CHAPTER 5

SOX antibodies in small cell lung cancer and Lambert-Eaton myasthenic syndrome

frequency and relation with survival

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Abstract

Introduction SOX1 antibodies are common in small-cell lung carcinoma (SCLC) with and without paraneoplastic syndrome (PNS) and can serve as serological tumour marker. Addition of other antibodies might improve its diagnostic power. We validated an ELISA to assess the diagnostic value of serum antibodies in SCLC and Lambert-Eaton myasthenic syndrome (LEMS). Clinical outcome with respect to SOX antibodies was evaluated, as the SOX-related anti-tumour immune response might help to control the tumour growth.

Patients and methods We used recombinant SOX1, SOX2, SOX3, SOX21, HuC, HuD or HelN1 proteins in an ELISA to titrate serum samples and validated the assay by western blot. We tested 136 consecutive SCLC patients, 86 LEMS patients (43 with SCLC), 14 patients with SCLC and PNS (paraneoplastic cerebellar degeneration or Hu syndrome), 62 polyneuropathy patients and 18 healthy controls.

Results Our ELISA was equally reliable as western blot. 43% of SCLC patients and 67% of SCLC-LEMS patients had antibodies to one of the SOX or Hu proteins. SOX antibodies had a sensitivity of 67% and a specificity of 95% to discriminate between LEMS with SCLC and non-tumour LEMS. No difference in survival was observed between SOX positive and SOX negative SCLC patients.

Conclusions SOX antibodies are specific serological markers for SCLC. Our assay is suitable for high throughput screening, detecting 43% of SCLC. SOX antibodies have diagnostic value in discriminating SCLC-LEMS from non-tumour LEMS, but have no relation to survival in patients with SCLC.

Introduction

Lung cancer is amongst the most frequent forms of cancer and is the most prevalent cause of cancer-related mortality in women and men.[1, 2] Small cell lung cancer (SCLC), representing 13-20%[3, 4] of lung cancers, is associated with aggressive clinical course and poor long term outcome.[4]

Searching for serological markers, SOX proteins were identified as immunogenic antigens in SCLC.[5] SOX proteins belong to the Sry-like high mobility group superfamily of developmental transcription factors[6] and can be subdivided in several families. SOX-B1 (SOX1, SOX2 and SOX3), SOX-B2 (SOX14 and SOX21) and SOX-C (SOX4, SOX11 and SOX12) families are thought to be important for neurogenesis. SOX-B1 may prevent differentiation of neural progenitor cells.[7] SOX21 is thought to repress SOX-B1 and promote differentiation.[8] Antibodies to SOX proteins were identified in 22-28% of SCLC patients,[9, 10] and in 64% of SCLC patients with Lambert-Eaton myasthenic syndrome (LEMS).[9]

LEMS is a disease of the neuromuscular junction, characterized by proximal muscle weakness, areflexia and autonomic dysfunction.[11, 12] It is caused by antibodies directed against P/Q-type voltage gated calcium channels (VGCC) in the presynaptic nerve terminal.[13] The same VGCCs are expressed by SCLC, suggesting that autoimmunization by the tumour is the cause of paraneoplastic LEMS.[14]

Patients with SCLC may also have antibodies against other antigens, like the Hu-family of DNA-binding proteins. Specific clinical syndromes, including paraneoplastic encephalomyelitis and paraneoplastic sensory neuronopathy have been associated with high titre Hu antibodies.[15] Low titre serum Hu antibodies, without accompanying clinical paraneoplastic syndrome (PNS), were found in 16% of SCLC patients.[16] Clearly, SCLC is an immunogenic tumour eliciting different serum antibody responses. Testing for the presence of one or more serum antibodies could provide a sensitive screening tool for early detection of SCLC. Aside from its diagnostic use, these antibodies might also identify patients with a more indolent tumour behaviour: SCLC patients with LEMS have a better prognosis than SCLC patients without LEMS.[17-19] Similarly, anti-Hu-antibodies have been associated with better prognosis in SCLC patients,[16] although this was not confirmed in other studies.[20, 21]

We recently reported a phage display technique to test for the presence of SOX1 antibodies.[9] High throughput screening purposes will require a more amenable assay method.[22] In this study, we developed and validated an ELISA to

test for both SOX and Hu antibodies. We assessed its diagnostic value in SCLC patients with or without LEMS, and studied the relation of these antibodies with survival, tumour stage and response to chemotherapy.

Materials and Methods

Patients

Sera from 136 consecutive Dutch SCLC patients, drawn before treatment, were described before.[19] Survival was noted as primary outcome measure, while tumour stage at diagnosis and response to chemotherapy were considered secondary outcomes.

Serum from 86 patients with LEMS (43 with SCLC, 43 without tumour) was ascertained from a nationwide Dutch study.[23] Internationally accepted diagnostic criteria for LEMS were used.[19] For paraneoplastic LEMS, histologic or cytologic diagnosis of SCLC was mandatory. LEMS was considered idiopathic (NT-LEMS) when no tumour was detected after at least three years follow-up after diagnosis of LEMS and screening had been adequate.[24] After obtaining informed consent, we interviewed and examined all patients using a structured checklist. We recorded demographic and clinical features related to LEMS and SCLC and reviewed clinical records. In 15 patients, who could not visit our outpatient clinic, we studied clinical records and interviewed the responsible neurologist.

