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Research paper

Significance of immune checkpoint proteins in *EGFR*-mutant non-small cell lung cancer

Ross A. Soo[a,b,d,*,1], Hye Ryun Kim[c,1], Bernadette Reyna Asuncion[d], Zul Fazreen[d], Mohamed Feroz Mohd Omar[d], Maria Cynthia Herrera[d], Joey Sze Yun Lim[d], Grace Sia[d], Richie Soong[d], Byoung-Chul Cho[c]

[a] *Department of Haematology-Oncology, National University Cancer Institute, Singapore, National University Health System, Singapore*
[b] *School of Surgery, University of Western Australia, Perth, Australia*
[c] *Division of Medical Oncology, Yonsei Cancer Center, Seoul, South Korea*
[d] *Cancer Science Institute of Singapore, National University of Singapore, Singapore, Singapore*

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ABSTRACT

Objectives: To characterize the expression of PD-L1, PD-1, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and T-cell immunoglobulin and mucin-domain containing-3 (TIM3) in epidermal growth factor receptor (*EGFR*) mutant non-small cell lung cancer (NSCLC).
Materials and methods: Samples from 90 patients with newly diagnosed advanced stage NSCLC harboring *EGFR* mutations and treated with first line EGFR tyrosine kinase inhibitors (TKI) within 3 months of diagnosis were stained for CTLA-4, PD-L1, PD-1, TIM-3 and CD3 expression by immunohistochemistry.
Results: PD-L1 was present in at least 1% of immune and tumor cells in 44% and 59% of samples, respectively. In multivariate analysis, increased CD3 immune shaped cell (ISC) counts (HR 2.805, $p = 0.034$) and high PD-L1 tumor H-score (HR 3.805, $p = 0.022$) was associated with a shorter progression free survival and high CTLA-4 ISC counts was associated with borderline overall survival significance (HR 1.054, $p = 0.061$).
Conclusion: Tumor PD-L1 expression was significantly associated with a shorter PFS whereas immune cell CTLA-4 may be prognostic for OS. Our findings support the ongoing development of CTLA-4 and PD1/PD-L1 inhibitors in this important molecularly defined subset of lung adenocarcinoma.

1. Background

First-line treatment with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) is now currently standard treatment for patients with advanced stage non-small cell lung cancer (NSCLC) harboring *EGFR* mutations [1]. Resistance however invariably occurs and novel therapeutic approaches are needed. The immune checkpoints molecules, programmed death-1 (PD-1) and its ligand, programmed death-1 ligand (PD-L1), have a key role in evading tumor immunosurveillance [2]. Treatment with monoclonal antibodies targeting the PD-1/PD-L1 pathway has been improve survival compared to docetaxel in the second-line setting in patients with advanced stage NSCLC [3,4]. In some studies, tumor PD-L1 expression was associated with improved efficacy [5,6] whereas PD-L1 expression in immune cells was associated with improved outcomes in others [7,8].

Several reports have highlighted a potential relationship between *EGFR* mutations and the PD-1/PD-L1 axis. PD-L1 expression has been reported to be associated with *EGFR* mutations in NSCLC [9,10]. In murine NSCLC models, EGFR signaling induced by *EGFR* mutations activated PD-L1 expression and induce immune escape, and PD-L1 expression was down-regulated by EGFR TKI treatment [11]. These observations have led to the initiation of studies of PD-1/PD-L1 inhibition in combination with EGFR TKIs [12,13].

In addition to PD-L1 and PD-1, numerous other proteins are involved in the regulation of tumour immunity. Of these, cytotoxic T-lymphocyte antigen-4 (CTLA-4), T-cell immunoglobulin-

* Corresponding author at: Department of Haematology-Oncology, National University Cancer Institute, National University Health System, 1E Kent Ridge Road, NUHS Tower Block Level 7 Singapore.
E-mail address: ross_soo@nuhs.edu.sg (R.A. Soo).
[1] These authors contributed equally to this article.

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Table 1
Clinical and molecular features of patients in the study series.

Variable	Amount (%)
Median Age (range, years)	62 (34–88)
Gender	
Female	64 (71%)
Male	25 (28%)
Smoking status	
Never	69 (77%)
Current/former	20 (23%)
AJCC Stage	
II	1 (1%)
III	4 (4%)
IV	84 (94%)
CNS metastases	
No	34 (38%)
Yes	55 (62%)
EGFR mutation type	
Exon 19 deletion	43 (48%)
L858R	41 (46%)
Other	5 (5%)
Response to EGFR TKI	
Complete Response	1 (1%)
Partial Response	54 (61%)
Stable disease	26 (29%)
Progressive disease	5 (6%)
Unknown	3 (3%)

and mucin domain-3-containing molecule 3 (TIM3) have emerged as potential therapeutic targets [14]. CTLA-4 is expressed on the surface of T cells and acts as a suppressor of T-cell activation. TIM3 is expressed on surface of immune cells that upon ligation, limit the duration and magnitude of Th1 and Tc1 T-cell responses [15]. However, little is known of their expression in human tumors, including NSCLC.

