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SSEA-4 is Expressed in Basaloid Lung Cancer and Associated with Poor Prognosis

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Abstract

Background: Basaloid carcinoma represents a rare variant of non-small cell lung cancer (NSCLC) which showed a poor prognosis in a couple of studies. Although it is considered to derive from a pluri- or multipotent pulmonary stem cell, little is known about the expression and clinical significance of stem cell antigens in this variant.

Patients and Methods: *Stage-specific embryonic antigen-4* (*SSEA-4*) was analyzed by immunohistochemistry in 38 patients with resected early-stage basaloid NSCLC who had a median follow-up of 72.9 months. The expression of *SSEA-4* was related to clinico-pathological characteristics, the expression of the adult stem cell antigens CD117, CD133 and *breast cancer resistance protein-1* (*BCRP1*) and prognosis.

Results: *SSEA-4* was positive in 37% of the specimens and showed no association with clinico-pathological characteristics or the expression of adult stem cell antigens. Cox proportional hazards regression analysis revealed a 6.0-fold increased risk of relapse ($p=0.001$) and a 4.2-fold increased risk of disease-related mortality ($p=0.017$) in *SSEA-4* positive patients, while *SSEA-4* negative patients showed a prognosis comparable to that of other early-stage NSCLC.

Conclusions: *SSEA-4* is expressed in a fraction of basaloid NSCLC and associated with poor prognosis.

Introduction

Basaloid lung cancer is a rare variant of pulmonary squamous cell (SCC) or large cell carcinoma (LCC) and accounts for 4.8%-6.3% of all non-small cell lung cancer (NSCLC) cases [1, 2]. In contrast to overall NSCLC which has a 5-year survival rate (SR) of average 40%-60% and a median survival time (MST) of about 50 months in stage I/II disease, analyses of Brambilla and coworkers in two European collectives comprising 38 and 90 patients with basaloid NSCLC revealed a significantly lower 5-year SR of 10%-27% and a shorter MST of 20-29 months [1, 3-6]. The basal cell phenotype of these carcinomas and their co-occurrence with virtually all histologic subtypes of NSCLC early lead to the assumption that this variant derives from a pluri- or multipotent pulmonary stem cell [1, 5, 7]. In recent years several *in vitro* and *in vivo* studies indicated the presence of stem-like cells in NSCLC. Those were characterized by the expression of stem cell antigens such as CD117, CD133 and/or *breast cancer resistance protein-1* (*BCRP1*), the ability to regenerate the primary tumour, an increased metastatic capability and drug-resistance [8-10]. Previous studies in early-stage disease indicated that expression of CD133 and *BRCP1* predicts an increased risk of relapse and disease-related mortality after pulmonary resection [11, 12]. In contrast to adult stem cell antigens, the knowledge about the expression and significance of embryonic stem cell (ESC) antigens in lung cancer is scanty. The markers most frequently used to identify ESC are the glycolipid carbohydrate epitopes *stage-specific embryonic antigen* (*SSEA*)*-3 and -4* and the transcription factor *Oct-4* [13]. Recent studies demonstrated expression of *Oct-4* in a subset of NSCLC cells with stem cell properties and an unfavourable prognostic significance of this antigen in adenocarcinomas with lepidic growth pattern [14-17]. In contrast, *SSEA-4* which characterizes very early ESC has yet been analyzed in teratocarcinomas, testicular germ cell tumours and a single study of ovarian cancer, but not in lung cancer [18-20]. Particularly in basaloid NSCLC which mostly suggests an association

with pulmonary stem cells, analyses on the expression of stem cell antigens are lacking so far. In this study, we analyzed tumour tissue of 38 completely resected stage I/II patients with basaloid NSCLC for the expression and prognostic significance of *SSEA-4*. Moreover, the results were related to clinico-pathological characteristics and the expression of the adult stem cell antigens CD117, CD133 and *BCRP1*.

