KRAS and ERBB-family genetic alterations affect response to PD-1 inhibitors in metastatic nonsquamous NSCLC

Marika Cinausero*, Noemi Laprovitera* , Giovanna De Maglio, Lorenzo Gerratana ,
Mattia Riefolo, Marianna Macerelli, Michelangelo Fiorentino, Elisa Porcellini,
Vanessa Buoro, Francesco Gelsomino, Anna Squadrilli, Gianpiero Fasola, Massimo Negrini,
Marcello Tiseo, Manuela Ferracin and Andrea Ardizzoni

Abstract

Background: Programmed cell death 1 (PD-1) and PD-ligand 1 (PD-L1) inhibitors represent novel therapeutic options for advanced non-small cell lung cancer (NSCLC). However, approximately 50% of patients do not benefit from therapy and experience rapid disease progression. PD-L1 expression is the only approved biomarker of benefit to anti-PD-1/PD-L1 therapy. However, its weakness has been evidenced in many studies. More recently, tumor mutational burden (TMB) has proved to be a suitable biomarker, but its calculation is difficult to obtain for all patients.

Methods: We tested specific NSCLC genetic alterations as potential immunotherapy biomarkers. Tumor DNA was obtained from advanced NSCLC patients treated with anti-PD-1 monoclonal antibody nivolumab ($n = 44$) or pembrolizumab ($n = 3$). The mutational status of 22 genes was assessed by targeted next-generation sequencing and the association with survival was tested in uni- and multivariate models. The association between gene mutations and clinical benefit was also investigated.

Results: The most frequently mutated genes were *TP53* (49%), *KRAS* (43%), *ERBB2* (13%), *SMAD4* (13%), *DDR2* (13%), *STK11* (9%), *ERBB4* (6%), *EGFR* (6%), *BRAF* (6%), and *MET* (6%). We confirmed that $KRAS_{mut}$ patients have a better response to PD-1 inhibitors, showing a longer progression-free survival (PFS) and overall survival (OS) than $KRAS_{wt}$ patients. In addition, we observed that patients with *ERBB*-family mutations, including *EGFR*, *ERBB2*, and *ERBB4* all failed to respond to PD-1 antibodies, independently of *KRAS* status.

Conclusions: This study suggests that the analysis of *KRAS* and *ERBB*-family gene mutational status is valuable when assessing the clinical practice for the selection of NSCLC patients to treat with PD-1 inhibitors.

Keywords: anti-PD-1, immunotherapy, nivolumab, non-small cell lung cancer, pembrolizumab

Correspondence to:
Manuela Ferracin
Department of
Experimental, Diagnostic
and Specialty Medicine
(DIMES), University of
Bologna, Via S. Giacomo,
14, Bologna, 20126, Italy
**manuela.ferracin@
unibo.it**

**Marika Cinausero
Lorenzo Gerratana
Vanessa Buoro**
Department of Medicine
(DAME), University of
Udine, Udine, Friuli-
Venezia Giulia, Italy

Department of Oncology,
University Hospital of
Udine, Udine, Friuli-
Venezia Giulia, Italy

**Noemi Laprovitera
Mattia Riefolo
Michelangelo Fiorentino
Elisa Porcellini**
Department of
Experimental, Diagnostic
and Specialty Medicine
(DIMES), University of
Bologna, Bologna, Italy

Giovanna De Maglio
Department of Pathology,
University Hospital of
Udine, Udine, Friuli-
Venezia Giulia, Italy

**Marianna Macerelli
Gianpiero Fasola**
Department of Oncology,
University Hospital of
Udine, Udine, Friuli-
Venezia Giulia, Italy

Francesco Gelsomino
Division of Oncology, S.
Orsola-Malpighi Hospital,
Bologna, Emilia-Romagna,
Italy

**Anna Squadrilli
Marcello Tiseo**
Department of Medicine
and Surgery, University
of Parma and Medical
Oncology Unit, University
Hospital of Parma, Parma,
Emilia-Romagna, Italy

Massimo Negrini
Department of
Morphology, Surgery and
Experimental Medicine,

Introduction

Worldwide, lung cancer is the leading cause of cancer-related death, representing a major global health concern.[1] Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancer diagnoses, and approximately 50% of NSCLC patients present with stage IV disease and a 5-year survival rate of approximately 5–10%.[2] Until recently, the standard-of-care treatment for advanced NSCLC was represented by targeted therapies, when a druggable oncogenic alteration is detected, or platinum-based chemotherapy in the first-line setting and docetaxel-based chemotherapy in the following lines.[3]

University of Ferrara, Ferrara, Emilia-Romagna, Italy

Andrea Ardizzoni
Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna, Bologna, Emilia-Romagna, Italy

Division of Oncology, S. Orsola-Malpighi Hospital, Bologna, Emilia-Romagna, Italy

*These authors contributed equally to this work

The recent arrival of immune checkpoint inhibitors (ICPIs) that target programmed cell death 1 (PD-1) or its ligand (PD-L1), has led to a major change in the management of metastatic NSCLC with no druggable molecular alterations. These agents are supposed to control antitumor immunity and have demonstrated unprecedented improvement in patient's survival and disease control.[4,5] Several randomized studies have demonstrated the superiority of the ICPIs (nivolumab, pembrolizumab, and atezolizumab) over docetaxel in second-line therapy for advanced NSCLC.[4–6] However, the majority of advanced NSCLC fail to respond to ICPIs.[7] Thus, the identification of predictive factors to identify responder and nonresponders patients represents a large unmet clinical need.