Further, sera were obtained from nine VGCC-positive SCLC patients with paraneoplastic cerebellar degeneration (PCD), five SCLC patients with anti-Hu syndrome, 62 patients with polyneuropathy and 18 healthy controls. Patients with polyneuropathy were screened for monoclonal gammopathy as part of our regular work-up. None had macroglobulinemia, paraproteins or an accompanying tumour. All sera were obtained during studies approved by medical-ethical committees.

Cloning of target genes

The coding region of human SOX2, SOX21, HuC, HuD and HelNI were amplified from either a total human muscle, a human fetal brain or a human cerebellum cDNA preparation via a nested primer PCR (For primers see supplemental data: Table S1). The use of the second primer pair introduced a 5'-prime *Nde*I and a 3'-prime *Xho*I restriction site that allowed direct cloning in the pET28b expression vector (EMD Biosciences, Novagen Brand, Madison, U.S.A.). Gel-purified PCR products of expected size were ligated in the TOPO-II blunt vector (Invitrogen, Breda, the Netherlands) and sequenced (LGTC, Leiden, the Netherlands).

Sequence-verified clones were digested with *Nde*I and *Xho*I and cloned into the pET28b vector, resulting in N- and C-terminally His_6-tagged proteins. SOX1 and SOX3 genes were synthesized *in vitro* (Mr Gene, Regensburg, Germany) after optimization of the DNA sequence for efficient mammalian and *E. coli* protein expression, with a 5'-prime *Nde*I and a 3'-prime *Xho*I restriction site for direct cloning into pET28b.

Expression and purification of SOX and Hu target proteins

The pET28b constructs were transformed in E. coli strain BL21(DE3)-RIL (Stratagene, La Jolla, U.S.A.) and production was induced according to standard procedures. In short, cells were grown at 37ºC to an OD_{600} of approximately 1.2 and production was induced with 1mM of IPTG for 4 hours. Cells were harvested in Bugbuster Mastermix (EMD Biosciences, Novagen Brand, Madison, U.S.A.) and incubated at room temperature for 20 minutes. The soluble fraction was separated from the insoluble fraction, which contained the target proteins, by centrifugation. Next, the insoluble fraction was solubilised in 8M urea, debris was removed by centrifugation and the target proteins were purified from the supernatant on the Profinia (Biorad, Veenendaal, the Netherlands) using IMAC cartridges (Biorad, Veenendaal, the Netherlands) according to the instructions of the manufacturer.

ELISA for detection of SOX and Hu specific antibodies

With recombinant SOX1, SOX2, SOX3, SOX21, HuC, HuD and HelN1 proteins, four serum samples with either SOX1 or HuD antibodies and two negative control sera were used to develop an ELISA. Dilution series of sera and coated antigen were performed to determine optimal signal to noise ratios and low background signals.

Purified His_6-tagged proteins were coated overnight at 4ºC on Maxisorp 96 wells plates (Nunc, Roskilde, Denmark) at a concentration of 2µg/ml in PBS. Next, wells were washed twice with PBS and blocked for one hour with 3% Marvel (skimmed milk powder) in PBS (mPBS). Blocked wells were incubated for one hour at room temperature with 1000x diluted patient serum. Thereafter, wells were washed four times with PBST [0.05% (v/v) Tween20 in PBS] and incubated for one hour with 0.1µg/ml mouse anti-human total IgG monoclonal antibody (Nordic Immunological Laboratories, Tilburg, the Netherlands). Wash steps were repeated and wells were incubated for one hour with 0.25µg/ml HRP-conjugated rabbit anti-mouse polyclonal antibody (Dako Netherlands B.V., Heverlee, Belgium). Next, wells were incubated with o-phenylenediamine (OPD) solution [3.7mM OPD; 50mM

Na_2HPO_4; 25mM citric acid; 0.03% (v/v) H_2O_2]. After 5 minutes the reaction was stopped with 1M H_2SO_4 and absorbance of each well was measured at 490nm using a Synergy HT microplate reader (Biotek, Winooski, U.S.A.).

Western blot for detection of SOX and Hu specific antibodies

Purified proteins were run on a 12% poly-acrylamide gel and transferred to PVDF membrane (Millipore, Amsterdam, the Netherlands). Membranes were incubated with 1000x diluted serum for one hour and subsequently washed five times with PBST. Antibodies recognizing the antigens on blot were detected with subsequent incubations of 0.1µg/ml mouse anti-human IgG monoclonal antibody, and 0.5µg/ml HRP-conjugated rabbit anti-mouse polyclonal antibody. As positive control, antigens were detected by their His_6-tag with 1000x diluted anti-His6 monoclonal antibody 27E8 (Cell Signaling Technology, Danvers, U.S.A.), followed by incubation with HRP-conjugated rabbit anti-mouse polyclonal antibody. Bound antibodies were visualized by enhanced chemo luminescence (GE Healthcare, Den Bosch, the Netherlands).

Cross-reactivity assay of SOX and Hu antibodies

We performed dilution series to determine the limiting dilution to use as starting concentration for depletion of SOX or Hu reactive antibodies. For depletion, diluted sera were incubated for one hour at room temperature in wells coated with 4µg/ml of the respective antigen. The non-bound fraction was transferred to wells coated with 4µg/ml SOX1, SOX2, SOX3, SOX21, HuC, HuD or HelN1, and further processed as described before. A serial dilution of the original serum was performed simultaneously on wells coated with 4µg/ml of the respective antigen and used as reference to calculate the amount of depletion of the respective antibodies.