Materials and Methods

Patient and sample characteristics

A total of 38 previously untreated patients with basaloid NSCLC who underwent complete pulmonary resection between 2002 and 2009 at the Department of Thoracic Surgery, Thoraxklinik, University of Heidelberg were analyzed. Tissue specimens and follow-up data were obtained from the tissue bank and the lung cancer registry of the Thoraxklinik and the tissue bank of the National Center for Tumour Diseases (NCT), University of Heidelberg. Patients had given informed consent following the 2000 revision of the guidelines of the declaration of Helsinki and the local Ethics Committee of the Medical Faculty Heidelberg. Preoperative and follow-up assessments were performed according to the guidelines of the German Respiratory Society last published in 2010 [21]. The stage was determined according to the 7th edition of the TNM classification of malignant tumours [3]. Detailed patient characteristics are given in **Tab. 1**.

Patient Characteristics				
Number of Patients	**Total** n=38	***SSEA-4* positive** n=14	***SSEA-4* negative** n=24	**p value**
Gender				
Female	7 (18%)	2 (14%)	5 (21%)	0.615
Male	31 (82%)	12 (86%)	19 (79%)	
Age [years]				
Mean ± SEM	38 (100%)	66.0 ± 8.2	61.9 ± 7.6	0.143
ECOG				
0	28 (74%)	11 (79%)	17 (71%)	0.601
≥ 1	10 (26%)	3 (21%)	7 (29%)	
Stage (UICC)				
IA	6 (16%)	2 (14%)	4 (17%)	0.899
IB	13 (34%)	4 (29%)	9 (37%)	
IIA	8 (21%)	3 (21%)	5 (21%)	
IIB	11 (29%)	5 (36%)	6 (25%)	
pT				
T1	10 (26%)	4 (29%)	6 (25%)	0.809
T2	28 (74%)	10 (71%)	18 (75%)	
pN				
N0	21 (55%)	7 (50%)	14 (58%)	0.618
N1	17 (45%)	7 (50%)	10 (42%)	
Type of Resection				
Pneumonectomy	6 (16%)	4 (29%)	2 (8%)	0.247
Bilobectomy	4 (10%)	1 (7%)	3 (13%)	
Lobectomy	28 (74%)	9 (64%)	19 (79%)	
Relapse [%]				
Total	16 (42%)	11 (79%)	5 (21%)	**0.000**
Local	1 (3%)	0 (0%)	1 (4%)	n.a.
Distant	15 (39%)	11 (79%)	4 (17%)	n.a.
Mortality [%]				
Total	14 (37%)	9 (64%)	5 (21%)	**0.011**
Disease-Related	13 (34%)	9 (64%)	4 (17%)	n.a.
Non-Disease-Related	1 (3%)	0 (0%)	1 (4%)	n.a.
Disease-Free Survival [months]				
Median [95% CI]	n.r.	11.1 [4.3-45.1]	n.r.	n.a.
Overall Survival [months]				
Median [95% CI]	n.r.	45.0 [14.5-n.r.]	n.r.	n.a.
Survival Rate [%]				
1-year [95% CI]	91.7 [82.7-100]	85.1 [66.0-100]	95.7 [87.3-100]	n.a.
2-year [95% CI]	83.0 [70.6-95.5]	69.6 [44.7-94.6]	90.9 [78.7-100]	
5-year [95% CI]	61.9 [44.4-79.4]	35.4 [8.1-62.7]	80.1 [64.4-97.8]	
Follow-up [months]				
Median [95% CI]	72.9 [42.7-91.5]	91.4 [42.7-96.3]	70.2 [37.5-84.2]	n.a.

Table 1: Patient characteristics. ECOG: Eastern Cooperative Oncology Group; UICC: Union internationale contre le cancer; SEM: standard error of the mean, CI: confidence interval, n.r.: not reached, n.a.: not applicable.

