Lung Cancer 121 (2018) 70–75

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Effect of mutant variants of the *KRAS* gene on PD-L1 expression and on the immune microenvironment and association with clinical outcome in lung adenocarcinoma patients

Alexander T. Falk[a,b], Nathalie Yazbeck[a], Nicolas Guibert[c], Emmanuel Chamorey[d], Agnès Paquet[e], Lydia Ribeyre[a], Coraline Bence[f], Katia Zahaf[f], Sylvie Leroy[g], Charles-Hugo Marquette[a,g], Charlotte Cohen[h], Baharia Mograbi[a], Julien Mazières[c], Véronique Hofman[a,f,i], Patrick Brest[a], Paul Hofman[a,f,i,*,1], Marius Ilié[a,f,i,**,1]

[a] Université Côte d'Azur, CNRS UMR7284, INSERM U1081, IRCAN Team 4, FHU OncoAge, Nice, France
[b] Antoine Lacassagne Comprehensive Cancer Center, FHU OncoAge, Department of Radiation Oncology, Nice, France
[c] University Hospital of Toulouse, Larrey Hospital, Thoracic Oncology Department, Toulouse, France
[d] Antoine Lacassagne Comprehensive Cancer Center, FHU OncoAge, Biostatistics Unit, Nice, France
[e] Université Côte d'Azur, CNRS, Institut de Pharmacologie Moléculaire et Cellulaire, FHU OncoAge, Sophia Antipolis, France
[f] Université Côte d'Azur, CHU Nice, FHU OncoAge, Laboratory of Clinical and Experimental Pathology, Nice, France
[g] Université Côte d'Azur, CHU Nice, FHU OncoAge, Department of Pulmonary Medicine and Oncology, Nice, France
[h] Université Côte d'Azur, CHU Nice, FHU OncoAge, Department of Thoracic Surgery, Nice, France
[i] Université Côte d'Azur, CHU Nice, FHU OncoAge, Hospital-Integrated Biobank (BB-0033-00025), Nice, France

ARTICLE INFO

Keywords:
KRAS
PD-L1
Immune microenvironment
Lung adenocarcinoma

ABSTRACT

Objectives: The effect of anti-PD-1/PD-L1 inhibitors on lung adenocarcinomas (LADCs) with *KRAS* mutations is debatable. We examined the association between specific mutant KRAS proteins and the immune infiltrates with the outcome of patients with LADCs.

Patients and methods: In 219 LADCs harboring either wild-type (WT) or mutated *KRAS* gene, we quantified the density of several immune markers by immunohistochemistry followed by automated digital image analysis. Data were correlated to clinicopathological parameters and outcome of patients.

Results: Tumors harboring mutant *KRAS*-G12 V had a significantly higher PD-L1 expression compared to other tumors ($p = 0.044$), while mutant *KRAS*-G12D tumors showed an increase in the density of CD66b+ cells ($p = 0.001$). High PD-L1 expression in tumor cells was associated to improved overall survival (OS) in *KRAS* mutant patients ($p = 0.012$), but not in the WT population ($p = 0.385$), whereas increased PD-L1 expression in immune cells correlated to poor OS of *KRAS*-WT patients ($p = 0.025$), with no difference in patients with KRAS mutations.

Conclusions: *KRAS* mutational status can affect the immune microenvironment and survival of LADC patients in a heterogeneous way, implying that specific mutant *KRAS* variants expressed by the tumor should be considered when stratifying patients for immunotherapy.

1. Introduction

Lung cancer is the most common and deadliest cancer worldwide [1]. Non-small-cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers, and lung adenocarcinoma (LADC), the most common histology subtype of NSCLC, is often accompanied by oncogenic driver mutations [2]. With the advent of clinical development of targeted therapies for molecular targets, some patients with LADC can benefit from specific drugs targeting *EGFR* or *BRAF* gene mutations and *ALK* or *ROS1* rearrangements [3]. Mutations in the *KRAS* gene affect ~30% of LADCs but unlike *EGFR* and *ALK*, altered in less than 20% of ADCs, mutant *KRAS* ADCs are resistant to anti-EGFR therapies and still cannot be targeted [4].

