariable analysis confirmed the significant association between PD-L1 and *EGFR* mutations ($P=0.002$) and between PD-L1 and adenocarcinoma histology ($P=0.10$) (Supplementary Table S1).

A formal comparison between primary tumours and metastasis was limited by the low number of samples (only 17 cases from metastatic sites). To clarify whether our results could be influenced by the simultaneous presence of primary and metastatic tumour samples, we reanalyzed the data by excluding all metastatic cases. In this analysis we confirmed that PD-1 positivity was significantly associated with current smoking status ($P=0.05$) and with presence of *KRAS* mutations ($P=0.02$) and that PD-L1 positivity status was significantly associated with adenocarcinoma histology ($P=0.007$) and with presence of *EGFR* mutations ($P<0.001$) (Supplementary Table S2).

PD1/PD-L1 expression was evaluable in 29 triple negative cases. In such subgroup, median PD-1 expression was 40, a result that was similar to the median PD-1 expression observed in *EGFR* mutants (median 20), *KRAS* mutants (median 60) or *ALK* translocated (median 15), as reported in Table 2. In the same subgroup of triple negative patients, median PD-L1 was 20, lower than the median PD-L1 levels observed in *EGFR* mutants (median 120) or in *ALK* translocated (median 115) and similar to *KRAS* mutants (median 55). As reported in Table 2, when compared with *KRAS* mutants, triple negative patients were more frequently PD-1 negative ($P=0.01$). When compared with patients with *EGFR* mutations, or with *KRAS* mutations or with *ALK* translocations, triple negative individuals were more frequently PD-L1 negative, with a *P*-value of <0.001, 0.02 and 0.06, respectively.

PD-1/PD-L1 expression and outcome of patients treated with EGFR-TKIs. Because of the strong association of PD-1/PD-L1

Table 1. Clinical and biological characteristic in the whole population

Characteristic	Total (no)	%
Total number of patients	125	100
Median age (years–range)	64	41–84
Gender		
Male	67	53.6
Female	58	46.4
Histology		
Adenocarcinoma	83	66.4
Squamous-cell carcinoma	23	18.4
Other[a]	19	15.2
Smoking history		
Never	37	29.6
Former	58	46.4
Current	17	13.6
Unknown	13	10.4
Sites of metastasis		
Lung	72	57.6
Lymph-nodes	55	44.0
Bone	37	29.6
Brain	18	14.4
Liver	13	10.4
Adrenal glands	8	6.4
***EGFR* status**		
Mutated[b]	56	44.8
Wild type	69	55.2
***KRAS* status**		
Mutated[c]	29	23.2
Wild type	96	76.8
***ALK* status**		
Translocated	10	8.0
Wild type	115	92.0
Triple negative[d]	30	24.0

Abbreviations: *ALK*=anaplastic lymphoma kinase; *EGFR*=epidermal growth factor receptor; *KRAS*=Kirsten rat sarcoma viral oncogene homologue.
[a]Other histologies included: large cell=4 (3.2%), NAS=3 (2.4%), mixed histology= 2 (1.6%), unknown=10 (8.0%).
[b]*EGFR* mutations included: exon 18=3 (2.4%); exon 19=30 (24.0%); exon 20=4 (3.2%); exon 21=14 (11.2%); other=5 (4.0%).
[c]*KRAS* mutations included: codon 12=26 (20.8%); codon 13=2 (1.6%); other=1 (0.8%).
[d]Triple negative included *EGFR/KRAS/ALK* wild-type patients.

