PD-L1 expression in small cell neuroendocrine carcinomas

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Abstract

Small cell lung cancer and extrapulmonary small cell carcinomas are the most aggressive type of neuroendocrine carcinomas. Clinical treatment relies on conventional chemotherapy and radiotherapy; relapses are frequent. The PD-1/PD-L1/PD-L2 pathway is a major target of anti-tumour immunotherapy. Aberrant PD-L1 or PD-L2 expression may cause local immune-suppression. Here we investigated expression of PD-1 and its ligands by immunohistochemistry and RNA-seq in small cell carcinomas.

PD-L1 and PD-1 protein expression were analysed in 94 clinical cases of small cell carcinomas (61 pulmonary, 33 extrapulmonary) by immunohistochemistry using two different monoclonal antibodies (5H1, E1L3N). RNA expression was profiled by RNA-seq in 43 clinical cases. None of the small cell carcinomas showed PD-L1 protein expression in tumour cells. PD-L1 and PD-1 expression was noticed in the stroma: Using immunohistochemistry, 18.5% of cases (17/92) showed PD-L1 expression in tumour-infiltrating macrophages and 48% showed PD-1 positive lymphocytes (45/94). RNA-seq showed moderate PD-L1 gene expression in 37.2% (16/43). PD-L1 was correlated with macrophage and T-cell markers. The second PD-1 ligand PD-L2 was expressed in 27.9% (12/43) and showed similar correlations.

Thus, the PD-1/PD-L1 pathway seems activated in a fraction of small cell carcinomas. The carcinoma cells were negative in all cases, PD-L1 was expressed in tumour-infiltrating macrophages and was correlated with tumour-infiltrating lymphocytes. Patients with stromal
PD-L1/PD-L2 expression may respond to anti-PD-1 treatment. Thus, evaluation of the composition of the tumour microenvironment should be included in clinical trials. Besides conventional immunohistochemistry, RNA-seq seems suitable for detection of PD-L1/PD-L2 expression and might prove to be more sensitive.

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1. Introduction

Small cell carcinoma is an aggressive neoplasm thought to be arising from neuroendocrine cells, most notably in the lung (small cell lung cancer, SCLC). SCLC represents 13–15% of primary lung cancers [1]. The median survival ranges from 23 months for limited-stage disease to eight months for extensive-stage disease [1]. Clinical treatment remained mainly unchanged for the last three decades and relies on conventional chemotherapy and radiotherapy [2].

A more recent therapeutic strategy is the use of anti-tumour immunotherapy such as blockade of co-inhibitory immune pathways, in particular PD-1/PD-L1. Aberrant activation of co-inhibitory pathways is a key determent of local immune-suppression, and counteracting PD-1/PD-L1 was demonstrated to yield strong and durable tumour regression in several solid tumours [3,4]. Monoclonal antibodies targeting PD-L1 or PD-1 are currently in phase III clinical trials with first positive results [4] in malignant melanoma, lung adenocarcinoma, non-Hodgkin lymphoma, renal cell carcinoma, and triple-negative breast cancer [5–8].

In the context of clinical trials, PD-L1 protein expression on tumour cells detected by immunohistochemistry (IHC) is currently the best predictive biomarker [5–8]. However, responses may occur in patients with PD-L1 IHC-negative tumours, and not all PD-L1 positive cases respond [3,9].

To date, the expression of PD-L1 in SCLC and extrapulmonary small cell carcinomas has not been explored. The aim of this study was to investigate PD-L1 and PD-1 expression patterns in small cell carcinomas and to compare conventional IHC with RNA-seq expression analysis.

2. Patients and methods

2.1. Patients

The study was approved by the local ethics committee. Informed consent was obtained from each patient. Clinical treatment was performed in the context of the molecular diagnostics programme ‘Network Genomic Medicine’, an academic multi-centre non-profit collaboration based at the Center for Integrated Oncology Köln-Bonn (www.ngml.de).