Histologic classification

The histologic classification of a basaloid NSCLC variant was based on the criteria published in the 2004 revision of the World Health Organization classification of tumours and included: (a) solid lobular or anastomotic trabecular pattern growing invasively in a finger-like fashion from the bronchial and/or glandular duct lining, (b) small cuboidal to fusiform cells of 12 µm - 15 µm mean diameter with scant, but visible cytoplasm, moderately hyperchromatic nuclei without nuclear molding or prominent nucleoli, (c) peripheral palisading with radially arranged cells at the periphery of the tumour lobules and (d) a mitotic index of $\geq$ 15-44 mitoses per 10 high power fields [22]. For histologic classification and grading hematoxylin-stained tissue sections were used. The diagnosis of a squamous variant was based on the presence of intercellular bridges or individual cell keratinization within the basal cell component in < 50% of the tumour area. Additionally, the expression of the neuroendocrine (NE) markers neuron-specific enolase (NSE), synaptophysin (SYP), chromogranin A (CHGA), CD56 and the high molecular weight cytokeratins (HMWCK) 1, 5, 10 and 14 was analyzed to exclude large cell neuroendocrine or small cell carcinomas. The diagnosis of a basaloid carcinoma was maintained when HMWCK were expressed and clear-cut staining for at least one specific NE marker was lacking.

Immunohistochemical and immunocytochemical analyses

Tissue microarrays (TMA) of formalin-fixed, paraffin-embedded tissue derived from pulmonary resections were prepared by the tissue bank of the NCT as previously described [23]. Of each patient two 1.2 mm diameter tissue cores each of the tumour center, the invasion

front and histologically normal lung tissue were spotted. In half of the cases additionally whole tissue sections were prepared. 2 μm sections of the TMA or the whole tissue were deparaffinized with xylene and rehydrated in graded alcohol series. For antigen retrieval, the sections were boiled in target retrieval buffer pH 6 (DAKO, Glostrup, Denmark) for 15 minutes (min). Sections for CD133 and *BCRP1* staining were additionally blocked by avidin/biotin treatment. Subsequent steps were performed in an immunostaining device (Autostainer, DAKO). Briefly, the sections were incubated with the primary antibody for 30 min, washed with phosphate buffered solution/Tween 20, incubated with the secondary antibody for 20 min and washed again. Endogenous peroxidase was blocked by incubation with peroxidase-blocking solution (DAKO) for 5 min. Detection was based on the avidin-biotin peroxidase principle using AEC as chromogen (DAKO REAL Detection System Peroxidase/AEC, Rabbit/Mouse). The sections were counterstained with Mayer's hematoxylin for 5 min andmounted with coverslips in Aquatex mounting medium (Merck KGaA, Darmstadt, Germany). The following primary antibodies, clones and dilutions were used: *SSEA-4* (clone MC-813-70, 1:100, Millipore, Billerica, MA, USA), CD117 (polyclonal, 1:50, DAKO), CD 133 (polyclonal [ab19898], 1:100, abcam plc, Cambridge, MA, USA), *BCRP1* (clone BXP-21, 1:100, abcam), Ki67 (clone MiB1, 1:200, DAKO), CD56 (clone 1B6, 1:50, Novocastra, A. Menarini Diagnostics Deutschland, Berlin, Germany), SYP (clone 27G12, 1:400, Novocastra), NSE (clone MIG-N3, 1:10,000, DCS Innovative Diagnostik-Systeme Dr. Christian Sartori GmbH & Co. KG, Hamburg, Germany), CHGA (clone LK2H10, 1:10, abcam) and HMWCK (clone 34βE12, 1:100, abcam) recognizing CK 1, 5, 10 and 14. For negative control, the primary antibody was omitted. Teratocarcinoma tissue derived from the tissue bank of the NCT was used as positive control for *SSEA-4*. For analysis of *SSEA-4* expression in non-tumoral conditions, lung tissue of healthy subjects with spontaneous pneumothorax was used and provided by the tissue bank of the NCT. The analysis was performed by two independent observers (E.H. and P.S.). Samples were

considered positive, if either dispersed expression or at least one focus with distinct staining was present. The TMA were scanned at 400x magnification using Aperio ImageScope v10.1.3.2028 software (Aperio Technologies Inc., Vista, CA, USA).

The human embryonic carcinoma cell line *NTERA-2* (Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and the basaloid NSCLC cell line *2427T* were cultured as indicated by the supplier or published, respectively [24, 25]. For immunocytochemical analyses, cytospins of each one million *NTERA-2* or *2427T* cells were prepared in a Shandon CytoSpin III cytocentrifuge (Fisher Scientific GmbH, Schwerte, Germany). The cells were fixed with neutral buffered 10% formalin for 5 min, boiled in target retrieval buffer pH 6 (DAKO) for 15 min and permeabilized with Triton X-100 for 10 min. All subsequent steps were performed as described above.