Initially, the evaluation of PD-L1 expression on cancer cells by immunohistochemistry (IHC) was proposed as a test to predict the efficacy of anti-PD1/PD-L1 immunotherapy.[8] However, the reduced sensitivity and specificity of PD-L1 expression in predicting immunotherapy efficacy has encouraged the search for other biomarkers.[9,10] Recently, a high tumor mutational burden (TMB) was found to be a positive predictive biomarker for response to immunotherapy in multiple tumor types, including lung cancer.[11–13] The accumulation of mutations in tumor cells generates novel immunogenic tumor antigens (neoantigens) and consequently induces a T cell-dependent immune response against the tumor.[11,12] However, the assessment of TMB by next-generation sequencing (NGS) is expensive and requires adequate pathologic material, which in advanced NSCLC is normally scarce. In addition, the use of TMB is affected by the dynamic changes that TMB can sustain during disease progression and therapies.[14]

Genetic alterations in specific driver genes activate tumor cell proliferation thus supporting tumor growth. It has been demonstrated that some oncogenic pathways also influence tumor recognition by the immune system, especially T cell-mediated recognition. Smoking-associated *KRAS* mutations are the most frequent oncogenic alterations in NSCLC.[15,16] Recent clinical evidence showed that *KRAS-TP53* comutated tumors, but not *KRAS-STK11* comutated tumors, have an immunogenic phenotype and are more sensitive to nivolumab.[16–18]

In this study, we assessed the mutational landscape of NSCLC patients treated with anti-PD-1 monoclonal antibodies pembrolizumab and nivolumab, to identify genetic alterations associated with a clinical benefit to ICPIs.

Materials and methods

Clinical samples
A retrospective consecutive series of 88 patients with locally advanced or metastatic nonsquamous NSCLC treated with ICPIs (anti-PD-1 nivolumab and pembrolizumab) were identified. Patients were treated at the Department of Oncology of Bologna, Udine and Parma (Italy), between January 2012 and December 2017. Tumor histology was confirmed using standardized diagnostic immunohistochemical workup (TTF-1, p40). After diagnostic testing, most of the residual samples were insufficient for retrospective PD-L1 IHC assessment. Demographic, clinicopathological, and outcome details for each patient were extracted from the electronic or paper medical records according to strict privacy standards. Among the total population, DNA of adequate volume and quality was available from archived formalin-fixed paraffin-embedded (FFPE) samples for 47 of the 88 patients (Table 1), whose tumors were, therefore, analyzed by target sequencing. DNA was extracted from 29 histological samples using Qiamp DNA FFPE kit (Qiagen, Venlo, Netherlands #56404) and from 18 cytological samples with Qiamp DNA Micro kit (Qiagen, Venlo, Netherlands #56304).

Target sequencing
Samples were analyzed for genetic alterations using Oncomine™ Solid Tumor DNA kit (Thermo Fisher CN, Waltham, MA, USA: A26761). This panel covers more than 500 cancer-related variants in 22 genes (*EGFR, ERBB2, ERBB4, MET, FGFR1, FGFR2, FGFR3, DDR2, ALK, KRAS, NRAS, PIK3CA, BRAF, PTEN MAP2K1, AKT1, TP53, STK11, CTNNB1, SMAD4, FBXW7*, and *NOTCH*). Genomic DNA quantity and quality was assessed using Quantifiler™ Human DNA Quantification kit (Thermo Fisher, Waltham, MA, USA #4343895) for *hTERT* gene on Applied Biosystems™, Waltham, MA, USA 7500 Real-Time PCR System (#4351105) following the manufacturer's instructions (PN 4344790F). Library preparation was performed according to the Oncomine™ Solid Tumor DNA kit protocol, following manufacturer's protocol (MAN0010935). Oncomine™ Solid Tumor panel's single pool of primers was used to perform multiplex PCR with a starting DNA quantity of

Table 1. Clinical and tumor features of NSCLC patients treated with anti-PD-1 inhibitors.

Clinicopathological features	n (%) (n = 47)
Sex	
Males	30 (63.83)
Females	17 (36.17)
Smoking habits	
Nonsmoker	6 (12.77)
Smoker	16 (34.04)
Former smoker	25 (53.19)
Stage at the beginning of immunotherapy	
IV	47 (100)
CNS metastases	
Yes	8 (17.02)
No	36 (76.60)
Unknown	3 (6.38)
ECOG PS at the beginning of immunotherapy	
0	15 (31.91)
1	25 (53.19)
2	7 (14.89)
Use of steroids	
Yes	15 (31.91)
No	29 (61.70)
Unknown	3 (6.38)
Type of immune checkpoint inhibitor	
Nivolumab	44 (93.62)
Pembrolizumab	3 (6.38)
Number of previous lines	
0	2 (4.26)
1	21 (44.68)
2	17 (36.17)
⩾3	7 (14.89)
Best response to previous line	
PD	17 (36.17)
PR	10 (21.28)
SD	16 (34.04)
Unknown	4 (8.51)

Table 1. (Continued)

Clinicopathological features	n (%) (n = 47)
Number of immunotherapy cycles	
⩽3	11 (23.40)
4–9	21 (44.68)
10–20	9 (19.15)
20–30	6 (12.77)
>30	0 (0.0)
Best response to immunotherapy	
CR	1 (2.13)
PR	4 (8.51)
SD	13 (27.65)
PD	29 (61.7)
Clinical benefit	
DCB	13 (27.66)
NDB	34 (72.34)
PD at database lock	
Yes	38 (80.85)
No	9 (19.15)
Immunotherapy beyond progression	
Yes	11 (28.95)
No	27 (71.05)
Post-progression treatment	
Yes	12 (31.58)
No	26 (68.42)
Death	
Yes	33 (70.21)
No	14 (29.79)
***KRAS* mutation**	
Yes	20 (42.55)
No	27 (57.45)
***EGFR* mutation**	
Yes	3 (6.38)
No	44 (93.62)

CNS, central nervous system; CR, complete response; DCB, durable clinical benefit; ECOG PS, Eastern Cooperative Oncology Group performance status; NDB, no durable benefit (SD, PR or CR < 6 months); NSCLC, non-small cell lung cancer; PD, progressive disease; (SD, PR or CR > 6 months); PR, partial response; SD, stable disease.