2.2. Sample selection and TMA preparation

Cases were selected from the archives of the Institute of Pathology, University Hospital Cologne, Germany. Formalin-fixed, paraffin-embedded tissue of 61 SCLCs and 33 extrapulmonary small cell carcinomas, including 45 primary tumours and 49 distant metastases (51 resection specimens, 43 biopsies), were arranged as tissue microarrays (Beecher Instruments, Sun Prairie, WI). Three regions of each paraffin-block were sampled with 1 mm core diameter, i.e. 2.36 mm² tissue per case. Each microarray contained tonsil-tissue as on-slide positive control. Thirteen cores containing lung adenocarcinomas were included as additional on-slide control. Thirteen cores containing lung adenocarcinomas were included as additional on-slide control.

Primary diagnoses were verified by three experienced pathologists (R.B., L.O., A.M.S.) according to current recommendations [1]: Morphologic features were evaluated on H&E stained slides, and immunohistochemical standard markers were used in each case (except for three cases in which the paraffin blocks were exhausted): Neuroendocrine markers (Chromogranin A, Synaptophysin, CD56, Neuron-Specific Enolase), Cytokeratins (Pancytokeratin AE1/AE3 or CK7), Ki-67 and TTF1. Cases were selected if positive for one or more neuroendocrine markers, and if the Ki-67 labeling index was \( \geq 60\% \) [10]. Eighty-seven samples (98%) were positive for cytokeratins. Information on the clinical cases are summarised in Supplementary Table 1.

2.3. Immunohistochemistry

PD-L1 immunohistochemistry was performed with either the non-commercial mouse monoclonal antibody 5H1 (kindly provided by Lieping Chen’s laboratory, Yale University, USA), or the commercial rabbit monoclonal antibody E1L3N (Cell Signaling Technology, Cambridge, UK). Both antibodies yielded similar staining patterns and intensities (Supplementary Fig. 1). For PD-1 mouse monoclonal antibody NAT105 (Abcam, Cambridge, UK) was used. Detection was performed on an automated staining system with polymer-based secondary antibody kit and DAB (Leica Bond Polymer Refine; Leica Biosystems, Wetzlar, Germany) (Representative microphotographs: Supplementary Fig. 2). Routine diagnostic immunohistochemistry was performed on an automated staining system according to standardised protocols (Leica Bond).
Three independent pathologists (A.M.S., A.H.S., L.O.) scored PD-L1 and PD-1 protein expression in analogy to the semiquantitative Allred system [11]: Intensity subdivided into four categories (0–3), proportion of PD-L1 positive cells subdivided into six categories (0–5), both values added to yield the Allred-Score (i.e. 0–8). Positivity of the individual cell was defined to be a circular membranous staining pattern. Occasional cytoplasmatic staining was considered unspecific and not scored. Tumour cells and adjacent stroma were scored separately. The stroma was subdivided into three regions: infiltrating cells within the carcinoma tissue, the tumour–stroma interface, and the stroma (Supplementary Table 2). No categorical differences in staining intensity and proportions were noticed among the three experiments.

2.4. RNA-seq data analysis

RNA-seq mRNA expression data are derived from two recent studies on SCLC. [12,13]. Forty-three resected primary SCLC cases and 22 SCLC cell lines were re-analysed in the current study. Paired-end RNA-seq data were processed as previously described. In brief, RNA-seq reads were aligned to the human reference genome (hg19). The transcriptional levels of annotated genes or exons were estimated with cufflinks and represented as FPKM (Fragments Per Kilobase of transcript per Million mapped reads). The maximal gene expression was chosen for those genes with multiple splice variants.

2.5. Statistical analysis

Statistics were performed using SPSS analytical software (IBM). IHC data were tested by $\chi^2$ tests with $\alpha = 5\%$. Correlations were quantified by Spearman’s rank correlation coefficient ($\rho$). RNA-seq data were tested by Wilcoxon rank-sum tests. Alpha was adjusted according to the Bonferroni method to $\alpha = 5\% * 1/n$ to correct for the cumulative error of multiple testing.

