

# A Comprehensive Analysis of Markers for Neuroendocrine Tumors of the Lungs Demonstrates Estrogen Receptor Beta to be a Prognostic Markers in SCLC Male Patients

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## Abstract

Knowledge about the biology and prognostic markers of neuroendocrine tumours (NET) of the lungs is scarce. As sex steroids contribute to cell proliferation in various human tissues, we aimed at characterising the expression of steroid receptors in a broad cohort of probes from NET of the lungs and evaluated their possible prognostic impact. A total of 192 tumour specimens of NET of the lungs were collected, with 58 surgical specimens of typical carcinoids, 42 of atypical carcinoids, 32 of large cell neuroendocrine carcinomas and 60 of small cell lung cancer (SCLC). Analysis by immunohistochemistry for the following antigens was performed: estrogen receptor  $\beta$  (ER $\beta$ ), ER $\alpha$ , PR (progesterone receptor), AR (androgen receptor), Thyroid transcription factor 1 (TTF1), synaptophysin and for chromograninA. ER $\beta$  was found to be expressed in more than 60% of tumor probes. Survival data were available from 126 patients. In SCLC patients a subgroup analysis showed a survival benefit in the group of male patients whose tumours expressed ER $\beta$  ( $p=0.008$ ) and all SCLC patients whose tumours expressed both ER $\beta$  and chromograninA ( $p=0.02$ ). Here we could demonstrate that ER $\beta$  expression is frequent through all NET of the lungs. Moreover, our results suggest that ER $\beta$  represents a favourable prognostic factor for SCLC male patients and all SCLC patients whose tumors co-expressed chromogranin A.

**Keywords:** Estrogen receptor; SCLC; Neuroendocrine tumors of the lung

## Introduction

Primary neuroendocrine tumours (NET) of the lungs include small cell lung cancer (SCLC), large cell neuroendocrine carcinomas (LCNEC), atypical carcinoids (AC), and typical carcinoids (TC). These tumors share the expression of neuroendocrine markers but present different biological features concerning e.g. mitotic rate, development of metastases and prognosis. The knowledge about the biology and the prognostic markers of lung NETs is scarce and in particular it is still unclear which tumour growth factors or pathways have significant prognostic impact for each tumour subtype. It is well known that sex steroid hormones play important roles in various human tissues contributing to cell proliferation through the interaction with intracellular receptors. To this regard, previous studies showed that a majority of non-small cell lung cancer (NSCLC) express estrogen receptors (ERs) [1-3] and that their growth can be stimulated by estrogen in nude mouse xenografts [4]. Moreover, the activation of the ER pathway has been shown to contribute to the development and progression of NSCLC and in vitro studies showed that its inhibition leads to tumor regression [5-7]. Androgens in androgen-receptor (AR) positive SCLC cell lines have been shown to elicit proliferative effects that could be counteracted by anti-androgens like flutamide or cyproteroneacetate [8,9]. Progesterone receptor (PR) and ER $\alpha$  have

been described to be expressed in a small cohort of patients with lung NET underlying the challenges for differential diagnosis between these tumours and metastases of neuroendocrine tumours from other origin [10]. Based on these data, we aimed to characterize the protein expression of the relevant steroid receptors in a multicentre cohort of lung NET tissue samples from surgical specimens and evaluated their possible use as prognostic markers.

## Materials and Methods

### Patient selection and Tissue Microarray (TMA) construction

Formalin-fixed and paraffin-embedded tissue blocks from surgical specimens of patients suffering from lung NET between 1993 and 2007 were retrieved from the archives at the Institutes of Surgical Pathology, University Hospital of Zurich, City Hospital Triemli Zurich, and Technical University Munich. All cases were reviewed by two pathologist (AS and DS) on full sections of a representative tumor block and classified following the definitions of the current 2004 WHO classification for each subtype of lung NET. In particular, carcinoid tumors were divided into typical and atypical carcinoid based on the number of mitosis per 2 mm<sup>2</sup> [11]. A TMA was built using 2 tissue cores of 0.6 mm diameter for each patient in a Ventana ES instrument (Ventana Medical Systems, Baar, Switzerland) as previously described [12]. Additional cores of control tissue, including

normal lung, normal tonsils as well as neuroendocrine tumors of uterus, ileo-caecum and appendix were added in duplicates at the end. The study was approved by the Institutional Ethical Review Board of the University Hospital Zurich under reference number StV 29-2009.