10 ng to generate 92 amplicons (115–120 bp long). Libraries were quantified using Ion Library TaqMan™ Quantification kit (Thermo Fisher, Waltham, MA, USA #4468802). After quantification, each library was diluted and pooled to obtain four equimolar library pools. Emulsion PCR and Ion Sphere Particles enrichment were performed according to the protocol Ion PGM™ Hi-Q™ View OT2 Kit (Thermo Fisher, Waltham, MA, USA #A29900; MAN0014579) using Ion OneTouch 2 system™ (#4474779). Sequencing was performed using Ion Torrent Personal Genome Machine™ (PGM™) sequencer (#4462921) with Ion PGM™ Hi-Q™ View Sequencing Kit (Thermo Fisher, Waltham, MA, USA #A30044; MAN0014583). Pools were loaded onto Ion 318 Chips. Data analysis was performed using Ion Reporter™ software selecting the AmpliSeq Colon and Lung Cancer v2 single sample Workflow. An average number of 900 variants was found for each sample. A filter chain was applied to select the variants belonging to the following types: single nucleotide variants, insertions, deletions, multiple nucleotide variants, and long deletions. A further selection was applied to remove variants below 3% of frequency, synonymous, and intronic variants. The common *TP53* polymorphism (P72R) was filtered out.

Statistical analysis

Clinicopathological characteristics were summarized using descriptive analysis. Continuous variables were reported using median and interquartile ranges, and categorical variables were described using frequency distribution. The association between clinicopathological and genomic features was explored using contingency tables with the chi-squared test.

Progression-free survival (PFS) was defined as the time elapsed between immunotherapy initiation and disease progression or death from any cause, whatever occurred first. Overall survival (OS) was defined as the time elapsed between immunotherapy initiation and death from any cause or the last follow-up. Durable clinical benefit (DCB) was defined as stable disease, partial response, or complete response lasting longer than 6 months. Patients that underwent progression to disease before 6 months were classified as no durable benefit (NDB).[19] Prognostic factors in terms of OS and PFS were tested both in uni and multivariate models by Cox regression with 95% confidence interval (CI 95%). The survival curves were estimated by the Kaplan–Meier method, and the log-rank test was performed to test differences between the survival curves. The association between gene mutations and DCB/NDB was investigated with Fisher's exact test. A value of two-sided $p \leqslant 0.05$ was considered significant. Statistical analysis was performed using STATA (StataCorp. (2015) Stata Statistical Software: Release 14.2. College Station, TX: StataCorp LP). The mutational plot was obtained using the Bioconductor package 'GenVisR'.[20] Only selected variants were plotted. When more than one mutation occurred in the same gene and patient, we plotted those with a higher frequency or higher PolyPhen2 score (suggesting damaging substitutions). Intronic mutations were evaluated using the online bioinformatic tool Human Splicing Finder.[21]