3. Results

3.1. PD-L1 protein expression in small cell neuroendocrine carcinomas

Immunohistochemistry for PD-L1 protein expression was performed in three separate experiments (antibody

Fig. 1. PD-1 and PD-L1 are expressed in the tumour stroma of small cell carcinomas. 94 cases of pulmonary small cell carcinomas (A) and extrapulmonary small cell carcinomas (B) did not show any PD-L1 staining in immunohistochemistry (representative microphotographs, 220x magnification, scalebar = 100 μm). However, PD-L1 is expressed in tumour-associated macrophages (C), which stain positive for CD68 (D). PD-1 positive T-lymphocytes were noticed in 48% of cases (E); lymphocytes reacted positive for CD3 epsilon (F).
5H1 twice, E1L3N once). None of the tested small cell lung cancers and extrapulmonary small cell carcinomas stained positive for PD-L1 (Fig. 1A, B; Table 1). The on-slide positive control showed the expected membranous pattern in the stratified tonsil epithelium in each staining (SI Fig. 2), and eight of the 13 spiked-in adenocarcinomas were positive (Allred scores 4–7; SI Table 2). No differences in intensity were noticed between the two different PD-L1 antibodies.

While the neoplastic cells were completely negative, 18.5% of the cases showed PD-L1 positive cells in the adjacent stroma (17/92): Cells with monocytic morphology located at the interface between neoplastic cells and desmoplastic stroma showed faint to moderate (intensity scores 1 or 2) circular membranous staining patterns (Fig. 1C). These cells were positive for the monocyte/macrophage IHC-marker CD68 (Fig. 1D) and were considered tumour-associated macrophages (TAMs).

The cases were subdivided by localisation of the primary tumour, origin of the tissue, and type of material (SI Table 3). No major differences were noticed among the subgroups, except for origin of the tissue: 13 of 17 cases with stromal PD-L1 expression were distant metastases, marking a significant enrichment ($p = 0.034$).

Table 1
Summary of Small Cell Cancer Specimens and IHC PD-1/PD-L1. (A) Summary of the tissue samples analysed. Ninety-four specimens of small cell neuroendocrine carcinomas were analysed on tissue microarrays. The samples included pulmonary and extrapulmonary tumours, primary tumours and metastases. Both resection and biopsy materials were included. (B) Summary of PD-1 and PD-L1 protein expression as determined by immunohistochemistry. None of the tumours showed PD-L1 or PD-1 protein expression. However, expression of both proteins was found in the stroma.

| A | Sample                | Pulmonary | 61 (65%) |
|   | Extrapulmonary        | 33 (35%) |
|   | Total                 | 94       |
| Origin | Primary           | 45 (48%) |
|       | Metastasis           | 49 (52%) |
|       | Total                | 94       |
| Specimen | Resection   | 51 (54%) |
|         | Biopsy              | 43 (46%) |
|         | Total               | 94       |

PD-1 PD-L1

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$p$(PD-1, PD-L1): 0.35

Fig. 2. RNA expression of PD-1, PD-L1 and PD-L2 in 43 clinical small cell lung cancer (SCLC) cases and 22 SCLC cell lines (A) (Normalised expression displayed as Fragments Per Kilobase of transcript per Million mapped reads, FPKM). Correlations were noticed between PD-L1 and the monocyte marker CD68 (B), as well as the common T-lymphocyte gene CD3E (encoding the epsilon chain of the CD3 receptor complex) (C). Subdividing the samples by PD-L1 expression into high (FPKM $\geq$ 1.5, expressed) and low (<1.5, not expressed) showed significant differences in the indicated genes (*). Expression of PD-L1 was not related to proliferation-related gene Mki67 (D).
3.2. PD-1 protein expression in small cell neuroendocrine carcinomas

The tissue microarrays were stained for PD-1 protein expression in a separate experiment. Again, on-slide tonsil tissue served as positive control (SI Fig. 2).

