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REPORT

CXCL12 expression is a bona fide predictor of recurrence in lung neuroendocrine tumours; a multicentric study with emphasis on atypical carcinoids - a short report

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Abstract

Purpose Neuroendocrine tumors of the lung (LNETs) encompass a heterogeneous group of lesions, including tumors with no or low metastatic potential, such as typical (TCs) and atypical (ACs) carcinoids, and highly aggressive neuroendocrine carcinomas. To date, only a few biomarkers with prognostic impact have been identified in LNETs. Previous experimental studies have suggested that the cytokine CXCL12 might have a role in stratifying the outcome of lung cancer as well as LNET patients. However, the reliability of immunohistochemical (IHC) tissue expression of CXCL12 in evaluating the prognosis of resected LNETs is currently not known.

Methods Here, we subjected a cohort of 112 resected LNETs specifically enriched for ACs to IHC for CXCL12 and Ki67 using routine procedures. The clinical value of CXCL12 was assessed by applying the Cox proportional-hazards model to overall and disease-free survival rates.

Results We found that CXCL12 was expressed in 8.3 to 38% of LNETs, depending on the different diagnostic categories. Upon survival analysis, when considering the whole cohort, we found that CXCL12-positive cases exhibited shorter disease-free survival rates compared to CXCL12-negative cases. Among ACs, tumors overexpressing CXCL12 showed significantly shorter disease-free survival rates. Finally, we found that the Ki67 index in ACs was higher in the CXCL12-positive cases.

Conclusion CXCL12 immunohistochemistry may serve as a potentially useful tool to better stratify LNETs, and more specifically ACs, in clinical practice.

Keywords Lung neuroendocrine tumours · Carcinoids · Prognostic biomarkers · CXCL12

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1 Introduction

Neuroendocrine tumors of the lung (LNETs) account for approximately 25% of primary lung neoplasms. These tumors are highly heterogeneous, ranging from well-differentiated tumors (i.e., low-grade typical carcinoids - TCs - and intermediate-grade atypical carcinoids - ACs) to poorly differentiated tumors (i.e., small cell carcinomas – SCLCs - and large cell neuroendocrine carcinomas - LCNECs), which are by far the most frequent ones (92%) [1]. Due to the increased availability and performance of imaging techniques, the detection of TCs and ACs has increased over the past few years [2].

The management of LNETs represents a clinical challenge given their diversity in clinical presentation and outcome [2]. Although carcinoid tumors generally show a good prognosis, ACs posses metastatic potential, thus making their early detection and correct classification crucial. However, the current

diagnosis of TCs and ACs remains strictly morphology-based. According to the World Health Organization (WHO), TCs and ACs should be distinguished on the basis of histopathologic features, including cell size, cell morphologic features, mitotic index, architectural growth patterns and the presence of necrosis [1]. Despite the updated 2015 WHO classification providing guidance on the use of the Ki67 index, no immunohistochemical (IHC) markers have been found to be reliable in predicting prognosis in ACs. In order to improve the clinical management of LNETs, additional tools to better stratify these patients are required.

Recently, we [3, 4] and others [5] have provided evidence on the prognostic impact of U3 small nucleolar ribonucleoprotein protein (IMP3) and $p16^{Ink4A}$ expression in LNETs. These biomarkers may represent promising adjuncts to the current diagnostic markers. Interestingly, a fraction of LNETs has recently been shown to express cytoplasmic and/or membranous CXCL12. Tumors with highest CXCL12 expression levels were found to have a worse prognosis [6]. This chemokine protein is constitutively expressed in the lung and, with its receptor C-X-C chemokine receptor type 4 (CXCR4), has been proposed to serve as a bona fide driver of non-small cell lung cancer (NSCLC) tumor progression [7]. Specifically, the CXCR4 receptor has been found to stimulate the growth of metastases at sites where its ligand, CXCL12, is present in large quantities. Recent lines of evidence suggest that in LNETs [8], as in other malignancies [9, 10], CXCL12 and CXCR4 form a functional network and signal to mTOR. In vitro, these proteins have been found to take part in the control of transcriptional, morphologic and functional modifications, which result in enhanced osteotropism of LNET cells. In particular, CXCL12 has been shown to be overexpressed in LNETs, with a particularly high intensity in SCLCs compared to carcinoid tumors [7]. The currently available data on the role of this chemokine in LNETs encompass, however, a very limited number of ACs.