With the development of anti-PD-1/PD-L1 inhibitors,

* Corresponding author.
** Corresponding author at: Laboratory of Clinical and Experimental Pathology, Pasteur Hospital, 30 Voie Romaine, BP69, 06001, Nice Cedex, France.
E-mail addresses: hofman.p@chu-nice.fr (P. Hofman), ilie.m@chu-nice.fr (M. Ilié).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.lungcan.2018.05.009
Received 6 March 2018; Received in revised form 29 April 2018; Accepted 13 May 2018

immunotherapy now plays a major role in NSCLC treatment [5]. Pembrolizumab, an anti-PD1 therapy has become standard care for first-line NSCLC expressing PD-L1 in tumor cells [5]. However, no specific studies have concerned NSCLC with specific *KRAS* mutations treated with anti-PD-1/PD-L1 immunotherapy. The OAK and Checkmate 057 trials reported superior efficacy compared to chemotherapy for 59–62% of patients presenting with a *KRAS* mutation [6,7]. A major issue concerns selection of patients that would benefit from this treatment, which raises the question of the specificity of PD-L1 expression in tumor cells as a reproducible predictive biomarker for clinical response in patients with *KRAS* mutations treated with anti-PD-1/PD-L1 inhibitors [5].

One mechanism of *KRAS* activation is a nonsense mutation on codon 12 with the substitution of glycine by another amino acid, the most frequent being cysteine p.G12C (40%), valine p.G12 V (22%) or aspartate p.G12D (16%) [8]. There is evidence that the subtype of the mutant KRAS amino acid substitution could affect patient survival, downstream signaling and patient response to treatments in a heterogeneous way [8,9]. Intriguingly, transgenic mice with *KRAS*-G12D-driven tumors did not show any significant response to treatment with an anti–PD-1 antibody, despite a high PD-L1 expression, suggesting that additional factors to PD-L1 may influence the therapeutic activity of PD-1 antibody blockade [10]. Thus, components of the tumor microenvironment, such as tumor-infiltrating immune cells, may contribute to immune evasion and therapeutic response to PD-1/PD-L1 inhibitors, suggesting that predictive biomarkers can be obtained by multiparametric profiling of the immune contexture [11]. The PD-1/PDL-1 interaction can inhibit the function and the viability of CD8+ cytotoxic lymphocytes and promote the differentiation of CD4+ T-cells into regulatory T-cells FOXP3+ [12]. Interferon-γ (IFN-γ) signaling activates the PD-1 signaling axis through direct upregulation of PD-L1 in tumor cells, in the immune infiltrate, and stromal cells, which interact with PD-1 on tumor-infiltrating T cells to downregulate their cytotoxic response [13]. Moreover, neutrophils activated by IFN-γ can also inhibit T-cells through PD-L1 expression [12]. However, our understanding of the diversity of PD-L1 expression and changes in the immune microenvironment in *KRAS* mutated LADC remains limited.

In our study, we investigated whether distinct mutant KRAS proteins bearing different amino acid substitutions correlate with immune infiltrates, clinico-pathological variables and patient outcome. We identified heterogeneity in PD-L1 expression and in the immune microenvironment of LADCs harboring different *KRAS* mutations, suggesting that more personalized immunotherapy may be needed for specific subsets of *KRAS* mutated LADC patients.

2. Materials and methods

2.1. Study population

This retrospective study included 219 patients with LADC surgically treated between August 2012 and April 2015 at the Pasteur Hospital, University Côte d'Azur, Nice, France. Tumor specimens were collected from the Hospital-Integrated Biobank BB-0033-00025 (Nice, France), while stored at 4 °C. Tumors included, balanced for the *KRAS* status, were either wild-type (WT) or harbored one of the three most frequent mutations in *KRAS* gene (p.G12C, p.G12D, p.G12 V), as determined by pyrosequencing (therascreen *KRAS* Pyro Kit, Qiagen, Hilden, Germany). They expressed no genomic alterations for the *EGFR*, *BRAF, MET, HER2, ALK*, or *ROS1* genes. Clinical data of patients were collected retrospectively from patients' files (Table 1). Stage IV patients who underwent surgery were oligometastatic patients who underwent multimodal treatment. The study complied with the REMARK recommendations for tumor marker prognostic studies using biological material. The study was approved by the local ethics committee and patients' informed consent was obtained.