Cut-off points between positive and negative ELISA signals

Each 96-wells ELISA plate contained sera in duplo from eight healthy controls. Mean titre and standard deviation were calculated. Sera from patients were defined as definitely positive if serum titre exceeded an OD value of 0.5. Sera were delineated as probably positive if titres exceeded an OD of 0.1 and in addition were above three standard deviations (SD) of normal sera. Titres were classified as negative if values were below three SD or below an OD value of 0.1. All samples with an OD value higher than the mean of healthy controls plus two SD and below

0.5 were measured by western blot, as well as a randomly chosen selection of other sera. In case of contradictory results, western blot was decisive.

Statistical analysis

Comparisons between groups were performed with Mann Whitney U test or Fisher's exact test when appropriate. Survival was determined from date of SCLC diagnosis to death. The relation of antibody status and survival was analyzed using Kaplan Meier plots and Log rank test. Statistical analysis was performed using SPSS 14.0 (Statistical Product and Services Solutions, Chicago, IL).

Results

Validity ELISA

We developed an ELISA with recombinant SOX1, SOX2, SOX3, SOX21, HuC, HuD and HelN1 proteins. To test the accuracy of the newly developed assay, 79 sera were analyzed blindly, containing 22 sera previously tested positive on SOX1 reactivity.[9] ELISA was 100% accurate in identifying all known SOX1 reactive sera. All but one positive sera gave signals on all four SOX proteins or all three Hu proteins, respectively. Western blot verified ELISA results (Figure 1). Interestingly, four additional sera that had previously been tested SOX1 and HuD negative, gave positive ELISA signals, albeit with low titres. In three of these sera, western blot confirmed the ELISA. The fourth sample was classified as low positive by ELISA for HuD and HelN1, but remained negative by western blot.

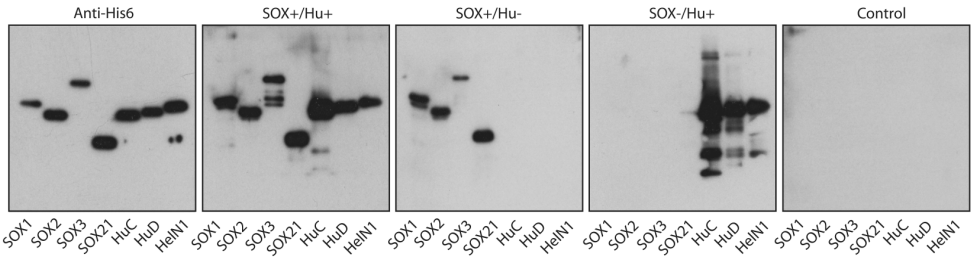

Figure 1 Western blot of SOX+/Hu+, SOX+/Hu-, SOX-/Hu+ and SOX-/Hu- sera, shown together with a positive control blot (anti-His6 antibody), which detects the His6-tagged recombinant proteins. Sera specifically recognize the respective proteins on western blot, which were positive in the ELISA, while no signal is obtained with SOX or Hu negative sera.

Cross-reactivity

There is high sequence homology between SOX proteins of different families. Therefore, cross-reactivity was studied by depleting three SOX positive sera from either SOX1 (SOX-B1 protein family member) or SOX21 (SOX-B2 protein family member) specific antibodies. After depletion, sera were tested for reactivity for SOX proteins (Supplementary data: Figure S1). SOX1 depletion resulted in almost complete loss of reactivity to all SOX proteins, while vice versa depletion with SOX21 resulted in only partial depletion of SOX-B1 activity. No cross-reactivity was observed between SOX and Hu family proteins (data not shown).

Frequency of SOX and Hu antibodies

SOX1, SOX2, SOX3 or SOX21 antibodies were found more frequently in SCLC patients with LEMS (67%) and with PCD (67%), than in SCLC patients without known PNS (36%; $p < 0.0001$ SCLC-LEMS vs SCLC and $p = 0.07$ SCLC-PCD vs SCLC). Only two elderly LEMS patients without SCLC (Supplementary text) tested positive for SOX antibodies ($p < 0.0001$ SCLC-LEMS vs NT-LEMS). SOX antibodies were present in two out of five Hu patients with SCLC. High levels of SOX antibodies were confined to SCLC patients, most of whom had a PNS (Figure 2). On average, frequency of positive sera and titres were comparable between different SOX-antigens. Between patient groups, SOX titres were higher in SCLC-LEMS patients than in SCLC patients without PNS ($p < 0.0005$). Only three out of 29 SOX positive SCLC patients with LEMS were positive for only one or three of the four SOX proteins. In SCLC patients without PNS, this incomplete pattern of antigen reactivity was present more frequently: SOX1, SOX2, SOX3 and SOX21 antibodies were detected in 32%, 24%, 22% and 25% of cases, respectively (Figure 3).

HuC, HuD or HelN1 was found in 30% of SCLC-LEMS patients, 5% of NT-LEMS patients, 15% of SCLC patients and in all Hu-SCLC patients ($p < 0.0001$ SCLC-Hu vs SCLC and $p=0.022$ SCLC-LEMS vs SCLC). After exclusion of two patients with SCLC-LEMS and concomitant anti-Hu syndrome, Hu antibodies were found in 27% of SCLC-LEMS patients ($p = 0.073$). All SCLC-LEMS patients with Hu antibodies also had SOX antibodies. NT-LEMS patients only rarely had Hu antibodies ($p = 0.003$ SCLC-LEMS vs NT-LEMS). Obviously, Hu titres were higher in SCLC-Hu patients than in other disease categories ($p < 0.0005$ SCLC-Hu vs SCLC-LEMS and SCLC-Hu vs SCLC).