Here, we sought to define the reliability of CXCL12 IHC as a prognostic biomarker in LNET patients. In this hypothesis-testing study, we retrospectively analyzed a multi-institutional set of LNETs, specifically enriched for the carcinoid subgroups.

2 Materials and methods

2.1 Patients, tissue specimens and tissue microarrays construction

A total of 112 patients who underwent surgery at Fondazione IRCCS Ca′ Granda - Ospedale Maggiore Policlinico (Milan, Italy), Città della Salute e della Scienza - Ospedale Molinette, (Turin, Italy), or San Gerardo Hospital (Monza, Italy) for LNET between 2000 and 2015 were enrolled in this study. This non-consecutive cohort included 21 LCNECs, 12 SCLCs, 46 TCs and 33 ACs after enrichment for carcinoids. The patients' clinicopathologic features are listed in Table 1. None of the patients received adjuvant or neoadjuvant therapy. Follow-up data were available in 86 cases. Archival formalin-fixed paraffin-embedded (FFPE) tissue blocks of all cases were retrieved from the respective pathology archives, with prior anonymization by a pathology staff member not involved in the study, to construct 5 tissue microarrays (TMAs). For each case, four samples from the tumor core and one sample from non-neoplastic lung tissue were incorporated into the TMAs using a semi-automatic arrayer (Alphelys Minicore2, Plaisir, France), with a diameter of 3 mm for each core as previously described [3, 4].

Table 1 Clinicopathological characteristics of the patient series

Histotype (n)	Mean Age Years	Sex Male	OS Alive	DFS No rec	CXCL12 Positive	T	N	M	Resection status R0
TC (46)	57	10	45	43	4	T1 = 32 T2 = 10 T3 = 3 T4 = 1	N0 = 42 N1 = 2 N2 = 2	M0 = 46	46
AC (33)	58.6	13	21	26	5	T1 = 18 T2 = 12 T3 = 2 T4 = 1	N0 = 22 N1 = 7 N2 = 4	M0 = 33	33
LCNEC (21)	61.2	9	6	5	8	T1 = 9 T2 = 11 T3 = 0 T4 = 1	N0 = 12 N1 = 6 N2 = 3	M0 = 21	21
SCC (12)	71.6	8	4	n/a	1	T1 = 5 T2 = 4 T3 = 3	N0 = 5 N1 = 2 N2 = 4 N3 = 1	M0 = 12	biopsies

2.2 Immunohistochemical analyses

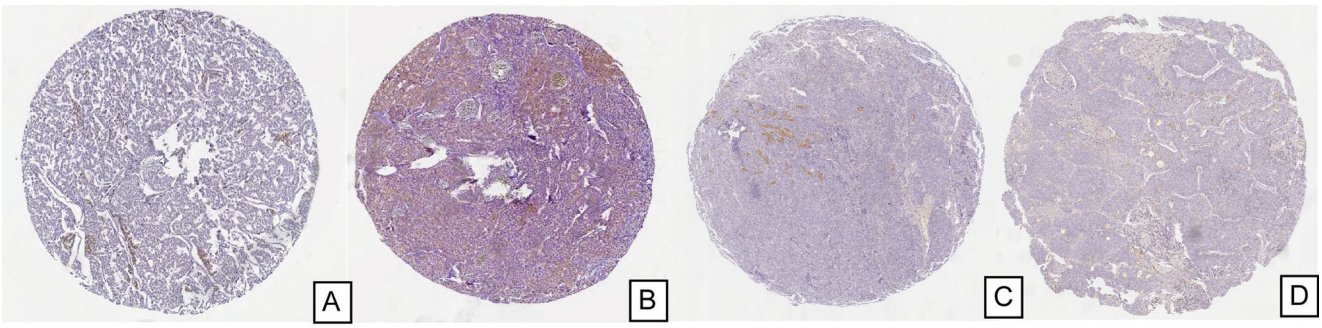
Consecutive 4 μm-thick sections were cut from each TMA block and subjected to IHC staining for CXCL12 (R&D Systems, clone #79018, 1:50) and Ki67 (Roche, clone 30–9) using an automatic BenchMark XT system (Ventana Medical Systems). Reactions were revealed using UltraViewTM Universal DAB, a biotin-free, multimer-based detection system, according to the manufacturer's instructions. Positive and negative controls were included in each run. Immunostaining was independently evaluated by two pathologists (ADG and SF). All discordant results were resolved on a multiheaded microscope. CXCL12 positivity was defined as strong cytoplasmic staining observed in more than 1% of the tumor cells. In all cases, the stromal compartment was unreactive. The Ki67 labeling index was assessed according to the percentage of tumor cells showing any degree of nuclear positivity.