Table 1
Patient characteristics for the whole population, the WT *KRAS* and mutant *KRAS* patients.

	Whole Population (n = 219)	WT *KRAS* (n = 111)	Mutant *KRAS* (n = 108)	*P*-value
Gender				
Male	138 (54.8%)	73 (65.7%)	59 (54.6%)	0.413
Female	86 (34.1%)	38 (34.2%)	49 (45.4%)	
Age (mean)		63	64	0.439
Never smoker	30 (16.6%)	20 (18%)	15 (13.8%)	0.613
Former or current smoker	151 (83.4%)	91(82%)	93 (86.2%)	
TNM stage				
I	70 (32,1%)	31 (28%)	39 (36.1%)	0.045
II	40 (18.2%)	30 (27%)	10 (9.3%)	
III	49 (22.4%)	23 (20.7%)	26 (24.1%)	
IV	60 (27.3%)	27 (24.3%)	33 (30.5%)	
KRAS mutation				
G12C	48 (21.9%)	N/A	48 (44%)	N/A
G12D	33 (15.1%)		33 (30.5%)	
G12V	27 (12.3%)		27 (25%)	

2.2. Immunohistochemical staining

Four-µm-thick sequential histological tumor sections were obtained from a representative formalin-fixed, paraffin embedded tumor block and used for immunohistochemistry (IHC) analysis. IHC was performed using an automated staining system (BenchMark ULTRA; Ventana Medical Systems, Tucson, AZ, USA) with antibodies against PD-L1 (clone 22C3, dilution 1:50; Dako Inc., Carpinteria, CA, USA) [14], CD8 (cytotoxic T cell; clone SP57, prediluted; Ventana), FOXP3 (regulatory T cells; clone D6O8R, dilution 1:100, Cell Signaling, Danvers, MA, USA), CD66b (polymorphonuclear myeloid-derived suppressor cells [PMN-MDSCs]; clone G10F5, dilution 1:500; BD Biosciences, San Diego, CA, USA), CD33 (PMN-MDSCs; clone 44M12D3, dilution 1:100; Novus Biologicals, Abingdon, UK). Expression of these proteins was detected using an OptiView Detection kit (Ventana) with a diaminobenzidine reaction to detect antibody labeling and hematoxylin counterstaining. Human tonsil FFPE tissues with and without the primary antibody were used as positive and negative controls, respectively, with each run IHC staining.

2.3. Image analysis

To measure the expression of the different markers with IHC, the stained sections were digitally scanned at x400 resolution using a NanoZoomer 2.0HT (Hamamatsu, Japan) slide scanner. PD-L1 expression was manually measured on tumor and immune cells and quantified as the tumor proportion score (TPS) or immune proportion score (IPS) (Supplementary Table S1). The densities of cells expressing CD8, CD66b, CD33, and FOXP3 were evaluated using CaloPix software (TribvnHealthcare, Châtillon, France) (Supplementary Fig. S1) and quantified in the intra-tumoral compartment (Supplementary Fig. S2). The three senior thoracic pathologists (MI, VH, and PH) who performed the image analysis were blinded to patients' outcome. The densities of cells expressing CD8, CD66b, CD33, and FOXP3 were evaluated using the CaloPix software (Tribvn Healthcare, Châtillon, France) (Supplementary Fig. S1) and quantified in the intra-tumor compartment (Supplementary Fig. S2) [15]. Densities were qualified as high or low depending on the superiority or inferiority to the median density of positive cells/mm^2.

2.4. Statistical analysis

Population characteristics were compared using the Student *t*-test