Results

Patient characteristics

Demographic, clinical, and pathological features of our cohort of 47 NSCLC patients are summarized in Table 1. A total of 30 males and 17 females were included, with a mean age of 66 years at first diagnosis (range 43–85, SD ± 9.08 years). The percentage of current/former smokers is 87.23%. All patients had stage IV disease at the beginning of immunotherapy and 17% of patients had central nervous system (CNS) metastases. The majority of patients received treatment with the PD-1 inhibitors nivolumab ($n = 44$) or pembrolizumab ($n = 3$) as the second or third therapeutic line. Only one patient interrupted the therapy due to toxicity (nivolumab-related pneumonitis).
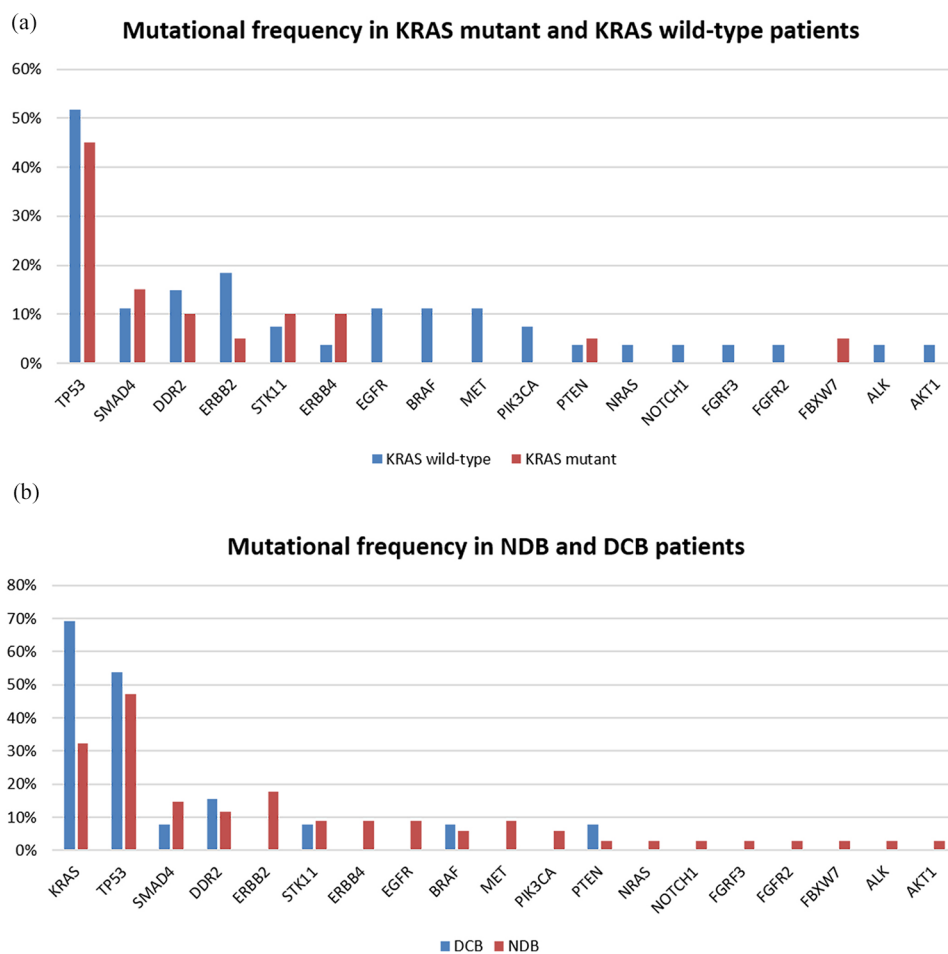
Median follow-up was 18.84 months (range 13.24–22.22 months). Median PFS and OS were 2.56 and 8.12 months, respectively. Disease progression (PD) occurred in 38 patients, and 28.95% of patients continued to receive immunotherapy beyond progression. A total of 13 patients (27.66%) had DCB, and 34 (72.34%) had NDB. A total of 33 patients (70.21%) died during the follow-up.

Mutational landscape of anti-PD-1 treatment resistance in NSCLC

NSCLC genetic alterations were assessed using NGS in 22 genes that are highly mutated in solid tumors. A median number of 2.27 nonsynonymous mutations/patient in 20 out of 22 genes were identified. All detected nonsynonymous alterations for each analyzed patient are presented in Figure 1. In addition to nonsynonymous
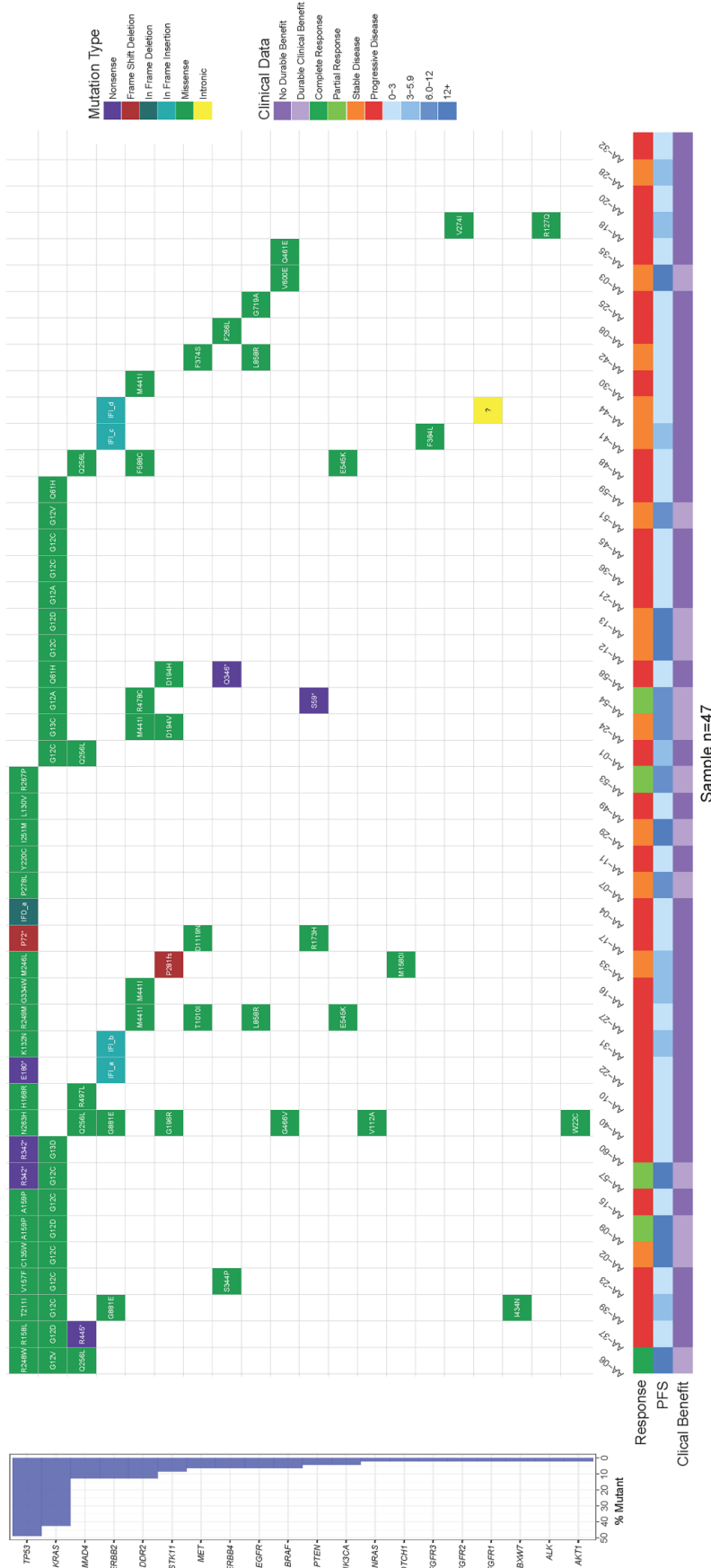
Figure 1. Nonsynonymous mutational landscape of NSCLC patients treated with anti-PD-1 immunotherapy. In this graph selected nonsynonymous mutations obtained through next-generation sequencing analysis are plotted. One intronic mutation (in yellow) was exceptionally plotted because of its potential impact on function. In the side bar on the left, the mutational frequency of each gene is shown, while in the lower bar chart the best response, progression-free survival (months) and clinical benefit to immunotherapy are displayed.