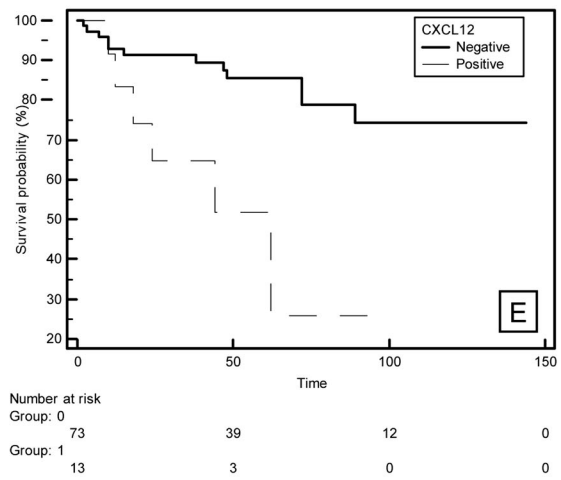
2.3 Statistical analysis

Associations with overall survival (OS) and disease-free survival (DFS) were assessed using the Cox proportional-hazards model. Hazard ratios (HR) and corresponding 95% confidence intervals (CIs) were also indicated. Survival curves were built according to the Kaplan-Meier method. A p value < 0.05 was considered statistically significant. Cut-off levels to cluster patients into low- or high-expression groups were generated using receiver operating characteristic (ROC) curves with a non-arbitrary criterion derived from the Youden (J) index. The J index is defined as sensitivity plus specificity minus 1. All analyses were performed using MEDCALC ® (MedCalc Software, Ostend, Belgium). Data were expressed as means ± standard deviation (SD), median (range) or n (%), as appropriate.

3 Results and discussion

Diagnosis, classification, and choice of appropriate treatment regimens for LNETs are matters of great controversy, particularly in TCs and ACs [11]. While aggressive treatment regimens have shown benefits in TCs and ACs, a proportion of these patients are at risk for disease progression [2, 12, 13]. To date, no biomarkers have been implemented in the clinic to properly stratify these patients and to allow for tailor-made





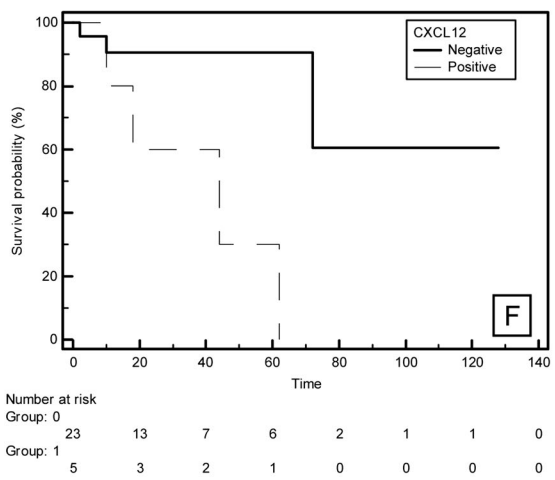


Fig. 1 Representative immunohistochemical micrograph of CXCL12 staining in (**a**) a typical carcinoid (TC) scored as 5%, (**b**) an atypical carcinoid (AC) scored as 40%, (**c**) a large cell neuroendocrine carcinoma (LCNEC) scored as 15% and (**d**) a small cell carcinoma scored as 5% positive. Original magnifications 50×. (**e**) Kaplan-Meyer disease-free survival (DFS) curve for the complete series ($p = 0.002$) and (**f**) for atypical carcinoids (ACs) only ($p = 0.004$)

management strategies. In this proof-of-principle study, we characterized the expression and clinical significance of the chemokine protein CXCL12 in LNETs, with a particular focus on TCs and ACs. We were able to readily detect CXCL12 expression by IHC at a low magnification (100×) in the cytoplasm of neoplastic cells, as illustrated in Fig. 1 (panels A-D). Overall, we found that 18 (16.1%) cases were positive, including 4/46 (8.7%) TCs, 5/33 (15.1%) ACs, 8/21 (38%) LCNECs and 1/12 (8.3%) SCLCs. No significant differences in the percentage of positive cells were observed among the different diagnostic groups.