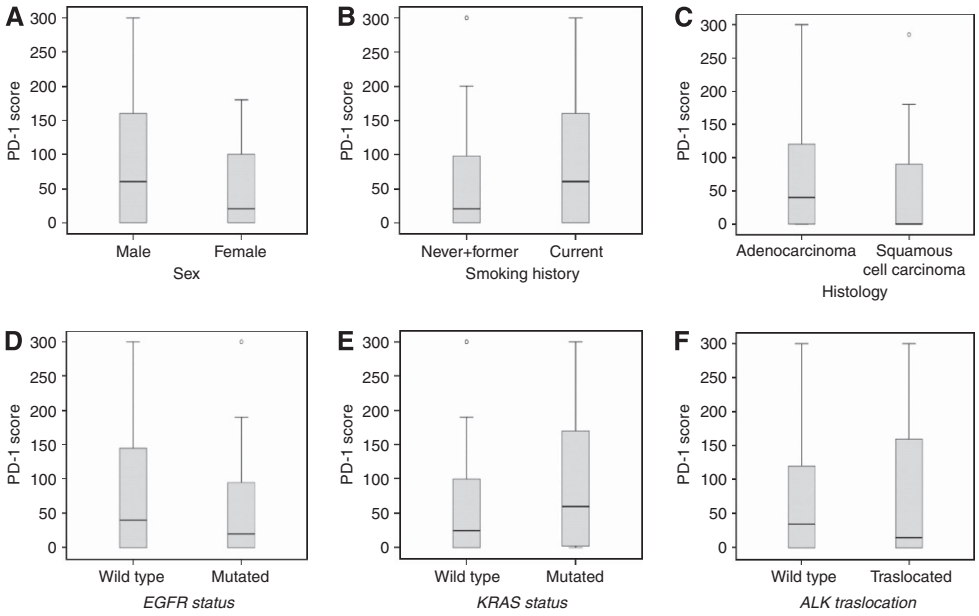

Figure 1. Levels of PD-1 expression and patient characteristics. This picture illustrates changes in the levels of PD-1 expression according to clinical (**A**–**C**) and biological (**D**–**F**) characteristics. Median levels of PD-1 score and interquartile ranges are showed. Median levels of PD-1 expression were higher in male (median score 60 *vs* 20) than in female (**A**), in current (median score 60 *vs* 20) than in never/former smokers (**B**), in adenocarcinoma (median score 40 *vs* 0) than in squamous-cell carcinoma histology (**C**), in *EGFR* wild type (median score 40 *vs* 20) than in *EGFR* mutated (**D**), in *KRAS* mutated (median score 60 *vs* 25) than in *KRAS* wild type (**E**) and in *ALK* wild type (median score 35 *vs* 15) than in *ALK* translocated (**F**) patients.

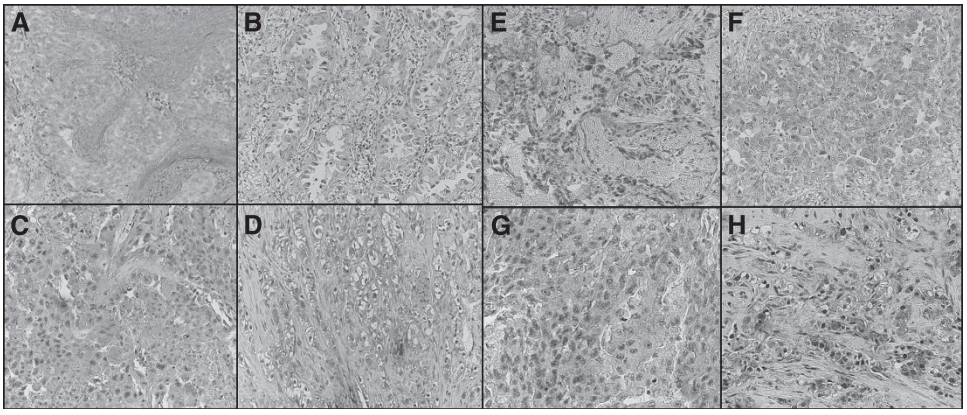

Figure 2. PD-1 and PD-L1 immunohistochemistry analysis. This figure illustrates four cases of PD-1 IHC analysis (**A**–**D**) and four cases of PD-L1 IHC analysis (**E**–**H**). Specifically, this picture showed: a PD-1 negative case (**A**), a PD-1 1+ case in 60% of tumour cells (**B**), a PD-1 2+ case in 80% of tumour cells (**C**), a PD-1 3+ case in 95% of tumour cells (**D**), a PD-L1 negative case (**E**), a PD-L1 1+ case in 10% of tumour cells (**F**), a PD-L1 2+ case in 50% of tumour cells (**G**) and a PD-L1 3+ case in 70% of tumour cells (**H**). The magnification used for the images was 20×.

expression and *KRAS* or *EGFR* mutations, we further investigated the potential effect of such biomarkers on sensitivity to anti-EGFR agents. With such purpose we analysed the outcome of 99 patients treated with gefitinib or erlotinib. EGFR-TKIs were given as front-line therapy in 29 patients including 26 *EGFR* mutated patients, and in second or subsequent lines of therapy in 70 individuals, including 29 *EGFR* mutated, 16 *KRAS* mutated and 25 triple negative. EGFR-TKIs were offered in the second or further lines of treatment in patients with *KRAS* mutations or in triple negative according to clinical practice (Kim *et al*, 2008; Shepherd *et al*, 2009; Cappuzzo *et al*, 2010). In the group of patients treated with erlotinib or gefitinib response rate (RR) was 47.5%, median TTP 7.9 months and median OS 18.0 months.