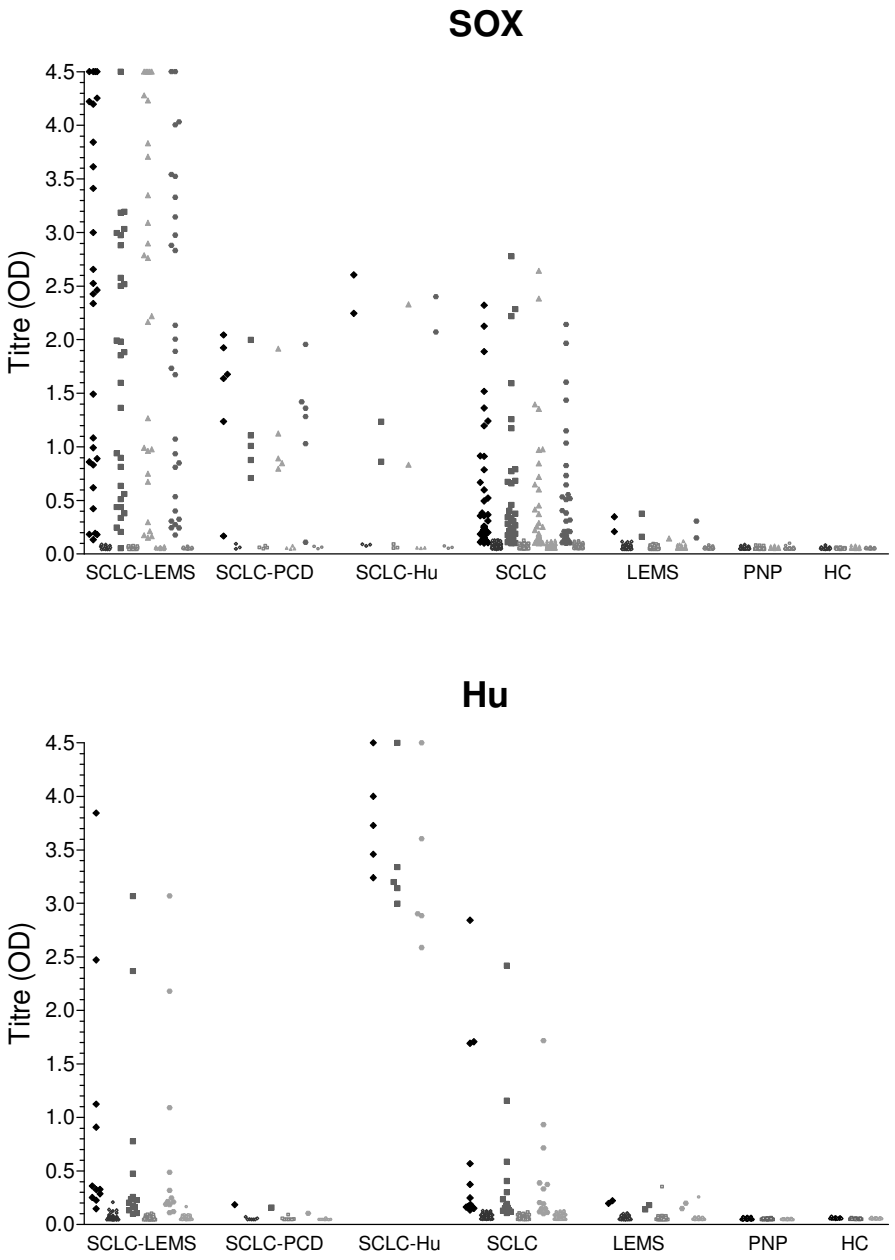

Figure 2 ELISA antibody titers of individual patients for four SOX (above) or three Hu (below) antigens. Results for each antigen are displayed in two columns, showing western blot positive (lefthand column) and negative (righthand column) samples. ♦ SOX1, ■ SOX2, ▲ SOX3, ⬣ SOX21; ♦ HuC, ■ HuD, ⬣ HelN1
PNP polyneuropathy; HC healthy controls

