



## Large cell neuroendocrine lung carcinoma induces peripheral T-cell repertoire alterations with predictive and prognostic significance



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### ABSTRACT

**Objectives:** This study was performed to evaluate for a potentially important role of T cells in the pathophysiology and treatment sensitivity of large cell neuroendocrine lung carcinoma (LCNEC), an orphan disease with poor prognosis and scarce data to guide novel therapeutic strategies.

**Materials and methods:** We performed T-cell receptor (TCR)  $\beta$ -chain spectratyping on blood samples of patients treated within the CRAD001KDE37 trial (n = 35) using age-matched current or former (n = 11) and never smokers (n = 10) as controls. The data were analyzed in conjunction with the complete blood counts of the probands as well as the data about response to treatment and overall survival in the clinical trial.

**Results and conclusion:** Untreated stage IV LCNEC patients had significant T-cell repertoire alterations (p < 0.001) compared to age-matched smokers. These changes correlated positively with blood lymphocyte counts (r = 0.49, p < 0.01), suggesting antigen-induced T-cell proliferation as the causative mechanism. At the same time, LCNEC patients showed mild lymphopenia (1.54 vs. 2.51/nl in median, p < 0.01), which reveals a second, antigen-independent mechanism of systemic immune dysregulation. More pronounced T-cell repertoire alterations and higher blood lymphocyte counts at diagnosis were associated with a better treatment response by RECIST and with a longer overall survival (441 vs. 157 days in median, p = 0.019). A higher degree of T-cell repertoire normalization after 3 months of therapy also distinguished a patient group with more favourable prognosis (median overall survival 617 vs. 316 days, p = 0.036) independent of radiological response. Thus, LCNEC induces clinically relevant changes of the T-cell repertoire, which are measurable in the blood and could be exploited for prognostic, predictive and therapeutic purposes. Their pathogenesis appears to involve antigen-induced oligoclonal T-cell expansions superimposed on TCR-independent lymphopenia.

### 1. Introduction

Large-cell neuroendocrine lung carcinoma (LCNEC) is a rare

pulmonary neoplasm (2–3%) first described in 1991 with poor prognosis and limited treatment options [1,2]. Currently actionable genetic alterations, like EGFR mutations or ALK translocations, are exceedingly

**Abbreviations:** LCNEC, large cell neuroendocrine lung carcinoma; TCR, T-cell receptor; CDR, complementarity-determining region; HD, Hamming distance; OS, overall survival; V $\beta$ , variable region of the T-cell receptor beta-chain

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uncommon in these patients [3]. Platinum-based chemotherapies have shown some activity, but outcomes remain poor with a median overall survival (OS) below one year for metastatic disease [4–7]. Due to the rarity of LCNEC, data to guide patient treatment or the development of novel therapeutic strategies are scarce [8]. In an effort to improve outcome, we recently explored the mTOR inhibitor everolimus in combination with carboplatin and paclitaxel as first-line treatment for stage IV LCNEC within the CRAD001KDE37 phase 2 trial (registered as EudraCT 2010-022273-34 and NCT01317615) [9]. Here, we analyze the antigen receptor diversity of circulating T cells in these patients in order to screen for an important immunologic component in the pathophysiology and treatment sensitivity of LCNEC. Spectratyping of the T-cell receptor (TCR)  $\beta$ -chain serves this purpose well, because it offers a comprehensive view of the entire T-cell compartment with relatively simple experimental procedures, low costs and easy data analysis [10]. Its basic measure is the complementarity-determining region (CDR)-3 length distribution of the TCR $\beta$  chains, which is Gaussian in naive T-cells, but becomes distorted during immune responses due to clonal expansions bearing CDR3 sequences specific for epitopes of the inciting antigens [11]. T cells are the most abundant lymphoid subset in the blood of healthy adults and lung cancer patients, accounting for 65–88% and 64–69% of circulating lymphocytes respectively [12,13].

## 2. Materials and methods

### 2.1. Patients and control probands

Included in this analysis were all stage IV LCNEC patients treated within the CRAD001KDE37 study with available PAXgene blood RNA samples at diagnosis (n = 35) and after 3 months of treatment (24/35 patients) as well as two age-matched control groups: one consisting of current and former smokers similar to the LCNEC patients (n = 11) and one of never-smokers (n = 10, Table 1). All probands provided written informed consent. The design, ethical approval, treatment regimen and outcome of the CRAD001KDE37 study have been reported elsewhere [9]. Response to treatment according to RECIST v1.1 [14] after 3 months (i.e. after four cycles of carboplatin/paclitaxel every 3 weeks combined with 5 mg everolimus daily) and overall survival (OS) from the treatment start were endpoints of the clinical study and available for all patients. Complete blood counts were available for 34/35 patients at baseline, for 26/35 patients at 3 months and for all control

donors from certified diagnostic laboratories by measurement of EDTA-anticoagulated blood samples drawn at the same time as the PAXgene tubes (Qiagen, Hilden, Germany). RNA was isolated using the PAXgene Blood RNA Kit, which together with the PAXgene Blood RNA Tubes is FDA-cleared for *in vitro* diagnostics (Qiagen).