Statistical analyses

The follow-up was defined as the Kaplan-Meier estimate with reversed status indicator. Death censored the true but unknown observation time, censoring was interpreted as endpoint. Thus, the unobservable follow-up time of a deceased patient was interpreted as the follow-up time that potentially would have been obtained if the patient had not died. Survival time was determined from the date of first diagnosis until last follow-up or reported death. Non disease-related death was censored. The disease-free survival (DFS) was determined from the date of first diagnosis until diagnosis of relapse or disease-related death. Survival times were analyzed using the Kaplan-Meier method and the log-rank test. For multivariate analysis the Chi-Square test and Wilcoxon rank sum test were used to evaluate the difference between groups. $P < 0.05$ was considered statistically significant. The statistical analyses were performed using SAS® version 9.2 (SAS Institute, Cary, NC, USA).

Results

Expression of *SSEA-4* in basaloid NSCLC

Tissue specimens of 38 patients with stage I/II basaloid NSCLC were analyzed for the expression of *SSEA-4*. The collective contained 24 pure basaloid carcinomas, 13 basaloid carcinomas with a SCC component and one basaloid carcinoma with an adenocarcinoma component. In all specimens the basaloid component comprised ≥ 60% of the tumour bulk. *SSEA-4* showed diffuse membranous/cytoplasmic expression in 37% (14/38) of the specimens **(Fig. 1A and Fig. 1B, picture A)**. The positive control, a teratocarcinoma, showed likewise diffuse cytoplasmic/membranous and in some cells also nuclear staining **(Fig. 1B, picture B)**. In the NSCLC specimens, positive staining was restricted to the basaloid component, while the well differentiated AC or SCC components were negative for *SSEA-4* **(Fig. 1B, picture C and D)**. The alveolar epithelium of patients and healthy subjects was negative for *SSEA-4* **(Fig. 1A, picture I; Fig. 1B, picture E)**, whereas the tracheobronchial epithelium displayed membranous/cytoplasmic staining of the basal cells and cytoplasmic accumulation at the apical cell pole of ciliated **cells (Fig. 1A, picture E; Fig. 1B, picture F)**.. All specimens displayed reactivity for HMWCK, while clear-cut expression of specific NE markers was absent **(Fig. 1C)**. Remarkably, the basaloid NSCLC cell line *2427T* which shows properties related to stem cells, i.e. high tumorigenicity in the animal model and reassembling of the original tumor histomorphology, displayed also expression of *SSEA-4*. Alike the positive control, the embryonic carcinoma cell line *NTERA-2*, *2427T* cells showed distinct membranous/cytoplasmic staining **(Fig. 1D)**.

SSEA-4, clinico-pathological characteristics and adult stem cell antigens

The expression of *SSEA-4* showed no association with clinico-pathological characteristics or the expression of adult stem cell antigens **(Tab. 1 and 2)**. CD117 was

positive in 47%, *BCRP1* in 50% and CD133 in 5% of the tumours. CD117 showed diffuse membranous and/or cytoplasmic staining, while *BCRP1* displayed additionally nuclear staining and predominantly focal expression **(Fig. 1, pictures B-D)**. The alveolar epithelium of patients was negative for adult stem cell antigens **(Fig. 1, pictures J-L)**, whereas the tracheobronchial epithelium showed cytoplasmic expression of CD117 in the basal cells and in some ciliated cells and nuclear expression of *BCRP1* in the majority of ciliated cells **(Fig. 1, pictures F-H)**.

***SSEA-4* and Adult Stem Cell Antigens**				
	Total n=38	***SSEA-4* positive** n=14	***SSEA-4* negative** n=24	**p value**
CD117	18 (47%)	6 (43%)	12 (50%)	0.671
CD133	3 (8%)	2 (14%)	1 (4%)	0.264
BCRP1	19 (50%)	8 (57%)	11 (46%)	0.501
CD117/*BCRP1*	11 (29%)	3 (21%)	8 (33%)	0.435

Table 2: Expression of *stage-specific embryonic antigen* (*SSEA-4*) and adult stem cell antigens. *SSEA-4* shows no association with the expression of the adult stem cell antigens CD117, CD133 and *breast cancer resistance protein-1* (*BCRP1*).