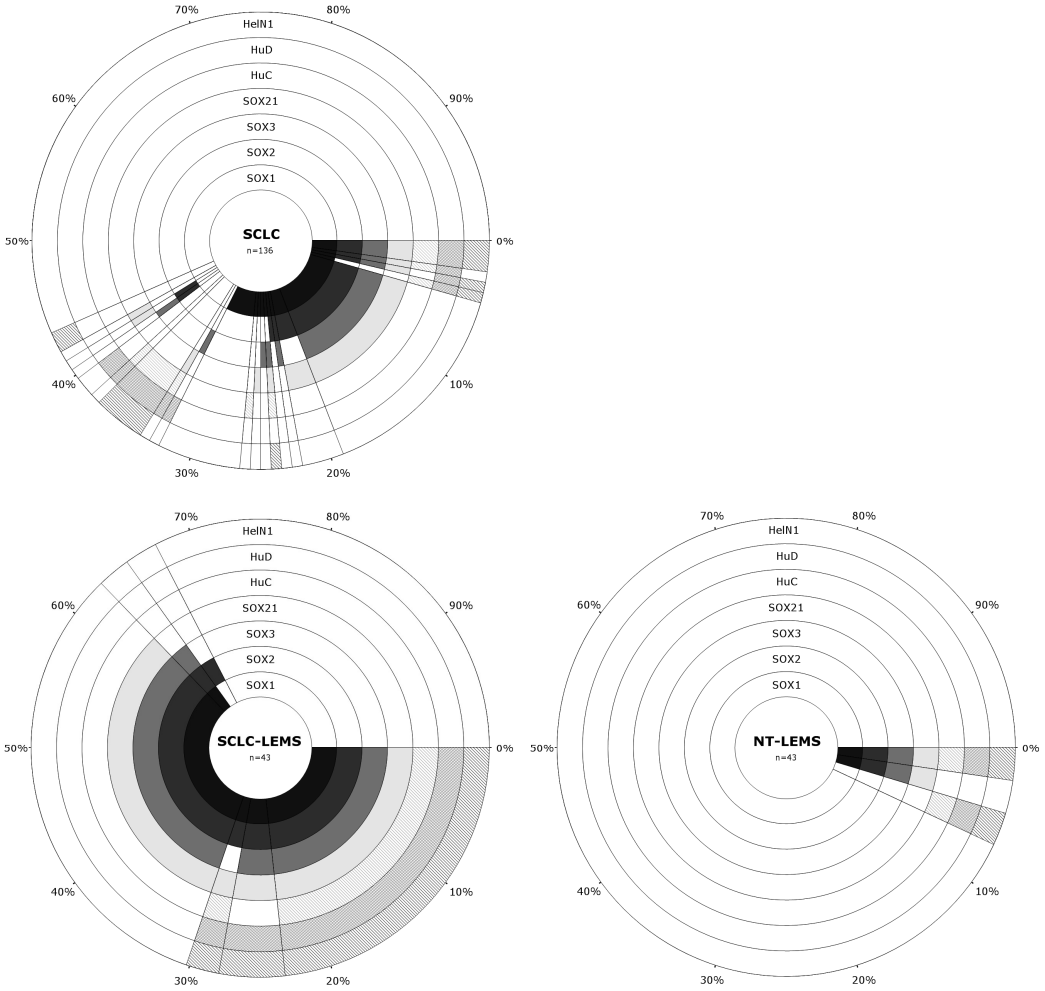

Figure 3 Composition of antibody responses in all individuals studied. Above SCLC patients (without PNS), left under SCLC-LEMS patients and right under NT-LEMS patients. SOX family antigens in solid bands, Hu family antigens in hatched bands.

Overall, SOX or Hu antibodies were present in 43% of SCLC patients without PNS. No healthy controls or patients with polyneuropathy had SOX or Hu-reactive antibodies.

	Western Blot			ELISA > 0.1 and > 3SD		
	SCLC-LEMS	NT-LEMS		SCLC-LEMS	NT-LEMS	
SOX1,2,3,21 (+)	29	2	67%	29	2	67%
SOX1,2,3,21 (-)	14	41	95%	14	41	95%
	43	43		43	43	

Table 1 Sensitivity and specificity of SOX antibodies to discriminate between SCLC-LEMS and NT- LEMS.

Sensitivity and specificity SCLC-LEMS vs NT-LEMS

ELISA of SOX1, SOX2, SOX3 and/or SOX21 showed a sensitivity of 67% and a specificity of 95% to discriminate between SCLC-LEMS and NT-LEMS (Table 1). Western blot analysis revealed identical sensitivity and specificity. Sensitivity in samples of 28 untreated SCLC patients with LEMS was comparable (68%). Analyzing SOX proteins separately, results were comparable to combination of SOX1, SOX2, SOX3 and/or SOX21.

In five SOX positive patients (positive for all four SOX proteins), samples were drawn before and after chemotherapeutic treatment. After treatment SOX titres were lower in all patients, but none became antibody-negative (Supplemental data: Figure S2).

Clinical parameters and survival in relation to SOX and Hu

NT-LEMS patients were significantly younger than SCLC patients with LEMS and more often female (Supplementary data: Table S2). SCLC-LEMS patients were comparable to SCLC patients without PNS with respect to age and sex. In SCLC patients with LEMS, limited disease was more prevalent compared to SCLC patients without PNS (64% vs 39%). SCLC patients without PNS showed no differences relating SOX1 or HuD positivity if clinical parameters were compared (Table 2). SCLC-LEMS patients also showed lack of clinical impact regarding extent of disease or success of chemotherapy. No differences were seen with regard to weight loss, lab abnormalities (LDH, leucocytes or haemoglobin) or time between onset of complaints and date of sample drawn.

Survival was better for patients with SCLC and LEMS than for SCLC patients without LEMS.[25] No difference in survival was observed with regard to presence or absence of SOX1 or HuD antibodies in SCLC patients, nor for any of the other