Variable levels of tumour-infiltrating lymphocytes (TILs) were noticed and 48% cases (45/94) showed PD-1 positive TILs (Fig. 1E, F; SI Tables 2 and 3). The staining intensity was usually moderate (intensity 2) and the TILs were most frequently located at the interface between carcinoma cells and stroma. Few cases showed TILs located between the carcinoma cells. The TILs were positive for CD3 (epsilon chain). Correlations between PD-L1+ TAMs and PD-1+ TILs were noticed; among all cases the correlation was 0.35 (Spearman’s rho) and ranged from 0.21 (primary carcinomas) to 0.56 (extrapulmonary carcinomas) in the subgroups (SI Table 3).

3.3. PD-L1, PD-L2 and PD-1 gene expression

RNA-seq expression data were analysed in a set of 43 primary SCLCs. Transcript levels of the PD-1 receptor and its ligands PD-L1 and PD-L2 were not highly abundant, with most samples revealing low or almost no expression (FPKM < 1.5). However, 23% (10/43) of the samples showed moderate PD-1 mRNA expression levels (FPKM > 1.5), while PD-L1 and PD-L2 were expressed in 37% (16/43) and 28% (12/43), respectively (Fig. 2A; Supplementary Fig. 3A, Supplementary Tables 4 and 5). Subdividing the samples by PD-L1 expression into PD-L1 high (FPKM > 1.5; expressed) and low (FPKM < 1.5, relating to almost no expression) showed differences in expression of the macrophage marker genes CD68 (Fig. 2B), CD163, CD11c and CSF1R (SI Fig. 3), and the common T-lymphocyte marker (epsilon chain) (Fig. 2C). This was similarly observed for PD-L2 and PD-1 (SI Fig. 3). The differences were significant in Wilcoxon-tests adjusted by the Bonferroni-method for multiple testing (p < 0.05x(1/11) = 0.0045). PD-1/PD-L1/PD-L2 expression was not associated with differences in the proliferation-related gene MKI67 (which corresponds to Ki-67 in IHC) (Fig. 2D, SI Fig. 3B). Twenty-two SCLC cell lines were likewise analysed and no expression of the investigated genes was noticed with two exceptions: CD68 was expressed in 36% (8/22) cell lines, and NCI_H196 cells were found to express PD-L2 (Fig. 2, SI Fig. 3, SI Table 5).

In four of the RNA-seq samples, sufficient tissue was available for IHC. IHC for PD-L1 was supplemented, and no protein expression was noticed in the neoplastic cells in all four cases. However PD-L1 positivity was noticed in monocytic cells in the stroma of two of the cases (data not shown).

4. Discussion

Tissue microarrays with 94 cases of SCLC and extrapulmonary small cell carcinomas were analysed by immunohistochemistry (IHC) for expression of PD-L1. Unexpectedly, none of the tumours showed PD-L1 protein expression in the tumour cells. However, expression of PD-L1 was noticed on tumour-associated macrophages (TAMs), while tumour-infiltrating lymphocytes (TILs) were positive for PD-1. Consistently, mRNA expression of PD-L1 and PD-L2 was detected in association of macrophage and T-cell markers by RNA-seq.

The success of blocking co-inhibitory pathways has sparked new interest in anti-tumour immunotherapy. Many solid tumours were found to express the B7-protein PD-L1 [7], which interacts with the PD-1 receptor and causes T-cell anergy. In non-small cell lung cancer, PD-L1 expression was found in up to 40% of cases [14]. Our study is the first to investigate PD-L1 in small cell lung cancer (SCLC).