IFD_a, in frame deletion p.I195_R196del; IFI_a, in frame insertion p.E770_A771insAYVM; IFI_b, in frame insertion p.G776insVC; IFI_c, in frame insertion p.E770_A771insAYVM; IFI_d, in frame insertion p.V777_G778insGSP.

(a)

Mutational frequency in KRAS mutant and KRAS wild-type patients

(b)

Mutational frequency in NDB and DCB patients

Figure 2. Histogram plots of mutational frequency distribution. This plot shows the percentage of mutations in (a) $KRAS_{mut}$ *versus* $KRAS_{wt}$ patients and in (b) DCB *versus* NDB subgroups.

mutations, we included as potentially pathogenic, an intronic mutation (FGFR1 - c.458-3T>G) that could affect splicing.

In our cohort ($n = 47$), the most mutated genes were *TP53* (49%), *KRAS* (43%), *SMAD4* (13%), *DDR2* (13%), *ERBB2* (13%), *STK11* (9%), *ERBB4* (6%), *EGFR* (6%), *BRAF* (6%), *MET* (6%), and other genes at a lower frequency (online supplementary Table 1). The mutational frequency distribution in $KRAS_{wt}/KRAS_{mut}$ patients and in responders (DCB) and nonresponders (NDB) patients is shown in Figure 2.

KRAS and ERBB-family mutations are associated with outcome in NSCLC

We investigated the association between the probability of DCB and molecular characteristics (Table 2). The results highlighted a significant difference

in *KRAS* mutation frequency between DCB/NDB groups ($p = 0.012$). We observed that mutations in ERBB genes *(EGFR, ERBB2, ERBB4), MET*, and in *SMAD4* were more frequent in NDB than in the DCB group, although no significant association was found when considering each gene mutation. When gene combinations were evaluated, we observed a significant difference in the frequency of pan-*ERBB*, including *EGFR, ERBB2*, and *ERBB4* mutations, between DCB/NDB groups ($p = 0.009$), showing a significant association with NDB status. In addition, when combining this panel with *MET* and *SMAD4* genes, this association became even stronger.

On univariate analysis for PFS (Table 3) patients with $KRAS_{mut}$ tumors had a better outcome than patients with $KRAS_{wt}$ disease (HR: 0.48, CI 95% 0.24–0.97 $p = 0.041$). However, patients that harbor mutations in *ERBB4* and *ERBB*-family genes

Table 2. Association between genetic alterations and clinical benefit.

Gene	Mutant in DCB n (%)	Mutant in NDB n (%)	p value[a]
KRAS	71	30	**0.012**
ERBB2	0	18	0.159
ERBB4	0	9	0.544
EGFR	0	9	0.544
MET	0	9	0.544
TP53	50	45	1
SMAD4	7	15	0.653
STK11	7	9	1
DDR2	14	12	1
BRAF	7	6	1
Pan-ERBB	0	36[b]	**0.009**
Pan-ERBB + MET	0	39[c]	**0.005**
Pan-ERBB + SMAD4	7	48[c]	**0.008**
Pan-ERBB + MET + SMAD4	7	51[c]	**0.007**

DCB, durable clinical benefit (SD, PR or CR >6 months); NDB, no durable benefit (SD, PR or CR <6 months).
[a]p value at Fisher's exact test.
[b]Mutually exclusive mutations.
[c]Samples with mutations in at least one of the indicated genes.
Significant p-values (<0.05) are in bold.

(*EGFR, ERBB2*, and *ERBB4*) were found to have a worse median PFS compared with *ERBB*-family wild-type patients (HR: 4.14, CI 95% 1.18–14.43 $p = 0.026$ and HR: 2.77, CI 95% 1.34–5.71 $p = 0.006$, respectively). Although a significantly worse PFS was associated with mutations in the *EGFR* (HR: 3.67, CI 95% 1.08–12.41 $p = 0.037$), *NRAS* (HR: 22.50, CI 95% 2.04–248.10 $p = 0.011$), *PIK3CA* (HR: 7.86, CI 95% 1.61–38.30 $p = 0.011$), or *AKT1* (HR: 14.82, CI 95% 1.54–142.55 $p = 0.011$) genes, their overall mutant allele frequency was low ($n = 1$–3). On multivariate analysis (Table 3), the presence of *KRAS* (HR: 0.42, CI 95% 0.19–0.92 $p = 0.033$), *ERBB4* (HR: 9.28, CI 95% 2.28–37.77 $p = 0.002$), and *AKT1* (HR: 29.12, CI 95% 2.47–344.05 $p = 0.007$) mutation maintained their statistical significance. Moreover, a negative impact in terms of PFS was also observed by combining the ERBB-family genes with *MET* (HR 2.32, CI 95% 1.08–4.99 $p = 0.031$), *SMAD4* (HR: 2.28, CI 95% 1.12–4.63 $p = 0.023$,) or both (HR: 2.49, CI 95% 1.23–5.04 $p = 0.011$).