Characteristics		SOX1 positive	SOX1 negative	p	HuD Positive	HuD negative	p	overall
SCLC patients without PNS (n)		44	92		17	119		136
median age	(years)	66	65	0.74	66	65	0.96	65
	range	(32-78)	(41-83)		(46-80)	(32-83)		(32-83)
sex	male	30	62	1.00	9	83	0.18	92
	female	14	30		8	36		44
VGCC	positive	2	4	1.00	1	5	0.56	6
	negative	42	88		16	114		130
extent of disease	limited	17	36	1.00	9	44	0.29	53
	extended	26	56		8	74		82
	unknown	1	0		0	1		1
treatment response	(partial) remission	27	61	1.00	10	78	0.57	88
	stable/progressive	12	28		6	34		40
	unknown	5	3		1	7		8
median survival	(months)	11	10	0.51	10	10	0.22	10
Patients with LEMS-SCLC (n)		28	15		13	30		43
median age	(years)	59.8	62.0	0.96	60.6	60.1	0.99	60.5
	range	(50-77)	(49-77)		(50-77)	(49-77)		(49-77)
sex	male	19	10	1.00	10	19	0.49	29
	female	9	5		3	11		14
VGCC	positive	26	15	0.53	12	29	0.52	41
	negative	2	0		1	1		2
ECOG	0-1	21	7	0.09	9	19	0.72	28
	2-4	6	8		3	11		14
	unknown	1	0		1	0		1
extent of disease	limited	15	12	0.18	8	19	1.00	27
	extended	12	3		4	11		15
	unknown	1	0		1	0		1
treatment response	(partial) remission	18	11	0.64	7	22	0.06	29
	stable/progressive	5	1		4	2		6
	unknown	1	3		1	3		4
	no chemo	4	0		1	3		4
median time to progression (months)		11	14	0.64	9	17	0.07	12
median survival	(months)	15	n.a.	0.10	13	23	0.041	17

Table 2 Clinical characteristics of patients

antibodies tested (Figure 4 and data not shown). In SCLC-LEMS patients survival was possibly worse if HuD antibodies were present (p=0.041).

Discussion

SOX antibodies are increasingly important markers for (early) diagnosis of cancer. Until recently, testing was elaborate and determination of antibody titres was difficult. Our newly developed ELISA has solved these issues and is amenable to high throughput screening. ELISA detected SOX or Hu serum antibodies in 43% of SCLC patients without clinical paraneoplastic disease and in 67% of SCLC patients with LEMS. Of all four SOX proteins, antibodies to SOX1 were found most frequently (32%) in SCLC patients without PNS. Testing for antibodies to HuC, HuD or HelN1 identified additional sera that were negative for SOX antibodies. Combination of antigens from each protein family, SOX1 and HuD, increased the diagnostic yield to 40%. This suggests the diagnostic value of the test to be further improved by including additional SCLC related antigens, which have been identified by studying paraneoplastic syndromes or serological analysis of expression cDNA libraries.[5] Examples are CRMP or ZIC protein families.[10, 26-28]

All samples from SCLC patients were ascertained before treatment. Presence of SOX or Hu antibodies was not related to extent of disease, nor to duration of symptoms of LEMS until SCLC diagnosis. This suggests that the antibody-mediated immune reaction was already present early in disease. It is unlikely that prolonged exposure or high antigen load completely explain the presence of SOX or Hu antibodies.

In SCLC patients without PNS, screening for HuC, HuD or HelN1 had additional diagnostic value, but not in SCLC-LEMS patients. Sera of these latter patients reacted to all members of the SOX or Hu protein families, while most SCLC sera only contained antibodies to part of the proteins of the SOX or Hu family.

Cross-reactivity among SOX proteins was studied in more detail. Absorption with SOX1 protein neutralized all SOX21 reactivity, but vice versa absorption with SOX21 only partially neutralized SOX-B1 (SOX1, SOX2 and SOX3) reactivity. This suggests that SOX-B1 antigens, possibly SOX1 itself, are more likely to be the primary antigen eliciting the initial immune response (supplemental data: Figure S1). In sera from SCLC patients without PNS, both in this study and in patients tested by Vural,[10] antibodies to SOX1 were detected more frequently than

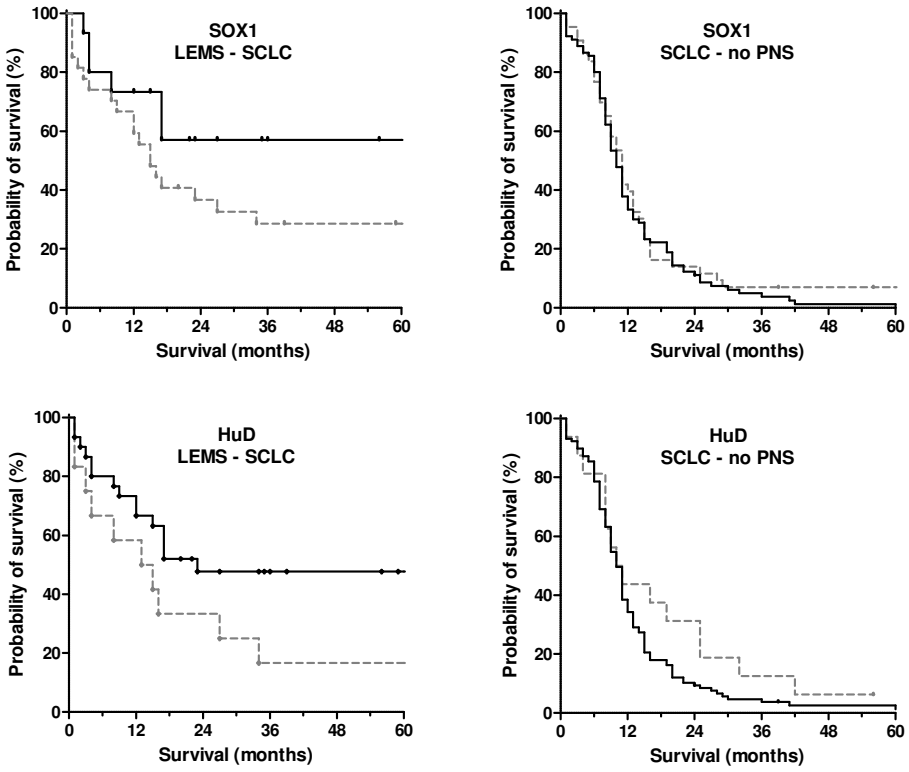

Figure 4 Survival of SOX1 (+) and SOX1 (-) and of HuD (+) and HuD (-) SCLC patients with LEMS (left) or SCLC patients without PNS (right). Solid lines represent antibody (-) and broken lines represent antibody (+) patients.