### 2.2. TCR $\beta$ spectratyping and data analysis

For TCR $\beta$  spectratyping, 500 ng of quality-controlled RNA (by measurement of the RNA integrity number with the Agilent 2100 Bioanalyzer, Agilent, Santa Clara, CA, Table 1) were reverse transcribed using oligo-dT and random hexamer primers (Transcriptor First Strand cDNA Synthesis Kit, Roche, Mannheim, Germany) followed by polymerase chain reaction (PCR) amplification for 40 cycles with primers specific for each of the 24 TCR $\beta$  variable region (V $\beta$ ) families (see supplementary material) [11]. PCR products were FAM-labeled by nested primer extension (see supplementary material) and analyzed by capillary electrophoresis (3100-Avant Genetic Analyzer and GeneScan Analysis Software v3.7, Applied Biosystems, Foster City, CA, USA). The relative frequency of transcripts with a specific CDR3 length in each spectratype profile was estimated by the area under the corresponding peak compared to the total area under the curve, which were both extracted from the raw data with the PeakScanner v2.0 software (ThermoFisher Scientific, Waltham, MA, USA). In order to quantify perturbations, a standard procedure was followed [15,16]: first a reference distribution was formed for each V $\beta$  family from the spectratype profiles of never-smokers by averaging the frequencies at each CDR3 length; subsequently, the Hamming distance (HD) of every other spectratype profile was calculated by subtracting its frequency at each CDR3 length from the frequency of the reference distribution for the given V $\beta$  family, and by subsequently adding the absolute values of these differences divided by two. The HD across all 24 V $\beta$  families were averaged for each proband (HDa) and compared between proband groups using a linear model with group as a fixed and V $\beta$  family as a random effect (SPSS v24, IBM Corp., Armonk, NY, USA).

### 2.3. Reverse transcription quantitative PCR (RT-qPCR)

For quantification of perforin (*PRF1*), granzyme B (*GZMB*) and Ki-67 (*MKI67*) expression in blood RNA of LCNEC patients and controls, RT-qPCR was performed in technical triplicates with internal plate

**Table 1**  
Age, smoking status, blood cell counts, RNA sample quality and spectratyping results of the study probands.

	LCNEC patients after 3 months of treatment (n = 24 <sup>a</sup> )	p-value <sup>b</sup>	LCNEC patients at diagnosis (n = 35 <sup>a</sup> )	p-value <sup>b</sup>	Current and former smokers (n = 11)	p-value <sup>b</sup>	Never- smokers (n = 10)
Age, years (mean $\pm$ SD)	61 $\pm$ 8		62 $\pm$ 9		65 $\pm$ 12		62 $\pm$ 13
cigarette pack-years (mean $\pm$ SD)	45 $\pm$ 18		44 $\pm$ 17		30 $\pm$ 20		0 $\pm$ 0
% positive smoking history	100%		100%		100%		0%
% current smokers	42%		49%		36%		0%
Lymphocytes (/nl, median;IQR)	1.44; 0.97	** <sup>d</sup>	1.54; 0.87		2.51; 1.70	ns <sup>e</sup>	2.59; 1.65
Neutrophils (/nl, median;IQR)	2.84; 1.67 <sup>c</sup>		5.75; 2.38	** <sup>e</sup>	4.82; 2.33	ns <sup>e</sup>	3.92; 1.33
Monocytes (/nl, median;IQR)	0.03; 0.03 <sup>c</sup>		0.6; 0.38	** <sup>e</sup>	0.49; 0.08	** <sup>e</sup>	0.36; 0.07
Eosinophils (/nl, median;IQR)	0.05; 0.05 <sup>c</sup>		0.2; 0.24	ns <sup>e</sup>	0.14; 0.08	ns <sup>e</sup>	0.09; 0.12
Basophils (/nl, median;IQR)	0.02; 0.02 <sup>c</sup>		0.04; 0.05	ns <sup>e</sup>	0.06; 0.03	** <sup>e</sup>	0.03; 0.02
Lymphocyte-to-neutrophil ratio (median;IQR)	0.57; 0.32 <sup>c</sup>		0.26; 0.19	*** <sup>e</sup>	0.62; 0.62	ns <sup>e</sup>	0.69; 0.37
RNA integrity number (mean $\pm$ SD)	8.7 $\pm$ 0.5		8.7 $\pm$ 0.5		8.9 $\pm$ 0.3		8.8 $\pm$ 0.2
HDa (% , mean $\pm$ SD)	20.5 $\pm$ 0.04	ns <sup>f</sup>	21.1 $\pm$ 0.04		16.6 $\pm$ 0.02		(reference)

SD: standard deviation; IQR: interquartile range; ns = p > 0.05; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001.

<sup>a</sup> At baseline, blood RNA samples were available for 35/35 and complete blood counts for 34/35 LCNEC patients. At 3 months, blood RNA samples were available for 24/35 and complete blood counts for 26/35 LCNEC patients.

<sup>b</sup> Each p-value refers to the comparison of data in the two adjacent columns.

<sup>c</sup> No comparison was performed, because patients were under myelosuppressive treatment.

<sup>d</sup> Obtained with the Wilcoxon matched-pairs signed rank test.

<sup>e</sup> Obtained with the Mann-Whitney test.

<sup>f</sup> Obtained using a linear model with proband group as a fixed and V $\beta$  family as a random effect; p < 0.001 for the comparison between LCNEC patients after 3 months and smokers.

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