On univariate analysis on OS, patients with $KRAS_{mut}$ tumors had a better OS than those with $KRAS_{wt}$ tumors (HR: 0.35, CI 95% 0.15–0.78 $p = 0.010$) (online supplementary Table 2). In contrast, the presence of *EGFR* (HR: 11.46, CI 95% 2.67–49.21 $p = 0.001$) or *PIK3CA* mutation (HR: 6.42, 95% CI: 1.35–30.52 $p = 0.019$), and *MET al*teration (HR 5.47, CI 95% 1.49–19.97 $p = 0.010$) were associated with worsened OS. In addition, patients that harbor mutations in ERBB-family genes were found to have a worse median OS compared with *ERBB*-family wild-type patients (HR: 2.19, CI 95% 1.02–4.70 $p = 0.044$). On multivariate analysis (online supplementary Table 2), the presence of *KRAS* mutation (HR: 0.39, CI 95%: 0.17–0.89 $p = 0.027$) and *EGFR* mutation (HR: 6.31, CI 95%: 1.22–32.77 $p = 0.028$) maintained its statistical significance, as well as the combined mutation of *ERBB*-family genes and *SMAD4* (HR: 2.22, CI 95% 1.04–4.73 $p = 0.039$), or both *MET* and *SMAD4* (HR: 2.37, CI 95% 1.11–5.03 $p = 0.025$).

Table 3. Univariate and multivariate analysis for PFS.

	Univariate analysis			Multivariate analysis		
	HR	**95% CI**	***p* value**	**HR**	**95% CI**	***p* value**
KRAS	**0.48**	**0.24–0.97**	**0.041**	**0.42**	**0.19–0.92**	**0.032**
STK11	1.44	0.51–4.08	0.495			
TP53	0.77	0.41–1.48	0.44			
SMAD4	1.78	0.69–4.59	0.234			
ERBB2	1.59	0.65–3.87	0.305			
ERBB4	**4.14**	**1.18–14.43**	**0.026**	**9.28**	**2.28– 37.77**	**0.002**
DDR2	1.13	0.44–2.91	0.804			
EGFR	**3.67**	**1.08–12.41**	**0.037**	1.96	0.41– 9.51	0.402
*NOTCH**	0.89	0.12–6.58	0.913			
BRAF	0.99	0.24–4.11	0.984			
*FBXW7**	1.07	0.15–7.89	0.947			
*NRAS**	**22.5**	**2.04–248.10**	**0.011**	NA		
*PTEN**	0.62	0.08–4.52	0.635			
*PIK3CA**	**7.86**	**1.61–38.30**	**0.011**	5.30	0.71– 39.31	0.103
MET	2.94	0.87–9.99	0.084			
*ALK**	1.01	0.14–7.42	0.993			
*FGFR2**	1.01	0.14–7.42	0.993			
*FGFR3**	0.84	0.11–6.19	0.866			
*AKT1**	**14.82**	**1.54– 142.55**	**0.011**	**29.19**	**2.47– 344.05**	**0.007**
Pan-ERBB§	**2.77**	**1.34–5.71**	**0.006**	2.09	0.96– 4.52	0.063
Pan-ERBB + MET§	**3.01**	**1.47– 6.17**	**0.003**	**2.32**	**1.08– 4.99**	**0.031**
Pan-ERBB + SMAD4§	**2.57**	**1.31– 5.03**	**0.006**	**2.28**	**1.12– 4.63**	**0.023**
Pan-ERBB MET + SMAD 4§	**2.79**	**1.43– 5.45**	**0.003**	**2.49**	**1.23– 5.04**	**0.011**

*Number of mutations detected <3.
§Corrected based on independent multivariate models.
CI, confidence interval; HR, hazard ratio; progression-free survival.
Significant associations are in bold.

Differences in PFS and OS were represented by Kaplan–Meier curves in Figure 3(a)–(c) and supplementary Figure 1(a)–(c), respectively.

Of note, no patient with nonsynonymous mutations in *ERBB*-family genes belonged to the group of DCB patients (n = 13). The 12 mutually exclusive mutations reported in these 3 genes were found to occur only in non-DCB patients, suggesting a potential implication of the *ERBB*-family in the mechanism of resistance to anti-PD1 blockers.

