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Clinicopathologic correlation of programmed death ligand-1 expression in non-small cell lung carcinomas: A report from India☆,☆☆

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ABSTRACT

Introduction: Increased expression of Programmed death ligand-1 (PD-L1) on cancer cells and immune cells predict response to PD-1/PDL1 inhibitors. Data regarding frequency and pattern of PD-L1 expression in NSCLC from India is not available.
Objectives: To analyse PD-L1 expression on tumour cells (TC) and immune cells (IC) and to correlate PD-L1 expression with baseline clinico-pathological characteristics, oncogenic drivers and outcome data.
Materials and methods: PD-L1 expression on tumour cells and immune cells was analysed.
Results: Eighty-nine cases of resected NSCLC were included. Squamous cell carcinoma was more common than adenocarcinoma. IC were present in almost all cases. Immunopositivity for PD-L1 in TC and IC was 27% and 18% respectively. PD-L1 immunopositivity in TC or IC did not correlate with age, sex, stage or mutation status however sarcomatoid carcinoma and solid predominant adenocarcinomas showed higher positivity rates. PD-L1 immunopositivity in ICs was found to correlate with better disease free survival.
Conclusion: PD-L1 immunopositivity was seen in a quarter of NSCLC patients in India. PDL1 positivity on immune cells may be associated with better prognosis in resected NSCLC. However the prognostic value of PD-L1 and clinical response to check point inhibitors in Indian population need to be validated in larger studies.

1. Introduction

Immune escape is considered as an important hallmark of cancer. The current area of interest in lung carcinoma has shifted to immunotherapy targeting the cellular immune checkpoints. The common targets under evaluation are cytotoxic T lymphocyte associated protein-4 (CTLA-4) and Programmed death-1 (PD-1). Binding of PD-1 with its ligand PD-L1 results in inhibition of T cells, leading to reduced immune response against tumour cells. Targeting either PD-1 or PD-L1 stimulates immune response against tumour cells and has demonstrated its superiority in terms of overall survival in comparison to standard chemotherapy in non-small cell lung carcinoma (NSCLC) patients [1-3].

Tumour cells (TC) express PD-L1 through adaptive immune resistance to escape T cell mediated anti-tumour immune response. PD-L1 expression on TC can also be constitutively expressed because of abnormal gene expression or PD-L1 gene amplification [4-8], which may be the reason of variable expression of immune checkpoints in oncogene-addicted NSCLC [9]. PD-L1 expression by TC is both dynamic and heterogenous. The dynamicity is in the form of different levels of expression at different points of time. Also, within the tumour PD-L1 expression is varied (heterogeneous) and has been seen to be higher in areas with increased tumour infiltrating lymphocytes [8].

Although PD-L1 immunohistochemistry (IHC) has not been validated as a predictive biomarker, it has been seen that cases with PD-L1 immunopositivity have better outcome with immunotherapy [10-13]. It has also been suggested that higher PD-L1 expression in tumour cell membrane or tumour infiltrating immune cells (IC) is associated with different clinico-pathologic features and clinical outcome in different

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tumour types [14]. However, the prognostic impact of this biomarker has not been established.

The data on the expression of PD-L1 on surgical resection specimens of lung cancer and especially from this part of the world is limited. In this study, we sought to characterize PD-L1 expression on TC and IC in resected specimens and to correlate it with clinicopathological features, status of oncogenic drivers and survival.

2. Material and methods

The study was both retrospective and prospective. Cases of NSCLC who underwent resection over a period of 7 years (2010–2016) were retrieved from the Institute's archives. Institutional Ethics Committee approval was obtained before data acquisition and tumour staining.

2.1. Clinical details

Detailed clinical history including age, sex, smoking history, receipt of neoadjuvant chemotherapy (NACT), type of resection, size, stage of the tumour and survival data was obtained from clinical files. The clinical files were reviewed and the data was entered into a predesigned proforma. The tumour was staged according to pathological TNM classification and were subgrouped into stage I & II and stage III & IV.

For survival analysis, disease free survival (DFS) and overall survival (OS) was calculated. DFS was defined as the time between diagnosis and clinical/radiological evidence of cancer recurrence (locoregional/distant) or last follow-up. OS was defined as time between diagnosis and death or last follow-up. The survival data was updated from clinical files or by contacting the patient over the telephone. The data was censored on 31st January 2017 or date of last follow up when patient was known to be alive.

2.2. Histopathological examination

Hematoxylin and Eosin (H & E) stained slides were reviewed by two pathologists (AGV and DJ) for confirming diagnosis of NSCLC and further histological subtyping was done according to WHO 2015 [15]. Presence of IC in the form of tumour infiltrating lymphocytes (TIL) and stromal (stroma contiguous to tumour) inflammation (SI) were noted on H & E slides. TIL was defined as immune cells that infiltrated into tumour islands (intraepithelial immune cells) [16]. SI was defined as the immune cells which are present in the stroma surrounding the tumour.