### Immunohistochemistry

For immunohistochemical (IHC) staining, 4-µm-thick paraffin sections were cut from the TMA block and mounted on silane-coated glass slides. The sections were stained on an automated IHC platform (Ventana Benchmark). The following antibodies were used for ERα (clone SP1, prediluted, Ventana, Roche), for ERβ (clone 14C8, dilution 1:150, Genetex), for AR (clone F39.4.1 Biogenex), for PR (clone 1E2, prediluted, Ventana, Roche), for TTF1 (clone 8G7G3/1, dilution 1:50, Dako), for Synaptophysin (clone 27G12, dilution 1:50, Novocastra) and for Chromogranin A (CgA) (clone DAK-A3, dilution 1:50, Dako). Neuroendocrine markers were analysed following the last WHO classification [11]. Biopsies were considered as positive for TTF-1 when >50% of tumor cells were stained. To determine the expression frequencies of ERβ, ERα, PR and AR the percentage of positive cells was recorded and cases with nuclear staining in ≥ 5% of cells were considered as positive for the respective antibody. The cut-off of 5% was chosen based on data from breast cancer patients. These patients benefit from an anti-estrogen therapy if ERβ is expressed in at least 5% of malignant cells [13]. As for SCLC no data concerning the prognostic or predictive value of ERβ have been shown to date, no further scoring system was applied to our cohort of patients. Each core was evaluated independently by two observers (A.C. and D.S.). Cases with less than 50 evaluable cells were excluded for evaluation.

### Clinical data and survival analysis

The following patient characteristics were collected: age at diagnosis, gender, histological subtype, stage at diagnosis and survival. Staging evaluation was based on the adjusted AJCC, TNM 7th edition for all tumor subtypes [14,15]. The Kaplan-Meier method was used to estimate tumor specific overall survival (OS). Tumor specific OS was determined from the date of histological diagnosis of NET of the lungs to the date of death from the same cancer up to 5 years follow-up. Patients who were alive at time of the last control or died from an unrelated death were considered as censored for OS analysis. Categorical data were compared between groups using chi-square test or Fisher's exact test when appropriate. Cox-regression was used to adjust the effect of ER beta for confounders. Continuous data were compared between groups using the Mann-Whitney test. Statistical analyses were performed using IBM SPSS Statistics 20.0 (SPSS Inc, Chicago IL).

### Results

#### Study population

A total of 192 NET tumours of the lung from 92 women and 100 men were included in the TMA and comprised 60 surgical specimens of SCLC, 32 of LCNEC, 42 of AC and 58 of TC. For patients' characteristics (Table 1). Immunohistochemical analysis of all markers was available for 51/60 SCLC, 25/32 LCNEC, 32/42 AC and 44/58 TC (Table 2). Data concerning the stage of disease was available from 47/60 SCLC, 32/32 LCNEC, 40/42 AC and 56/58 TC. Patients' age varied between 16 and 79 years (average of 56y).

Subtype	Stage								Gender (f/m)	Age mean (std dev)	Total
	IA	IB	IIA	IIB	IIIA	IIIB	IV	n.a.			
SCLC	9	11	8		11	1	7	13	19/41	62 (9)	60
LCNEC	9	6	4	3	6	2	2	0	10/22	63 (9)	32
AC	15	5	12	1	7			2	28/14	52 (15)	42
TC	56							2	35/23	48 (16)	58

**Table 1:** Clinical data: staging is reported according to the AJCC 7th edition. n.a.: not available; small-cell lung cancer (SCLC), large-cell neuroendocrine tumors of the lung (LCNEC), atypical carcinoids (AC), typical carcinoids (TC).