PD-1 was assessed in 96 specimens and a positive expression was observed in 28 (29.2%) cases. Among the 93 patients evaluable for response, no differences in terms of RR (46.2 *vs* 50.7%, $P=0.70$), TTP (7.0 months *vs* 8.0 months, $P=0.97$) and OS (17.8 months *vs* 18.9 months, $P=0.82$) were identified in PD-1 positive ($N=26$, 28.0%) *vs* PD-1 negative ($N=67$, 72.0%) cases, as illustrated in Figure 4A and B.

PD-L1 was successfully evaluated in 98 specimens and PD-L1 positive expression was observed in 52 (53.1%) cases. Among the 95 patients treated with gefitinib or erlotinib and evaluable for response, PD-L1 positive ($N=49$, 51.6%) patients had significantly higher RR (61.2% *vs* 34.8%, $P=0.01$), significant longer TTP (11.7 months *vs* 5.7 months, $P<0.0001$) and longer OS (21.9 months *vs* 12.5 months, $P=0.09$) than PD-L1 negative ($N=46$, 48.4%) patients, as illustrated in Figure 4C and D.

In order to explore whether higher sensitivity to PD-L1 positive patients was related only to the concomitant presence of *EGFR* mutations or whether PD-L1 positive patients had a more indolent disease outcome when treated with EGFR-TKIs in presence of

Table 2. PD-1/PD-L1 expression and patient characteristics

Characteristic	PD-1 + (no/%)	PD-1–(no/%)	*P*-value	Median PD-1 levels	PD-L1 + (no/%)	PD-L1–(no/%)	*P*-value	Median PD-L1 levels
Gender								
Male	24/55.8	41/51.9	0.68	60	36/52.9	30/54.5	0.86	60
Female	19/44.2	38/48.1		20	32/47.1	25/45.5		80
Smoking history								
Never/former smokers	27/73.0	65/90.3	0.02	20	53/86.9	41/82.0	0.48	60
Current smokers	10/27.0	7/9.7		60	8/13.1	9/18.0		30
Histology								
Adenocarcinoma	29/85.3	52/75.4	0.25	40	52/88.1	30/65.2	0.005	100
Squamous-cell carcinoma	5/14.7	17/24.6		0	7/11.9	16/34.8		0
***EGFR* status**								
EGFR mutated	17/39.5	38/48.1	0.36	20	40/58.8	16/29.1	0.001	120
EGFR wild type	26/60.5	41/51.9		40	28/41.2	39/70.9		20
***KRAS* status**								
KRAS mutated	16/37.2	12/15.2	0.006	60	15/22.1	13/23.6	0.84	55
KRAS wild type	27/62.8	67/84.8		25	53/77.9	42/76.4		80
***ALK* status**								
ALK translocated	3/7.0	7/8.9	1.00	15	6/8.8	4/7.3	1.00	115
ALK wild type	40/93.0	72/91.1		35	62/91.2	51/92.7		70
***EGFR* status**[a]								
EGFR mutated	17/70.8	38/63.3	0.51	20	40/85.1	16/42.1	<0.001	120
Triple negative[b]	7/29.2	22/36.7		40	7/14.9	22/57.9		20
***KRAS* status**[a]								
KRAS mutated	16/69.6	12/35.3	0.01	60	15/68.2	13/37.1	0.02	55
Triple negative[b]	7/30.4	22/64.7		40	7/31.8	22/62.9		20
***ALK* status**[a]								
ALK translocated	3/30.0	7/24.1	0.70	15	6/46.2	4/15.4	0.06	115
Triple negative[b]	7/70.0	22/75.9		40	7/53.8	22/84.6		20

Abbreviations: *ALK*= anaplastic lymphoma kinase; *EGFR*=epidermal growth factor receptor; *KRAS*=Kirsten rat sarcoma viral oncogene homologue; PD-1=programmed death-1; PD-L1=programmed death-ligand 1; PD-1 + =PD-1 positive; PD-1–=PD-1 negative; PD-L1 + =PD-L1 positive; PD-L1–=PD-L1 negative.
[a]Analysis performed *vs* triple negative cases.
[b]Triple negative included *EGFR/KRAS/ALK* wild-type patients.