Discussion

In this study, we retrospectively analyzed by targeted sequencing a consecutive cohort of patients
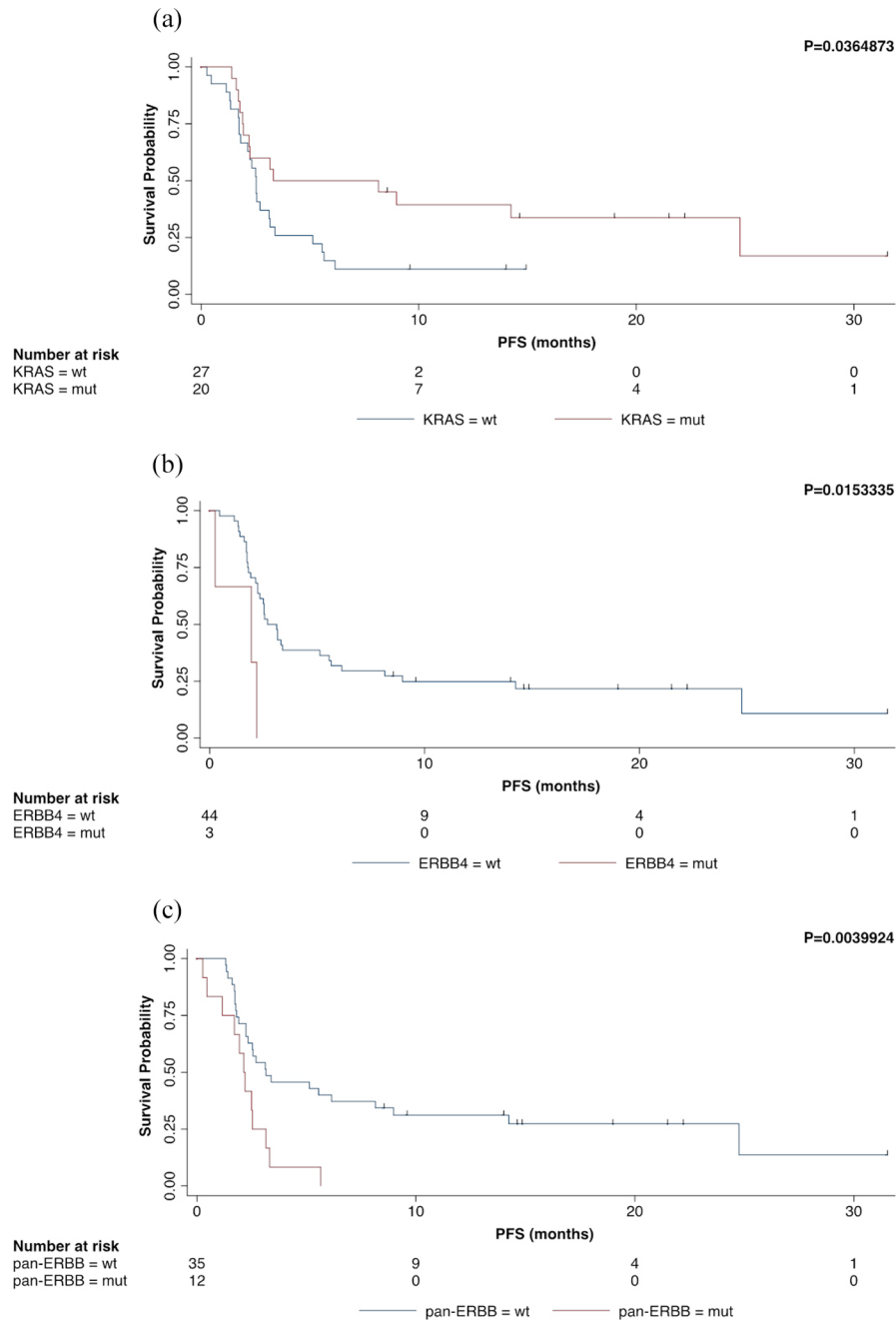
Figure 3. Kaplan–Meier plots for progression-free survival (PFS). The plots show significantly different PFS curves in (a) *KRAS*_{wt} *versus KRAS*_{mut}, (b) *ERBB4*_{wt} *versus ERBB4*_{mut} and (c) pan-*ERBB*_{wt} *versus* pan-*ERBB*_{mut} (including *ERBB2, ERBB4,* and *EGFR* genes) NSCLC patients.

with locally advanced or metastatic nonsquamous NSCLC, who were treated with the anti-PD1 inhibitors nivolumab or pembrolizumab. The aim was to highlight some of the genetic determinants of anti-PD-1 resistance.

The arrival of ICPIs, targeting PD-1 and its ligand PD-L1, has led to a major change in the treatment of metastatic nononcogene addicted NSCLC, with a significant improvement of survival and disease control. The anti-PD-1 and anti-PD-L1 inhibitors are currently the treatment of choice after first-line chemotherapy (CT). Specifically, the anti-PD-1 nivolumab and anti-PD-L1 atezolizumab are approved as second and later therapeutic lines for advanced NSCLC,

regardless of PD-L1 expression. Pembrolizumab has been approved as a first-line monotherapy for patients with advanced NSCLC and PD-L1 score ≥50%,[9] and is the standard treatment for NSCLC patients who progressed to first-line CT with PD-L1 score ≥1%.[5] Recently, positive results from several clinical trials led to the marketing approval of a first-line combination of pembrolizumab plus CT for patients with advanced NSCLC,[22–24] and atezolizumab plus bevacizumab plus CT as first-line treatment for advanced non-squamous NSCLC.[25] In addition, nivolumab plus ipilimumab represents another promising therapeutic options for patients with untreated, advanced NSCLC with high TMB.[26]

Despite unprecedented improvement in outcomes with the use of anti-PD-1 and anti-PD-L1 inhibitors, the majority of NSCLC patients fail to respond to ICPIs. Thus, the identification of predictive clinical factors and biomarkers of clinical response or resistance to this therapy in metastatic NSCLC represents a significant need for the appropriate selection of responders from nonresponders.

The extreme heterogeneity characterizing advanced nonsquamous NSCLC represents a clinical challenge and affects the development of effective therapeutic strategies for patients with such diagnosis. Simultaneously, the high incidence of *KRAS* mutations and the lack of specific and effective agents targeting *KRAS* have led to a growing interest in *KRAS*-mutated NSCLC.