Prognostic significance of *SSEA-4*

Within the median follow-up time of 91.4 months for *SSEA-4* positive and 70.2 months for *SSEA-4* negative patients, significantly more individuals with *SSEA-4* positive than *SSEA-4* negative tumours experienced relapse (79% *vs.* 21%, p=0.0005). All but one relapse were distant relapses **(Tab. 1)**. The 5-year survival rate of *SSEA-4* positive patients was 35% and that of *SSEA-4* negative patients 80%. Cox proportional regression analysis revealed a 6.0-fold increased risk of relapse (HR: 6.0, 95% CI: 2.1-17.4, p=0.001) and a 4.2-fold increased risk of disease-related mortality (HR: 4.2, 95% CI: 1.3-13.7, p=0.017) in patients with *SSEA-4* positive tumours **(Fig. 2A and Fig. 2B)**. These patients showed a

median disease-free survival of 11.1 months and median overall survival of 45.0 months, while the median DFS and OS of patients with *SSEA-4* negative tumours were not reached.

Discussion

Basaloid lung cancer represents a rare variant of NSCLC and showed an unfavourable prognosis in a couple of studies [1, 5, 6]. Brambilla *et al.* who were the first to describe this variant speculated as early as 1992 that basaloid NSCLC might derive from a pluri- or multipotent pulmonary stem cell [5]. Although a few studies indicated the expression and unfavourable prognostic significance of embryonic and adult stem cell antigens such as *SSEA-1*, *Oct-4*, CD133 and *BCRP1* in NSCLC, analyses particularly in basaloid lung cancer are lacking so far [11, 12, 16, 17, 26, 27]. In this study analyzing 38 patients with early-stage basaloid NSCLC we found expression of the early ESC antigen *SSEA-4* in 37% of the specimens and an association with a 6.0-fold increased risk of relapse and a 4.2-fold increased risk of disease-related mortality. While patients with *SSEA-4* negative tumours showed a relapse rate of 21% and a 5-year SR of 80%, patients with *SSEA-4* positive tumours were recurrent in 79% of the cases and showed a 5-year SR of 35%. Without stratification for *SSEA-4*, the relapse rate (42%) and 5-year SR (62%) of the entire collective was similar to that reported for overall early-stage NSCLC or poorly differentiated SCC (PDSC) [1-6]. This is in contrast to the results of Brambilla and coworkers who reported a significantly worse outcome of basaloid carcinomas as compared to other NSCLC or PDSC: While the latter displayed a 5-year survival rate of 44% and 47%-55% in stage I/II disease, respectively, the 5-year survival rate of early-stage basaloid NSCLC was 10%-27% [1, 5, 6]. On the other hand, previous studies of Kim *et al.* and Wang *et al.* in two Asian collectives likewise demonstrated comparable relapse rates (33%-55%) and 5-year SR (50%-57%) for basaloid carcinoma and PDSC [2, 28]. The results of our study might provide one of the reasons for the inconsistent findings of previous studies as a random accumulation of patients with *SSEA-4* positive tumours might result in worse prognosis of the entire study population with basaloid NSCLC. However, several other factors have to be considered for these discrepancies between the

studies including technical aspects, investigator's variability in the identification of basaloid NSCLC, changes of the staging system, differences of the surgical procedure and post-operative treatment phase, differences of the interval and type of clinical assessments as well as differences of the number of analyzed subjects, the follow-up time and clinico-pathological characteristics of the study population. Moreover, the better treatment options for relapsed patients since 2000 might have leveled discrete survival differences in the more recent studies of Kim *et al.* and Wang *et al.* [2, 28, 29].