EGFR mutations, we further analysed the outcome of 54 *EGFR* mutated patients treated with gefitinib or erlotinib and evaluable for response. In this subgroup, although no difference in RR was detected (76.3% *vs* 75.0%, $P=1.00$), PD-L1 positive ($N=38$, 70.4%) patients had a significantly longer TTP (13.1 months *vs* 8.5 months, $P=0.01$) and a non significantly longer OS (29.5 months *vs* 21.1 months, $P=0.75$) than PD-L1 negative ($N=16$, 29.6%) patients, as reported in Figure 5A and B.

DISCUSSION

In the present study, the first specifically conducted in a cohort of molecularly selected NSCLC, we showed that expression of immune checkpoints differs according to tumour biology and patient characteristics. Patients harbouring *KRAS* mutations had higher levels of PD-1 expression when compared to the *KRAS* wild-type population and presence of *EGFR* mutations or *ALK* translocations were associated with increased PD-L1 protein levels. Moreover, the clinical profile of patients expressing PD-1 was different from the profile of individuals expressing PD-L1. Patients expressing PD-1 were more frequently male, smokers with adenocarcinoma, whereas patients expressing PD-L1 were more frequently female, never/former smokers with adenocarcinoma, the typical clinical features of *KRAS* mutants or *EGFR* mutants, respectively.

During the past few years, a growing interest surrounded immunotherapy and several agents targeting molecules involved in regulation of the immune system are under development. Agents targeting PD-1 and PD-L1 showed promising results in patients with NSCLC, particularly in individuals with high PD-L1 expression. PD-L1 is frequently found highly expressed in many human cancer types including NSCLC, being up-regulated in tumours by activation of key oncogenic pathways, such as the PI3KCA-AKT and the RAS-RAF-MAPK pathways (Parsa *et al*, 2007). These findings suggested that high mutational rates may contribute to increased immunogenicity (Chen *et al*, 2012) indirectly predicting sensitivity to checkpoint inhibitors (Soria *et al*, 2013). In a recent study, Antonia *et al*, 2013 showed that PD-L1 levels were numerically higher in individuals with *EGFR* mutations. Nevertheless, the very low number of patients with *EGFR* mutations included in this study, only four cases, precluded any firm conclusion.

Importantly, we described for the first time PD-1 expression on tumour cells. This finding could have therapeutic implications and need additional confirmation. The lack of additional tumour tissue precluded the possibility to perform further experiments.

Interestingly, it seems that mechanisms of immune system deregulation differ according to the type of mutation, with potential therapeutic implications. PD-1 expression was significantly associated with presence of *KRAS* mutations, while PD-L1 was strongly associated with presence of *EGFR* mutations, potentially modulating sensitivity to anti-EGFR agents. In a recent study, Akbay *et al*, 2013, showed that activation of the EGFR pathway induces PD-L1 expression to facilitate evasion of the host anti-tumour immune response. This role of EGFR signalling was independent of its effects on cell proliferation and survival, suggesting an active role for the *EGFR* oncogene in