Upon survival analysis, when considering all LNETs together, we found that the CXCL12-positive cases exhibited shorter DFS rates (HR 3.99 [95% CI 0.91–17.35] $p = 0.002$) compared to the negative cases (Fig. 1e). Interestingly, when considering the AC group alone, we found that the CXCL12-positive cases showed a significantly poorer prognosis. Indeed, ACs expressing CXCL12 exhibited a significantly shorter DFS compared to the CXCL12-negative cases (HR 6.55 [95% CI 0.90–47.37] $p = 0.004$) (Fig. 1f). After detailed analyses, no correlations with T or M stage were noted, both in the whole series and when considering ACs alone, using univariate Cox proportional hazards regression analyses. We found that a higher nodal status (N stage) correlated with a shorter DFS in the whole series ($p = 0.04$), but not in the AC subgroup ($p = 0.29$). When performing multivariate analysis considering the node status and CXCL12 expression in the whole series, both parameters retained a statistically significant association with DFS ($p = 0.03$ for N2 stage cases and $p = 0.0009$ for CXCL12-positive cases). No correlation between increased CXCL12 expression and the OS rate was observed in the study group. Finally, we set out to determine the optimal cut-off value for CXCL12 expression and to investigate the presence of a correlation between survival and CXCL12 expression in ACs. However, no statistically significant values were observed using ROC curves or the Youden index associated criterion (J Index). The Ki67 proliferation index was found to be higher in CXCL12-positive ACs compared to the negative cases as expected, with mean values of 10% ($n = 5$) and 7.5% ($n = 28$), respectively.

CXCL12 is a well-known prognostic biomarker in NSCLCs, with expression rates that reach 80% for squamous cell carcinomas and 25% for adenocarcinomas [14, 15]. There are several lines of evidence suggesting that a high expression of CXCL12 may be associated with an increased risk of death in patients with esophagogastric and lung cancer [15]. On the contrary, in breast cancer patients high levels of CXCL12 expression seem to indicate an OS advantage [16]. To the best of our knowledge, this is the first attempt to investigate the prognostic role of tissue CXCL12 expression by IHC in a multicenter cohort of surgically resected LNETs. Our findings provide evidence to suggest that the expression of this small cytokine may serve as a bona fide risk indicator of recurrence in ACs. In particular, we found that CXCL12 expression is a poor prognostic factor in terms of DFS for all LNETs. Next, we decided to focus on ACs and to assess whether CXCL12 expression could be used to further stratify this enigmatic group of LNETs. We found that CXCL12 may serve as a bona fide negative prognostic biomarker in ACs. Indeed, the CXCL12-positive ACs showed a six times greater risk of recurrence than the negative cases, although with a wide 95% confidence interval. The Ki67 proliferative index has previously been found to show substantial differences among the three LNET categories. Marchio et al. reported that the majority of LNETs (61.5%) showed a Ki67 index of < 4%, while 25 and 13% of them showed a proliferation index of 4–9% and ≥ 10%, respectively [17]. We observed a provisional Ki67 index difference between the CXCL12-positive (10%) and CXCL12-negative (7.5%) ACs. Given the retrospective nature of our study cohort, the role of CXCL12 as a prognostic biomarker in LNETs needs further validation. Our work does, however, provide novel information on CXCL12 expression in LNETs and should be considered a proof-of-principle study. Additional large multicentric prospective studies coupled with high-throughput molecular analyses are warranted.

In conclusion, our data indicate that CXCL12 expression analysis by IHC can potentially be employed to improve LNET risk stratification, thus representing a step forward in the follow-up management of these patients and the realization of potential precision medicine regimens.

Compliance with ethical standards

Disclosure The authors declare no conflict of interest.

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