Besides clinical significance, the expression of the early ESC antigen *SSEA-4* in basaloid carcinoma and in basal cells of the tracheobronchial epithelium represents an important pathophysiologic aspect and supports the stem cell hypothesis of Brambilla *et al*. [5]. In line with rapid down-regulation of this glycolipid antigen in the embryonic carcinoma cell lines *2102Ep*, *BG01V*, *NTERA-2* and in blastocyst-derived ESC upon differentiation, we found no expression of *SSEA-4* in well differentiated tumor components of basaloid NSCLC [30-34]. Moreover, a recently established basaloid NSCLC cell line which shows properties related to stem cells, i.e. high tumorigenicity in the animal model and reassembling of the original tumor histomorphology, displayed also expression of *SSEA-4* [25]. However, as defined model systems and conditions for the assessment of multilineage differentiation capacity of pulmonary cells are lacking so far, the nature of *SSEA-4* positive cells in the tracheobronchial epithelium and in some basaloid NSCLC remains to be elucidated. As for the current study, the results suggest that a part of the basaloid carcinomas retain the embryonic antigen expression of their putative cell of origin, while others lack either any stem cell antigens or show exclusively expression of adult stem cell markers. It may be speculated whether these carcinomas derive from more committed (stem) cells or undergo differentiation during tumour growth. The lacking expression of CD133 and *BCRP1* in the basal cells of the tracheobronchial epithelium, but focal expression of *BCRP1* in some *SSEA-4* positive tumours suggests the capability of intratumoral differentiation.

Since the representativeness of the TMA plays a critical role particularly in focally expressed antigens such as *BCRP1*, the TMA was prepared according to recent validation data in lung cancer and mesothelioma which demonstrated that 3-4 tissue cores per sample are sufficient to produce reliable results [35-37]. Moreover, in half of the cases the results of the TMA were compared to corresponding whole tissue sections and showed full concordance.

With expression of the early ESC antigen *SSEA-4* in basaloid carcinoma and in the basal cells of the tracheobronchial epithelium, the transcription factor and proto-oncogene *c-myc* has to be discussed as a potential molecular driver of this NSCLC variant. *C-myc* represents one of the factors which is crucially involved in the early steps of somatic cell reprogramming and is the center of a regulatory network that induces an embryonic gene expression profile, albeit no pluripotency in cancer cells [38, 39]. Depending on the method, 5.6%-80% of all NSCLC show an amplification of this gene and 48%-58% an overexpression [40-42]. Remarkably, in normal tracheobronchial epithelium the highest expression values were reported for basal cells and tumours arising from these natural progenitor cells might *per se* display ESC features [42]. So far no study or subgroup analysis of basaloid lung cancer is known to the authors which would have analyzed the status of *c-myc* or the genetic profile of this variant. An own comparative genomic hybridization (CGH) and multiplex fluorescence *in situ* hybridization (m-FISH) analysis of two primary tumours and one lymph node metastasis of basaloid NSCLC which was conducted in the context of cell line establishment demonstrated a consistent amplification of 8q24, the gene locus of *c-myc* [25].

Anyhow, the presence of tumour cells with ESC characteristics might provide novel therapeutic options and targets for NSCLC: Glycolipid antigens such as *SSEA-4* are highly immunogenic and might be suitable for antibody-based therapy approaches, whereas embryonic signaling pathways such as Wnt/β-catenin, hedgehog or notch could be a target for small molecule inhibitors [43, 44].

In summary, this study demonstrated expression of the ESC antigen *SSEA-4* in a fraction of basaloid NSCLC and in the basal cells of the tracheobronchial epithelium. Patients with *SSEA*-4 positive tumours showed a significantly increased risk of relapse and disease-related mortality. With respect to the limited sample size of this study which debars a definitive statement, *SSEA-4* might represent a promising candidate for the identification of patients with basaloid NSCLC who will benefit from an adjuvant therapy after surgery.