Similar to previous reports,[4,6,27] we observed that patients with *KRAS* mutations benefit most from PD-1 blockade. Indeed, we found that *KRAS*-mutant patients had statistically significant longer OS and PFS as compared with patients with *KRAS* wild-type disease. Furthermore, we also showed that the presence of nonsynonymous *KRAS* mutations is associated with a DCB. The mechanisms underlying this higher sensitivity to immunotherapy of *KRAS*-mutant tumors are still under investigation.[28] It is known that *KRAS* mutations are generally associated with smoking and high TMB which, in turn, results in the generation of immunogenic neoantigens that could stimulate immune response.[29]

The most innovative finding of our analysis is the strong association between *HER/ERBB* pathway mutations and the lack of response to anti-PD-1 inhibitors. Previous reports suggested using *EGFR* mutations as biomarkers of resistance to ICIs.[4,27,30,31] In our cohort, the patients harboring mutations in *EGFR* were found to have NDB from immunotherapy.

When we analyzed all *ERBB*-family genes contained in the NGS panel (*EGFR, ERBB2,* and *ERBB4*), we obtained evidence of a statistically significant negative impact of *ERBB*-family mutations on patient's outcomes. Of note, we found that patients with mutations in *ERBB* pathway genes had worse PFS and OS than patients with *ERBB*-family wild-type disease. Our findings suggest that ICPIs with anti-PD-1 may not be effective in patients with *EGFR* or any *ERBB*-gene mutant nonsquamous NSCLC.

The *HER/ERBB* pathway is frequently mutated in NSCLC and has been linked to PD-L1 upregulation and reduced antigen presentation to CD8+ T cells.[32] Specifically, *EGFR* and *ERBB2* pathway activation was shown to be responsible for PD-L1 increased expression on the surface of lung cancer cells[33] and for the impairment in MHC class I antigen presentation.[34,35] In addition, *EGFR*-mutant patients have a high frequency of inactive tumor infiltrating lymphocytes.[36] These mechanistic studies provide a biological explanation of the results we obtained in the clinic.

Recently, it was also demonstrated that *KRAS*-mutant NSCLC include different subtypes with different biology, prognosis, and response to ICPIs.[16,17] Specifically, Skoulidis and colleagues proved that alterations in *STK11* are associated with de novo resistance to anti-PD-1 therapy despite the presence of intermediate/high TMB. This is partially due to the reported association between lack of PD-L1 expression and *STK11* inactivation. In addition, *KRAS/TP53* comutation was reported to be associated with increased TMB and PD-L1 expression[16] and a more favorable response to anti-PD-1 blockade.[31] In our series, we observed nine (45%) *KRAS*-mutant tumors with intact *STK11/LKB1* and *TP53* (KO or K only), nine *KRAS*-mutant harboring mutations in *TP53* (KP), and two (10%) *KRAS*-mutant bearing mutations in *STK11/LKB1* (KL). No triple-mutant (*KRAS/ TP53/ STK11*) tumors were detected. These frequencies were different from the ones reported in the Skoulidis study. Unfortunately, the small number of cases belonging to the KO, KP and especially KL subgroups prevented us from verifying the association

between *KRAS* comutations and outcome. However, it is worth noting that of the four patients with *STK11* mutations, one had a durable benefit lasting longer than 6 months. On the bases of these results, we cannot draw any definitive conclusion about the impact of these alterations on PFS and OS of our population. It is likely that the type of molecular assay used (NGS panel *versus* WES) may account for the different findings.

Finally, we assessed the impact on prognosis of other mutations such as *SMAD4, NRAS, PIK3CA, AKT*, and *MET* mutation. *SMAD4* and *MET* mutations were correlated with worse outcomes on univariate analysis in our series. However, larger prospective studies are required to confirm these findings.

Our study has some limitations, including the retrospective observational design and the small sample size. However, it provides a real-world scenario of genomic analysis in a consecutive series of advanced nonsquamous NSCLC patients receiving immunotherapy with anti-PD-1 in three large Italian hospitals, in the context of standard clinical practice, using a commercially available NGS panel.

Conclusion
Our findings suggest that the presence of *KRAS* mutations and the absence of *ERBB*-family gene mutations should be further evaluated as biomarkers of benefit to nivolumab and pembrolizumab treatments.

Author contributions
AA designed the study; MC, MT, MM, VB, AS, GF, and FG selected patients and collected clinical information; MF, NL, and EP performed NGS experiments and analyzed results; LG and MR performed statistical analyses; MFi and GDM performed the histo-pathological examinations; MN contributed to data interpretation; AA, MF, MC, NL, and LG interpreted and discussed the data and wrote the manuscript. All authors revised and approved the manuscript.

Conflict of interest statement
AA reports grants and personal fees from BMS, personal fees from MSD, personal fees from Eli-Lilly, personal fees from Boehringer, personal fees from Pfizer, and grants from Celgene outside the submitted work. MT reports advisory boards and/or speaker's fee for BMS and MSD. The other authors declare that they have no conflicting interests.

Ethics approval and consent to participate
The study was approved by Comitato Etico Unico Regionale (C.E.U.R.; approval ID: CEUR-2017-0s-123-ASUIUD), Friuli-Venezia Giulia Region. Before study entry, all patients provided written and voluntary informed consent for inclusion, collection and use of clinicopathological data and samples.

ORCID iDs
Noemi Laprovitera [iD] https://orcid.org/0000-0002-2737-4665

Lorenzo Gerratana [iD] https://orcid.org/0000-0002-8313-4834

Manuela Ferracin [iD] https://orcid.org/0000-0002-1595-6887

Supplemental material
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