#### Expression of neuroendocrine and lung specific markers

Neuroendocrine markers were detected at high frequency in all tumour subtypes: Synaptophysin in 96%, CgA in 75% (both with cytoplasmatic staining) and TTF1 in 63% of all tumour samples (with nuclear staining), the latter in line with previously reported data [16,17]. At least two markers were expressed in each sample. For detailed expression data in each subtype (Table 2).

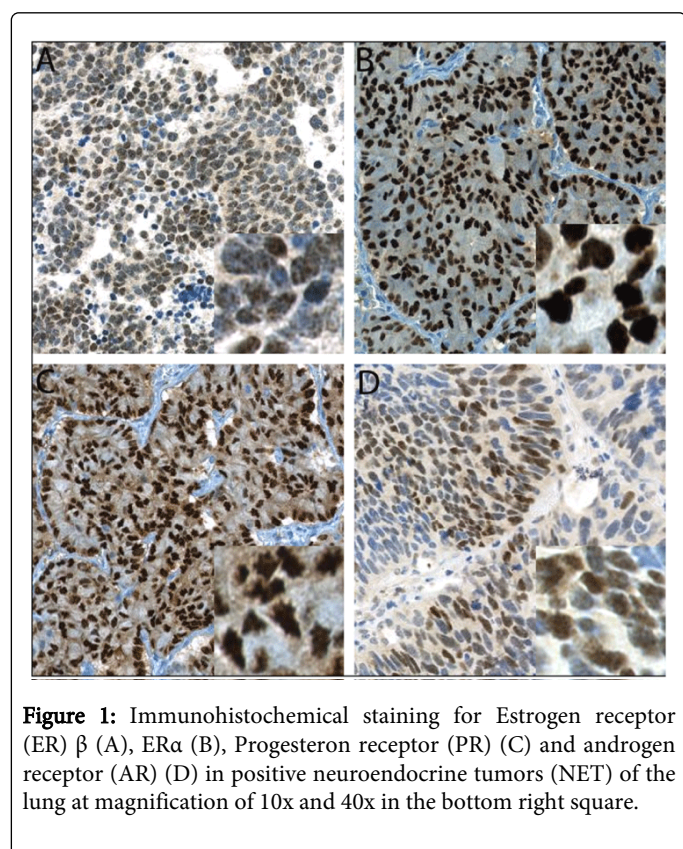
#### Expression of steroid receptors

ERβ was detected in 30/53 SCLC, 17/27 LCNEC, 21/33 atypical carcinoid and 31/47 typical carcinoid samples (Table 2 and Figure 1a)

with nuclear with or without cytoplasmatic expression. ERα was detected in 2/58 TC (Table 2 and Figure 1b) with unique nuclear expression. Progesterone receptor was expressed in 7/53 SCLC, in 4/27 LCNEC, in 3/33 AC and in 7/47 TC, here only nuclear expression was found (Table 2 and Figure 1c). Androgen receptor was expressed in 1/53 SCLC, in 2/27 LCNEC and in 1/33 AC. All 47 TC cases were negative; expression was detected in the nucleus and cytoplasm (Table 2 and Figure 1d). For all markers the percentage of positive malignant cells in the tumor samples was ≥ 5%.