2.3. Evaluation of oncogenic drivers

Adenocarcinomas were evaluated for presence of epidermal growth factor receptor (EGFR) (exons 18, 19, 20 and 21), V-Ki-ras2 rat sarcoma viral oncogene (KRAS) (exon 2) and HER-2/neu (exon 20) mutations by Sanger sequencing while ALK and ROS-1 rearrangement and c-MET overexpression was tested by IHC. FGFR-1 and c-MET IHC was done in squamous cell carcinomas.

2.4. Immunohistochemistry for PD-L1 and oncogenic drivers

To measure PD-L1 expression, we used Rabbit Anti-Human PD-L1/CD274 antibody (Monoclonal Antibody, clone SP142, Spring Bioscience) at a dilution of 1:100.

Briefly, 3-μm sections were deparaffinized, rehydrated, and washed with Tris chloride buffer (pH 7.5). Sections were rehydrated and antigen retrieval was done by boiling in citric acid buffer (pH 6) for 30 min. After antigen retrieval using citrate buffer, endogenous peroxidase activity was blocked using H2O2. The samples were blocked for peroxidase endogenous activity prior to incubation with the primary antibody overnight at 4 °C. Bound antibodies were detected with UltraTek Reagent (ScyTek, Logan, USA). Amplification was performed with 3,3 diaminobenzidine as the chromogen for the purposes of detection along with mild counterstaining with hematoxylin.

During staining of each batch, appropriate positive (human placenta and tonsil) and negative controls (obtained by omitting the primary antibody) were used.

Due to patchy expression of PD-L1, the tumour on the slides being assessed was divided into areas of equal amount of tumour at low magnification and each area was evaluated for PD-L1 positivity. Slides were viewed at high magnification to look for weak membrane staining. The average percentage of positivity from all areas of the tumour was taken for the overall percentage tumour proportion score for PD-L1 positivity in TCs. Normal (alveolar macrophages) and necrotic cells were excluded from scoring. For ICs, the area of tumour infiltrated by PD-L1 expressing ICs was expressed as a percentage [6]. Only stroma that is contiguous to individual tumour nests was included in the tumour-area definition.

Membranous (complete circumferential or partial linear plasma membrane) and cytoplasmic granular staining at any intensity in > 5% tumour cells or immune cells (IC) were considered positive [8,16-20]. PD-L1 expression at the tumour–stroma interface was recorded separately.

Same protocol of IHC staining was followed for other antibodies namely FGF Receptor 1/FGR1 (D8E4, XP Rabbit monoclonal antibody, Cell Signalling), c-MET (SP44, Rabbit Anti-Human C-Met Monoclonal Antibody) and ROS1 (Cell Signalling D4D6).

ALK IHC staining was performed on a Ventana Bench Mark XT automated slide-processing system at Ventana Medical Systems, Inc. (VMSI) using D5F3.

2.5. Mutation analysis for EGFR, KRAS and HER-2/neu

2.5.1. DNA isolation and quantification

DNA extraction was performed using 50-μm-thick sections of formalin-fixed and paraffin-embedded (FFPE) tissue samples. For all samples, morphological review was performed to confirm a tumour cell content of > 75%. The tumour area was determined by comparing it with the corresponding Hematoxylin & Eosin stained slide. A Promega FFPE DNA Tissue extraction kit Reliaprep FFPE Gdna Miniprep System cat. No. A2352 (USA) was used for DNA extraction with slight modifications in manufacturer's instructions. EGFR, KRAS and HER-2/neu mutation analysis was performed using Sanger sequencing.

2.5.2. PCR and sanger sequencing for EGFR (exons 18, 19, 20 and 21), KRAS (exon 2) and HER-2/neu (exon 20)

Primers were designed using NCBI primer designing tool like Primer3 and checked by *insilico* PCR at UCSC genome bioinformatics. The standardized protocol was used to amplify EGFR, KRAS and HER-2/neu genes from the isolated genomic DNA using primers. Forward primer for EGFR exon 18: *CTGGCACTGCTTTCCAGCAT* and reverse primer: *GCTTGCAAGGACTCTGGGCT*. Forward primer for EGFR exon 19: *GCATCGCTGGTAACATCCAC* and reverse primer: *AGATGAGCAGGGTCTAGAGC*. Forward primer for EGFR exon 20: *TGACTCCGACTCCTCCTTTA* and reverse primer: *ATCTCC CTTCCCTGATTAC*. Forward primer for EGFR exon 21: *TGACCCTGAATTCGGATGC* and reverse primer: *ATACAGC TAGTGGGAAGGC*. Forward primer for KRAS (Exon 2): *CGTCTGCAGTCAACTGGAATT* and reverse primer (Exon 2): *GAAACCCAAGGTACATTTCAG*. Forward primer for HER-2/neu (exon 20): *GCCTTTGTCAAATGGGGATAATG* and reverse primer for HER-2/neu (exon 20): *TGGAAGAGTGGGTGGGAAGGG*.

For Sanger sequencing, 3 μl purified PCR product was used. The sequencing was done using both forward and reverse primers for greater accuracy and the results were analyzed using SeqMan II software (DNASTAR).