antibodies to other SOX proteins. Furthermore, sensitivity and specificity to discriminate between SCLC-LEMS and NT-LEMS were comparable for all SOX proteins in our study: two-third of patients with an SCLC and 5% of NT-LEMS patients tested positive. In conclusion, testing for Hu antibodies has additive value in antibody screening tests for SCLC without PNS.

SOX antibodies are tumour markers and are considered to be exclusively present in patients with a tumour. Hu antibodies are sometimes discovered in patients with anti-Hu syndrome, while long term follow-up does not reveal any tumour. As expected, in healthy controls and patients with polyneuropathy no SOX or Hu antibodies could be found. Interestingly, all three LEMS patients without SCLC who tested positive were of older age. In two, their clinical profile suggested high risk for underlying SCLC.[25, 29] Although speculative, the immune response

might have eradicated an occult SCLC without treatment, as reported before in patients with SCLC and PNS.[30-33]

Both SOX and Hu antibodies are directed against intracellular nuclear proteins and both were described in a consistent part of SCLC patients without clinical PNS,[16] which makes a direct pathogenic role in paraneoplastic syndromes unlikely. This is supported by lack of clinical differences between SOX or Hu positive and negative patients. In SCLC patients without PNS, no differences in survival were found between antibody positive or negative patients.

Lead time bias or enhanced anti-tumour immune response would explain the improved survival of SCLC-LEMS patients compared to SCLC patients without PNS. As most patients had no pulmonary complaints at time of diagnosis of LEMS, lead time bias is probable. The larger amount of patients with SCLC-LEMS with limited disease SCLC (64% vs 39%) might be attributed to lead time bias, which suggests a beneficial effect on survival of future screening. On the other hand, better tumour control might also retard progression to extensive disease. Three clues hint towards improved anti-tumour response: SCLC-LEMS patients with extended disease had a median survival of 16 months, considerably better than SCLC patients (median 9 months). Secondly, part of our patients already had pulmonary complaints at time of diagnosis of LEMS, without worse prognosis compared to other SCLC-LEMS patients. It is unlikely that lead time bias plays a unique role in these patients. Finally, improved survival was described in independent prospective studies previously.[17-19]

In SCLC patients without PNS, no significant effect on survival of SOX or Hu seropositivity was observed. Similarly, Vural *et al.* found no significant effect of SOX or Zic antibodies, although possible better survival for SOX1, especially for high titres and in combination with presence of ZIC2 antibodies, was reported.[10] In our cohort of SCLC patients, no titre-related survival effect for SOX or Hu was found. In contrast, both SOX1 and HuD positive SCLC-LEMS patients showed a tendency towards worse prognosis, although this only reached borderline significance for HuD (median survival 13 vs 23 months, $p_{uncorrected} = 0.041$). After exclusion of two patients with concomitant clinical paraneoplastic anti-Hu syndrome, which is known to worsen prognosis,[21] median survival of HuD positive or negative patients remained 13 vs 23 months ($p = 0.053$). No significant difference was found between SOX1 positive or negative SCLC-LEMS patients ($p = 0.10$). Furthermore, all Hu positive SCLC-LEMS patients were SOX1 positive, which biased SOX1 survival.

In summary, we developed a highly reliable ELISA for the early detection of SCLC, which is amenable to high throughput screening. Its current sensitivity reaches 43%. Adding other SCLC-associated antigens, we expect this sensitivity to be further improved and implemented in a diagnostic setting or be used for population screening.

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Supplementary data

Clinical information of 3 LEMS patients without SCLC, but with SOX or Hu antibodies

Patient 1 had low antibody titres against all seven proteins. This 61 year old woman developed LEMS with cerebellar ataxia, but an SCLC was never found. She had a smoking history of 45 pack years. Seven years after diagnosis of LEMS, she developed an endometrium carcinoma without any relapse of LEMS symptoms. The blood sample was drawn just one month before. The carcinoma was surgically removed; fourteen years after initial diagnosis of LEMS, she is still in good health.

Patient 2 had low titres of HuC, HuD and HelN1. He was a 52 years old male patient. He developed rapidly progressive LEMS in December 2003. He had clubbing fingers and weight loss (6% in 3 months). His smoking history revealed 20 pack years. Despite strong suspicion, five CT-thorax investigations and one FDG-PET scan did not reveal any sign of SCLC.

Patient 3 had antibodies against SOX1, SOX2 and SOX21. This male was 66 years old, when he developed a mild phenotype of LEMS. He had no signs at all of a tumour. He had never smoked. He died at the age of 83 because of a small intestinal perforation despite surgery.