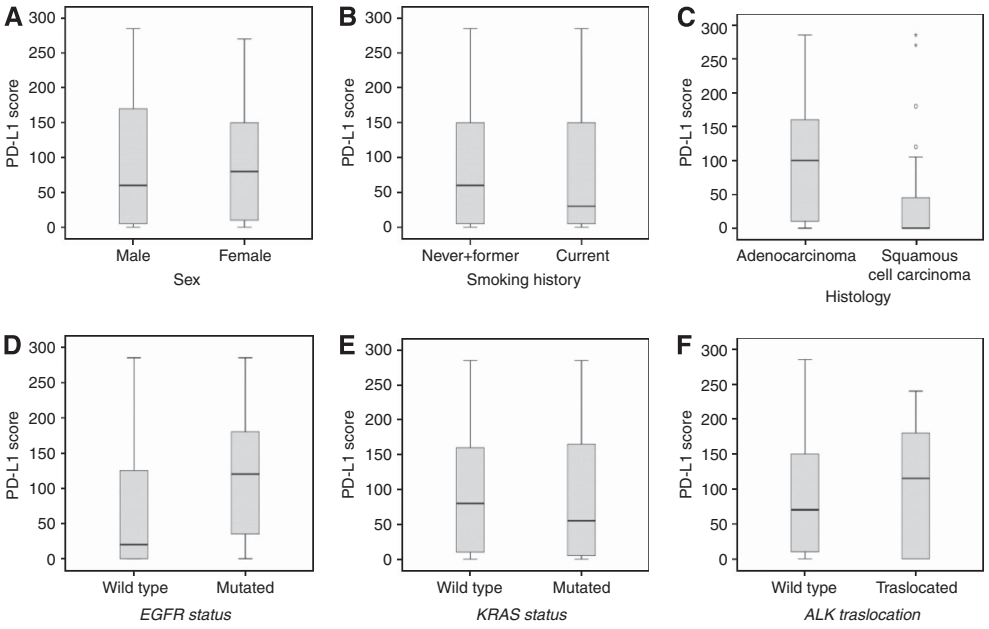

Figure 3. Levels of PD-L1 expression and patient characteristics. This picture illustrates changes in the levels of PD-L1 expression according to clinical (**A**–**C**) and biological (**D**–**F**) characteristics. Median levels of PD-1 score and interquartile ranges are showed. Median levels of PD-L1 expression were higher in female (median score 80 *vs* 60) than in male (**A**), in never/former (median score 60 *vs* 30) than in current smokers (**B**), in adenocarcinoma (median score 100 *vs* 0) than in squamous-cell carcinoma histology (**C**), in *EGFR* mutated (median score 120 *vs* 20) than in *EGFR* wild type (**D**), in *KRAS* wild type (median score 80 *vs* 55) than in *KRAS* mutated (**E**) and in *ALK* translocated (median score 115 *vs* 70) than in *ALK* wild type (**F**).

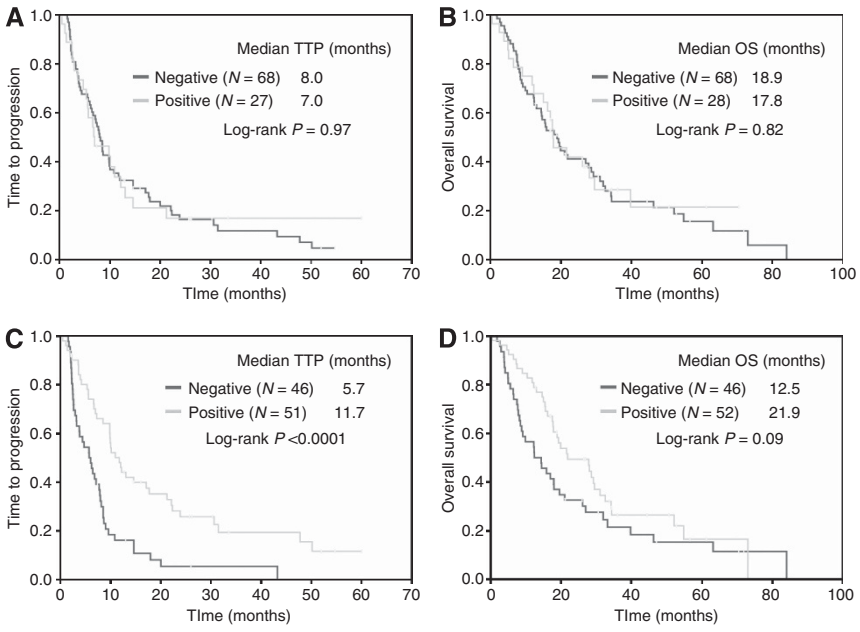

Figure 4. Kaplan–Meier curves of time to progression (TTP) (A–C) and overall survival (OS) (B–D) in patients treated with EGFR-TKIs. In the subgroup of patients ($N = 99$) treated with EGFR-TKIs no differences in terms of TTP (7.0 months *vs* 8.0 months, $P = 0.97$) (**A**) and OS (17.8 months *vs* 18.9 months, $P = 0.82$) (**B**) were identified in PD-1 positive ($N = 26$, 28.0%) *vs* PD-1 negative ($N = 67$, 72.0%) cases. PD-L1 positive ($N = 49$, 51.6%) patients had a significant longer TTP (11.7 months *vs* 5.7 months, $P < 0.0001$) (**C**) and a longer OS (21.9 months *vs* 12.5 months, $P = 0.09$) (**D**) than PD-L1 negative ($N = 46$, 48.4%) patients.