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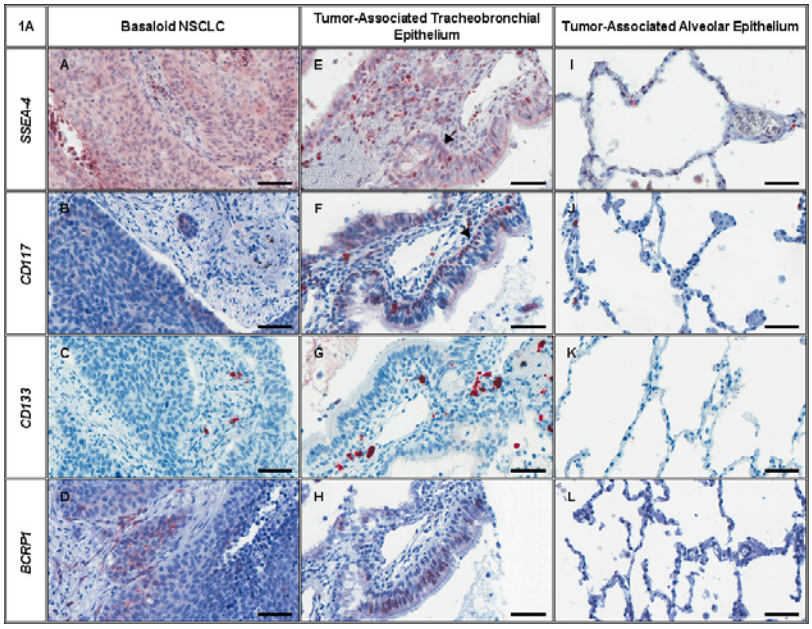
1A
Basaloid NSCLC
Tumor-Associated Tracheobronchial Epithelium
Tumor-Associated Alveolar Epithelium
SSEA-4
CD117
CD133
BCRP1
A
B
C
D
E
F
G
H
I
J
K
L

Figure 1 A

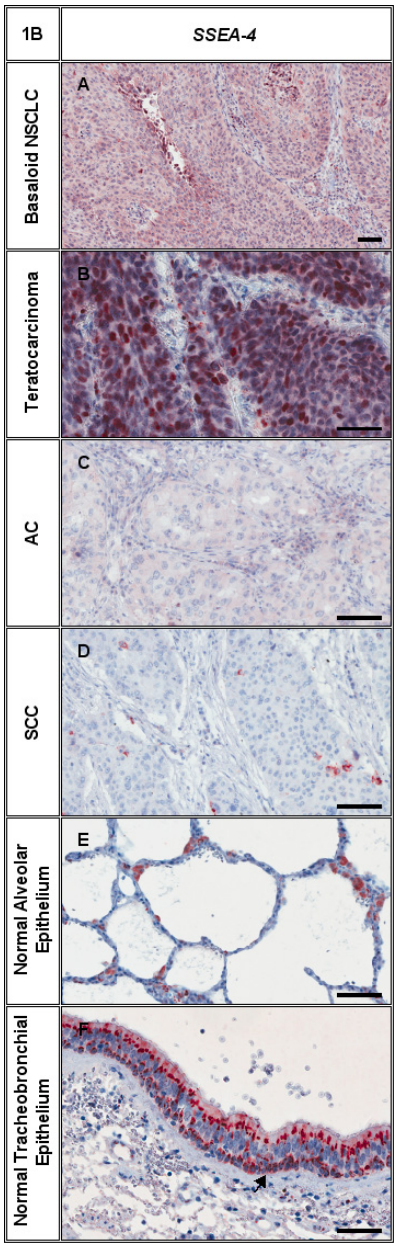
1B
SSEA-4
Basaloid NSCLC
A
Teratocarcinoma
B
AC
C
SCC
D
Normal Alveolar Epithelium
E
Normal Tracheobronchial Epithelium
F