In this study, the expression patterns and prognostic significance of PD-L1, PD-1, CTLA-4 and TIM3 were analyzed in tumors from NSCLC patients with *EGFR* mutations and treated with first-line EGFR TKIs in order to gain insight into the role of these proteins and the potential value of immune checkpoint inhibition in this setting.

2. Methods

2.1. Samples

Tumor samples were obtained from 90 patients with newly diagnosed advanced stage NSCLC harboring *EGFR* mutations and treated with first line EGFR TKI within 3 months of diagnosis at the Yonsei Cancer Centre, Seoul, Korea between 2011 and 2014. The *EGFR* mutation status of the tumors had previously been determined by Sanger sequencing or real-time PCR of exons 18 through 21. The study was approved by institutional review boards.

2.2. Immunohistochemistry

Immunohistochemistry (IHC) staining of 4 μm thick tumor sections was performed using the HRP Bond Refine Detection Kit (Leica, Wetzlar, Germany) according to recommended protocols. To maximize tissue use, double staining was used to examine PD-L1 (rabbit monoclonal antibody SP142 at 1:200 dilution; Spring Bioscience, Pleasanton, CA) and CD3 (mouse monoclonal antibodiy LN10 at 1:50 dilution; Leica), as well as TIM3 (rabbit polyclonal 2E2 at 1:150 dilution; Abcam) and PD-1 (mouse monoclonal antibody NAT105 at 1:100 dilution; Abcam, Cambridge, UK) using DAB brown (Leica) and Vina green (Biocare Medical, Irvine, CA) chromagens respectively for each double stain on the Bondmax autostainer (Leica). CTLA-4 (mouse monoclonal 14D3 at 1:100 dilution; eBioscience, San Diego, CA) was examined as a single IHC stain using DAB on the BondRx autostainer (Leica). Post-treatment steps after each antibody incubation comprised three rinses of Bond-Wash solution and two subsequent polymer incubation steps [16]. Haematoxylin was incubated for ten minutes. Positive and negative controls were included in each run, consisting of control tissue with known expression, and sections stained without primary antibody respectively.

Table 2
Distribution of PD-L1 expression patterns in tumor (TC) and tumor infiltrating immune cells (IC).

	IC0	IC1	IC2	IC3	Total
TC0	34 (41%)	1 (1%)	0 (0%)	0 (0%)	35 (43%)
TC1	1 (1%)	3 (4%)	1 (1%)	0 (0%)	5 (6%)
TC2	13 (16%)	15 (18%)	6 (7%)	0 (0%)	34 (41%)
TC3	1 (1%)	5 (1%)	2 (2%)	0 (0%)	8 (10%)
Total	49 (60%)	24 (29%)	9 (11%)	0 (0%)	82 (100%)

2.3. Immunohistochemistry assessment

Assessment of IHC stains was performed by two pathologists (BRA, MCH) blinded to the results of the study. Manual assessment was performed in tumor and ISCs according to recently described scoring systems [17]. Tumor staining were scored as TC0 if <1% tumor cells had staining, TC1 for ≥1% to <5%, TC2 ≥5% to <50% and TC3 ≥50%. ISCs were scored as IC0 if <1% ISCs had staining, IC1 for ≥1% to <5%, IC2 ≥5% to <10% and IC3 ≥10%. Digital slide imaging assessment was performed using the Vectra slide imaging system and InForm software (Perkin Elmer, Waltham, MA), consistent with methods described previously [18]. The system was trained by the pathologists to segment tumor and intratumoral stroma areas, as well as cell membrane and immune-shaped cells (ISC) in these areas (see Supplementary Fig. S1 in the online version at DOI: 10.1016/j.lungcan.2017.01.008). The assessment of the expression of single proteins in samples with double stains was achieved by spectral filtering for relevant chromagens. Expression in tumor cells was represented by an average H-Score [19], and for immune cells by an average from three standardized image fields selected by the pathologists at 20x magnification.

2.4. Statistical analysis

Assessment of the correlation in scores between manual and digital assessment of PD-L1 was performed by a one-way ANOVA post test for linear trend using Prism Software 7.0 (GraphPad Software, La Jolla, CA). Associations between clinical and molecular features was tested using Spearman's rho test for variables that were both continuous, Mann-Whitney *U* test for continuous and categorical variables, and either Chi-Square analysis or Fisher's exact test (when events were less than 5) for variables that were both categorical. Cox's proportional hazard model method was used to test the association with survival of features on a univariate basis, and as continuous variables where possible. Progression free survival (PFS) was defined as the time from starting treatment with an EGFR TKI until the onset of disease progression or death. Overall survival (OS) was defined as the time from starting treatment with an EGFR TKI until death due to any cause. Patients who had not progressed or died at the time of analysis were censored on the date of last assessment. Survival associations were also assessed for categorical variables using the Kaplan-Meier method, with the log rank test to assess significance. Multivariate analysis was performed through Cox's Proportional Hazard Model method using backward conditional selection. All statistical tests