Subtype	N	Neuroendocrine markers and steroid receptors expression						
		Synaptophysin	CgA	TTF1	ERβ	ERα	PR	AR
		pos (%)	pos (%)	pos (%)	pos (%)	pos (%)	pos (%)	pos (%)
SCLC	51	48 (94%)	25 (49%)	39 (76%)	30 (59%)	0 (0%)	7(14%)	1 (2%)
LCNEC	25	23 (92%)	16 (64%)	23 (92%)	15 (60%)	0 (0%)	4 (16%)	2 (8%)
AC	32	32 (100%)	31 (96%)	15 (46%)	21 (66%)	0 (0%)	3 (9%)	1 (3%)
TC	44	44 (100%)	43 (97%)	20 (45%)	30 (68%)	2 (2%)	6 (14%)	0 (0%)
total	152	147 (96%)	115 (75%)	97 (63%)	96 (63%)	2 (1%)	20 (13%)	4 (3%)

**Table 2:** Immunohistochemical analysis in lung NET subtypes. Only cases with data for all markers are described. Chromogranin A (CgA); Thyroid transcription factor 1 (TTF1); Estrogen Receptor (ER); Progesteron receptor (PR); Androgen receptor (AR). Small-cell lung cancer (SCLC), large-cell neuroendocrine tumors of the lung (LCNEC), atypical carcinoids (AC), typical carcinoids (TC).



**Figure 1:** Immunohistochemical staining for Estrogen receptor (ER) β (A), ERα (B), Progesteron receptor (PR) (C) and androgen receptor (AR) (D) in positive neuroendocrine tumors (NET) of the lung at magnification of 10x and 40x in the bottom right square.

### Association of estrogen receptor β expression with gender, neuroendocrine and lung specific markers

ERβ expression was analysed with regard to gender, Synaptophysin, CgA and TTF1 and no statistically significant associations were found (resp.  $p=0.12$ ,  $p=0.3$ ,  $p=0.29$ ,  $p=0.12$  and  $p=0.13$ ) (Table 3).

### Association of estrogen receptor β expression and survival

Survival data were available for 126 patients. Survival analysis of all patients showed a significant difference in survival between tumours subtypes in accordance to the literature, with a 5-years tumour related

survival of 18% for SCLC, of 20% for LCNEC, of 96% for AC, of 96% for TC. Further survival analysis was performed only for ERβ, as the number of tumour samples positive for ERα, AR and PR was not sufficient for statistical analysis. Neither marker nor gender did represent an independent prognostic marker for any tumour subtype. However, a subgroup analysis on SCLC patients, showed a survival benefit in the subgroup of male patients whose tumours expressed ERβ ( $p=0.008$ ) compared to male and female (figure 2 resp. b and a) and a benefit in SCLC patients whose tumours expressed both ERβ and CgA ( $p=0.02$ ) (Figure 2 resp. b, d). This results were valid for SCLC but not for other tumour types. Moreover, in the subgroup of male SCLC patients, a multiple Cox-regression demonstrated that ERβ expression is associated with improved survival (hazard ratio (HR) 0.16, 95% CI 0.05 to 0.54) independently from age (HR 1.09, 95% CI 1.03 to 1.15) and CgA (HR 1.10, 95% CI 0.44 to 2.2). Stage could not be included as variable in the multiple Cox-regression, due to the paucity of cases in advanced stage.