Figure 1 B

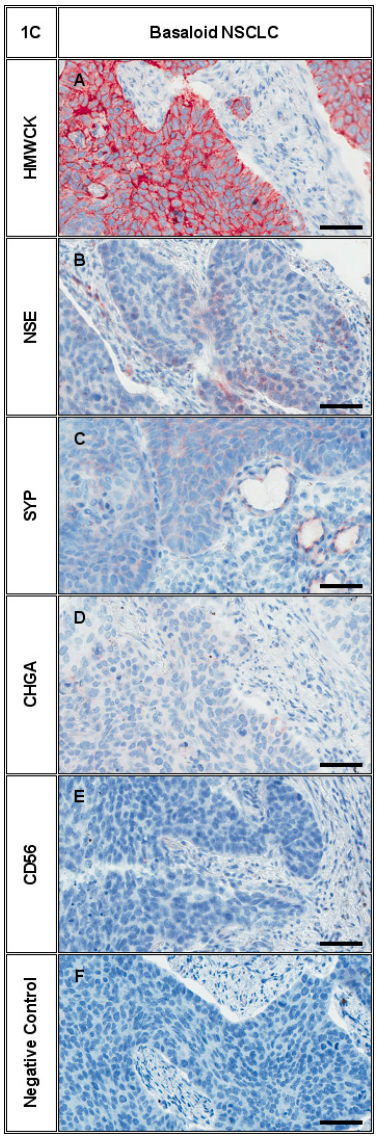

Figure 1C

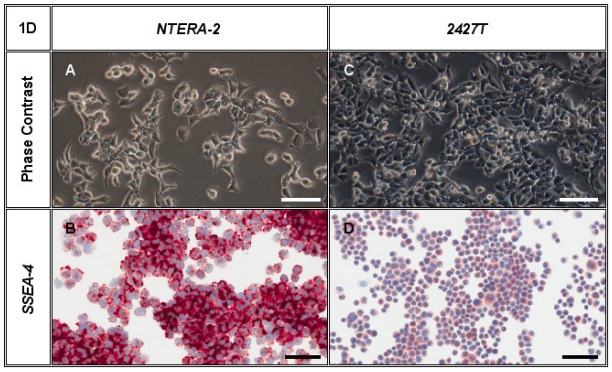

Figure 1D

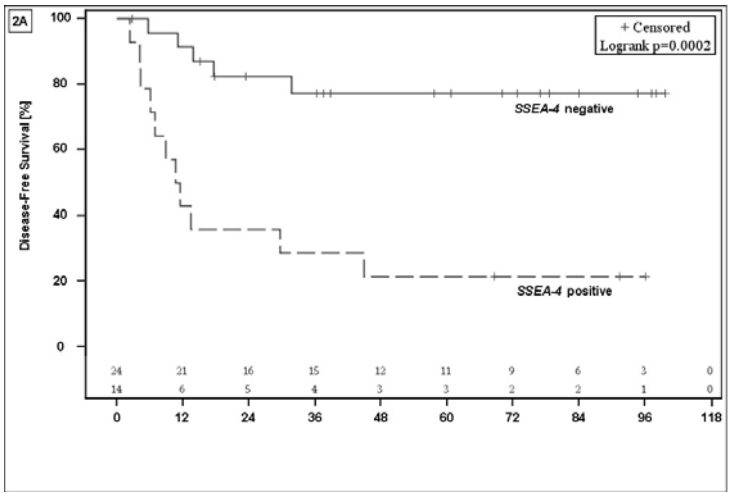

Figure 2A

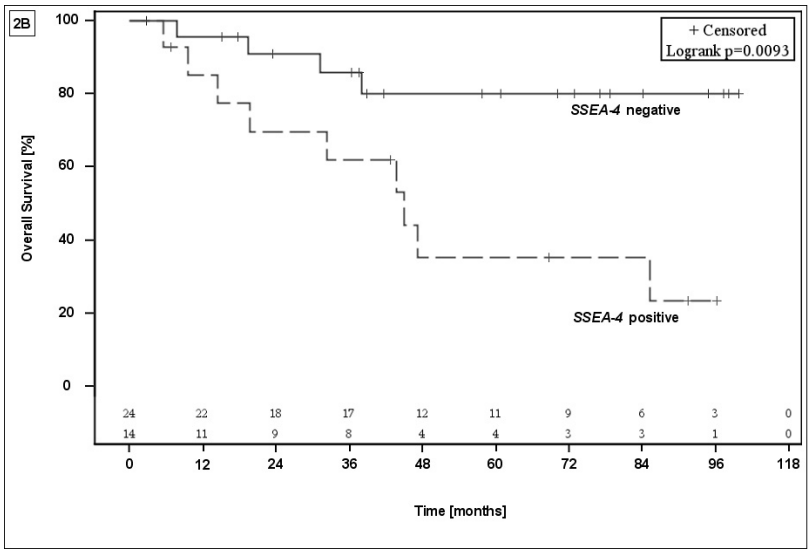

Figure 2B