Gene	*UTR specific primers*	*ORF specific primers*
SOX2	5'-gtttgcaaaaggggaaagt-3'	5'-gaccatatgtacaacatgatggagacg-3'
	5'-gtcatttgctgtgggtgatg-3'	5'-tgcctcgagcatgtgtgagaggggcagtg-3'
SOX21	5'-gcagccaacattgatttcct-3'	5'-gaccatatgtccaagccggtggaccac-3'
	5'-gcagcgctcgtacctataca-3'	5'-tgcctcgagtagcgcggcagcgtaggccgc-3'
HuC	5'-gccttcatcgccacatct-3'	5'-gaccatatggtcactcagatactgggg-3'
	5'-tgcctgtgctgtctctcttg-3'	5'-tgcctcgagcgccttgtgctgtttgctgg-3'
HuD	5'-aagctctgcgagacccaata-3'	5'-gaccatatggttatgataattagcacc-3'
	5'-cgcgtgtgttttgttcgtat-3'	5'-tgcctcgagggacttgtgggctttgttgg-3'
HelN1	5'-gtttcccctcccctttcata-3'	5'-gaccatatggaaacacaactgtctaatg-3'
	5'-tccccatctcaacactgactt-3'	5'-tgcctcgagggctttgtgcgttttgtttg-3'

Table S1 Primers used for amplification of the genes SOX2, SOX21, HuC, HuD and HelN1. Restriction sites in the ORF specific primers that were used to clone the gene in the pET28b vector are underscored.

		SCLC-LEMS		NT-LEMS		SCLC		
number		43		43		136		
median age (years)		60.5	(49.5-77.7)	54.1	(24.5-74.9)	65	(32-83)	< 0.0005 * and 0.16 #
sex	male	29	67%	19	45%	92	68%	0.05 *
	female	14	33%	23	55%	44	32%	
extent of disease	limited	27	64%			53	39%	0.007 #
	extended	15	36%			82	61%	
	unknown	1				1 not determined		

Table S2 Basic clinical characteristics of the patients.
* SCLC-LEMS vs NT-LEMS; # SCLC-LEMS vs SCLC

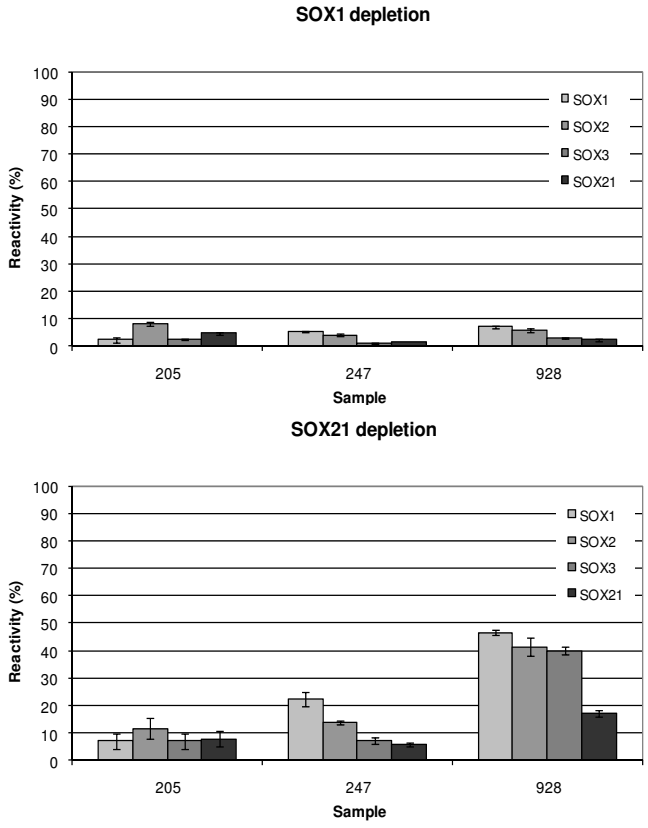

Figure S1 Reactivity of sera for SOX1, SOX2, SOX3 and SOX21 after depletion with SOX1, a SOX-B1 protein family member (above) or SOX21, a SOX-B2 protein family member (below).

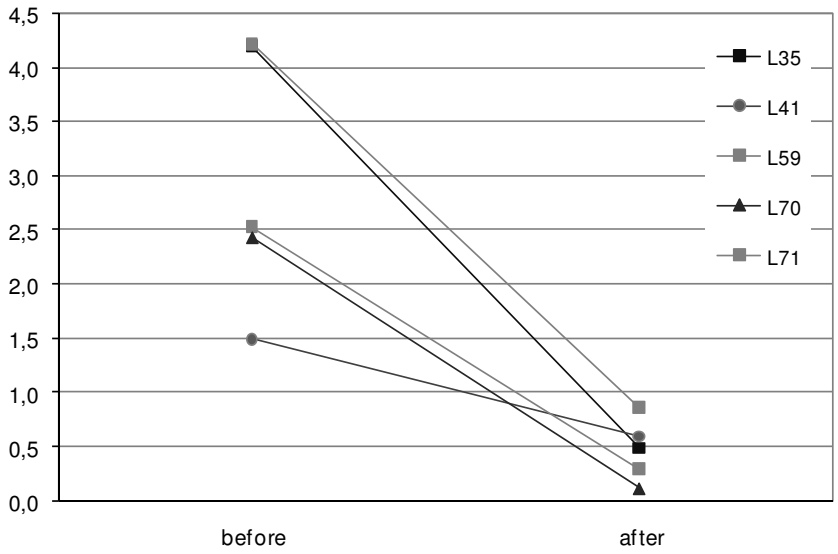

Figure S2 SOX1 titres in five SOX1 positive patients before and after treatment.