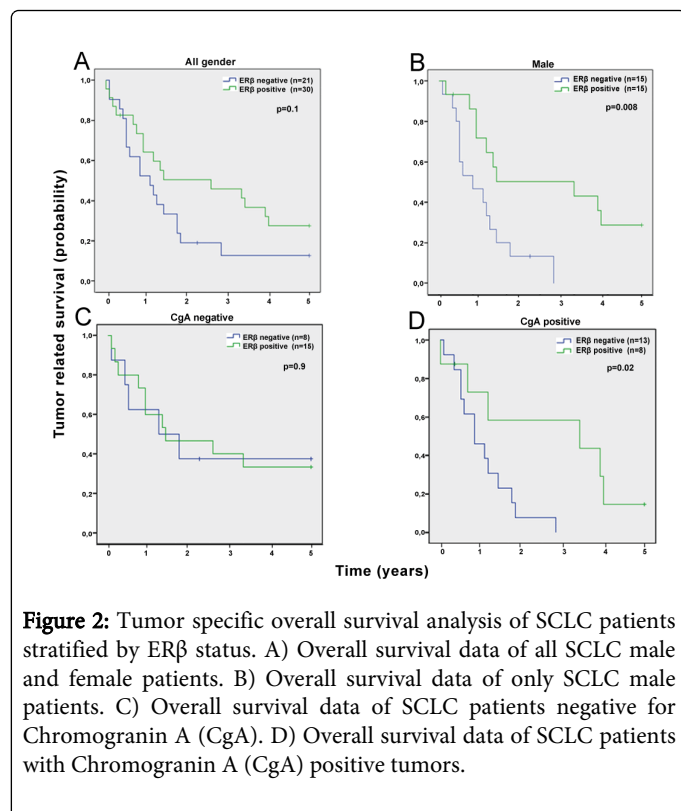
ERβ	N	Male/Female	Synaptophysin (+)	CgA (+)	TTF1 (+)
ERβ pos	96	54/42	92/96 (95%)	71/96 (74%)	62/96 (64%)
	56	30/26	55/56 (98%)	44/56 (78%)	35/56 (62%)
ERβ neg					
p value		0.12	0.29	0.12	0.13

**Table 3:** Analysis of all tumor types for estrogen receptor (ER) ERβ expression associated with gender, Synaptophysin, Chromogranin A (CgA) and Thyroid transcription factor 1 (TTF1)

### Discussion

Knowledge about markers with biological relevance for NET of the lung is scarce. In our cohort of 192 NET, we detected a protein expression of ERβ in 63% of cases of all subtypes, while ERα, PR and AR expression was rare (resp. 1%, 13%, 3%). The reason for frequent ERβ expression and the mechanisms of signalling in cancer are not entirely understood, especially in NSCLC, where data on ERs

expression remain controversial. In NSCLC, where several pathways have been explored, ER can interact directly with EGFR, leading to transcription of target genes [18,19], but the role of an activated ER $\beta$ /EGFR pathway in SCLC is to date unknown. Although EGFR expression has been described in up to 22% of tumor samples from SCLC patients [20-22], we detected only one case out of 51 (2%) expressing EGFR (the analysis was performed according to Pirker [23], data not shown). With this suggest that alternative mechanisms are involved in the carcinogenesis of SCLC.



**Figure 2:** Tumor specific overall survival analysis of SCLC patients stratified by ER $\beta$  status. A) Overall survival data of all SCLC male and female patients. B) Overall survival data of only SCLC male patients. C) Overall survival data of SCLC patients negative for Chromogranin A (CgA). D) Overall survival data of SCLC patients with Chromogranin A (CgA) positive tumors.

Of relevance is that ER $\beta$  expression in our patients represents a favourable prognostic marker for SCLC. In particular this is true for two subgroups of patients: male patients and all patients whose tumors co-expressed ER $\beta$  and CgA.

The different prognostic impact of ER $\beta$  between the male and female patients might reflect the double role of estrogens in carcinogenesis, as estrogens can stimulate tumor growth [24] but also have a protective role through estrogen-receptor- $\beta$ -mediated mechanisms and stimulation of the immune system [25]. In women, the good prognostic role of ER $\beta$  might be masked due to higher estrogens levels compared to male patients. Concerning the positive outcome of the subgroup of patients whose tumors co-expressed ER $\beta$  and CgA, this result might reflect the fact that these tumors are more differentiated. In fact CgA is more frequently elevated in well-differentiated tumors compared to poorly differentiate ones [26]; nevertheless, CgA alone does not represent a prognostic marker, as previously reported. This moreover underlines the importance of this marker in this disease and opens new perspectives for future studies. Importantly, this is the first report of the prognostic role of ER $\beta$  in SCLC and a confirmatory analysis is planned on a larger cohort of patient, as our results suggest a functional role of ER $\beta$  in SCLC which is worthwhile to further investigate.

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