A common clinical course of disease in SCLC is good initial response to chemoradiotherapy, followed by relapse few months later. The end of chemoradiotherapy might be a window of opportunity for consecutive immunotherapy. Chemotherapy stimulates anti-tumour immune response [15], and an additional boost by immunotherapeutics might help eradicate residual tumour cells, increasing the duration of the remission. On the other hand, immunologic paraneoplastic syndromes are not uncommon in SCLC [16]. It might be sensible to screen for SCLC-associated autoantibodies in possible clinical trials to reduce the risk of adverse effects. Of importance, brain metastases from SCLC are frequent and require immunosuppressive corticosteroids for management, which might interfere with immunotherapies. In clinical trials with PD-1/PD-L1 inhibitors some patients responded with strong tumour-regression and excellent outcomes, while others failed to respond [3–8]. Many parameters of the neoplastic tissue and peripheral blood were tested for their predictive value to develop companion diagnostic tests for the new drugs. Currently PD-L1 expression on the tumour cells is the best predictive marker [8], however, the test characteristics (sensitivity, specificity) of PD-L1 IHC have not been evaluated comprehensively.

In our small cell carcinoma cases no PD-L1 expression was found on the neoplastic cells, and the cases would be classified ‘negative’ along this line. However, the presence of PD-L1+ TAMs and PD-1+ TILs indicates that the pathway may be activated in 20–35% of cases: PD-1 is expressed on CD4+ and CD8+ T-lymphocytes upon antigen receptor signalling [16].

The PD-L1/PD-1 correlation is in concordance with a recent study by Taube et al., which demonstrated a geographic association between PD-L1 expression and the presence of PD-1+ TILs in five different types of
solid tumours [8]. Taube et al. showed that both PD-L1 expression in tumour cells, and the presence of PD-1+ TILs, have predictive value for anti-PD-1 therapy.

We would like to suggest the hypothesis that patients with small cell carcinoma and stromal PD-L1/PD-L2 expression might respond to anti-PD-1 therapy. Clinical trials should include a celltype-specific analysis of the tumour microenvironment to investigate the significance of PD-L1 expression in tumour-associated cells. Response to anti-PD-L1 therapy seems less likely given that PD-L2 might still activate PD-1.

RNA-seq and IHC yielded highly comparable results in the present study. Using IHC, macrophages as origin of the PD-L1 expression could be directly observed. In RNA-seq PD-L1 and PD-L2 mRNA expression was correlated to macrophage and T-cell expression signatures. RNA-seq of 22 SCLC cell lines confirmed that the employed marker genes are not expressed by SCLC and can be attributed to cells in the stroma of the tumour tissue (except for CD68). RNA-seq yielded more PD-L1 positive cases than immunohistochemistry (35.7% versus 18.5% of cases). The result is not surprising given that tissue microarrays were used for IHC, while RNA-seq was performed on larger tumour sections of resection specimens. TAMs and TILs often show heterogeneous distributions in and around the neoplastic tissue and might be underrepresented in tissue microarrays. Furthermore, the sequencing depth yielded by RNA-seq might be more sensitive than conventional IHC. Thus, guided by the knowledge of IHC–protein expression patterns, RNA-seq or similar high-throughput techniques might be used to screen for PD-L1 expression.

Conflict of interest statement

AMS received travel grants from Roche. RB is a founder and co-owner of Targos Molecular Pathology, Inc. (Kassel, Germany), he received research support from Pfizer and honoraria for Advisory Board contributions from Roche, Boehringer Ingelheim, AstraZeneca, Merck, Lilly, Merck-Serono, Bayer Healthcare and Qiagen. JW has served as an advisory board member of Novartis and AstraZeneca and has received research support from Novartis and honoraria from Novartis and AstraZeneca. RKT is founder and shareholder of Blackfield AG, patent applications: genotype-specific drug discovery/assays and biomarkers, he received research support from AstraZeneca, EOS and Merck, is consultant for Blackfield, K&J, received honoraria from Roche, Boehringer Ingelheim, AstraZeneca, Merck, Lilly, Sanofi-Aventis, Merck KgaA, Bayer, Puma, MSD and Clovis and travel grants from Pfizer. JG received consulting fees from Blackfield AG. All other authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2014.12.006.

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