remodelling the immune microenvironment. Moreover, pharmacological blockade of the PD-1 pathway using EGFR-TKIs reduced PD-L1 expression, leading to tumour reduction, with a positive impact on overall survival (Akbay *et al*, 2013). The same effect was observed in our study. Patients harbouring *EGFR* mutations with high PD-L1 expression resulted more sensitive to gefitinib or erlotinib probably because of PD-L1 downregulation induced by the *EGFR* inhibition. The evidence that PD-L1 expression is a potential negative prognostic factor in several cancers further supports the hypothesis that the observed improvement in TTP was related to an indirect effect of the therapy on checkpoint expression (Zou and Chen, 2008). Overall,

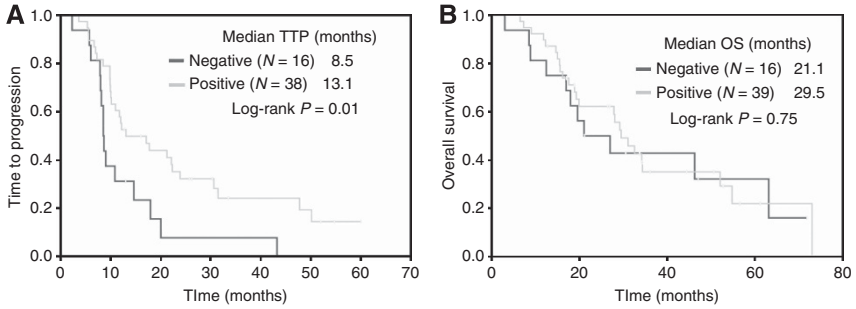

Figure 5. Kaplan–Meier curves of time to progression (TTP) (A) and overall survival (OS) (B) in PD-L1 positive *vs* PD-L1 negative *EGFR* mutated patients treated with EGFR-TKIs. In *EGFR* mutated patients ($N = 54$) treated with EGFR-TKIs, PD-L1 positive ($N = 38$, 70.4%) cases had a significantly longer TTP (13.1 months *vs* 8.5 months, $P = 0.01$) (**A**) and a non-significantly longer OS (29.5 months *vs* 21.1 months, $P = 0.75$) (**B**) than PD-L1 negative ($N = 16$, 29.6%) cases.

our and other data suggest that combination of PD-1 blockade with EGFR TKIs may be a promising therapeutic strategy to extend the duration of treatment response and delay development of resistance (Akbay *et al*, 2013).

Another interesting finding was the evidence that levels of PD-L1 were higher in patients with *ALK* translocations. Although the association was not statistically significant and although we were not able to evaluate whether a different checkpoint expression impacted on efficacy of anti-*ALK* agents, median PD-L1 levels were 5 times higher in *ALK* positive when compared with triple negative patients. Similarly to *EGFR* mutations, a potential effect of *ALK* translocation on checkpoint expression cannot be excluded and additional studies are recommended in this particular NSCLC subpopulation.

In the present study, we adopted two different methods for PD-1 or PD-L1 expression analyses, including a semiquantitative method largely adopted by our group in previous studies with biomarkers in NSCLC (Cappuzzo *et al*, 2005a, b). Although the usage of a new scoring system could represent a potential limitation, such method allowed us to assess more properly the association of checkpoint expression with patient characteristics.

In conclusion, our study showed that immune checkpoints are differently expressed in oncogene-addicted NSCLC potentially modulating sensitivity to targeted agents. Our findings represent the rationale to choose a different checkpoint inhibitor according to the tumour driver and to combine targeted therapies with anti-PD-L1 or anti-PD-1 agents. Considering the retrospective nature of our investigation, additional prospective studies are warranted.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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