The 180 splice variant of NCAM—containing exon 18—is specifically expressed in small cell lung cancer cells

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Background: The Neural Cell Adhesion Molecule (NCAM) is a glycoprotein expressed as 120, 140 and/or 180 kDa isoforms, all derived through alternative splicing of a single gene. NCAM 120 contains no intracellular domain, whereas NCAM 140 and 180 have different intracellular domains determined by alternative splicing of exon 18. NCAM has been described as a biomarker to discriminate small cell lung cancer (SCLC) from non-SCLC (NSCLC). However, peripheral blood mononuclear cells (PBMC) also express NCAM. We studied the expression of NCAM splice variants in cell lines, tumor tissues and control cells.

Methods: Using reverse transcriptase-PCR we evaluated the expression of NCAM exon 18 splice variants in lung cancers cell lines, control cell lines, PBMC of healthy controls and SCLC tissue. In addition we studied the expression of the NCAM exon 18 encoded protein (E18) in SCLC by immunocytochemistry and flow cytometry using an E18-specific monoclonal antibody obtained by hybridoma fusion of E18-immunized mouse spleen cells. Finally we looked at immune responses to E18 in mice.

Results: We found expression of RNA encoding the NCAM 180 variant in all SCLC cell lines. NCAM exon 18 was not expressed in 23/28 (82%) of the other tumor and leukemia cell lines tested and PBMC. Next, we also evaluated the expression of NCAM exon 18 in human SCLC tissue. Expression of NCAM exon 18 in 8 of the 10 (80%) SCLC biopsy samples was found. The newly raised E18-specific antibodies stained NCAM at the adherent junctions between adjacent cells in SCLC cell lines. The data demonstrate the intracellular location of E18 in SCLC. Furthermore, a specific cytotoxic T cell (CTL) response and significant antibody titers were found in mice upon immunization with recombinant E18 and its encoding DNA.

Conclusions: The results of this study can be applied in the diagnosis and immunotherapy of SCLC. A larger study investigating E18 as a marker for SCLC is indicated.

Keywords: Immunotherapy; small cell lung cancer (SCLC); tumor marker; alternative splicing; NCAM exon 18

Introduction

Small cell lung cancer (SCLC) represents 10% to 15% of clinical lung cancer cases. It is an aggressive malignancy strongly associated with smoking, annually killing an estimated 250,000 people worldwide. SCLC is decreasing in industrialized countries due to smoking cessation programs, but it is increasing in low- and middle-income countries (1).

In about two thirds of the cases SCLC is detected when metastatic lesions are already present (2). In those cases patients respond well to a first round of chemotherapy, but then invariably resistance to chemotherapy develops and the fast growth rate of resistant tumor cells will lead to death within a year from the initial diagnosis (1,3). Over the last decades survival rates for SCLC have not improved (4).

A limiting factor for the much needed improvement of therapies is the lack of biomarkers for SCLC. These markers, combined with serial 'liquid biopsies', would both allow early diagnosis and the monitoring of the effects of chemotherapy. Biomarkers could also be used to monitor the effect of new therapies.

In the past Neural Cell Adhesion Molecule (NCAM) has been investigated as a marker for SCLC and the wider group of neuroendocrine cancers to which SCLC belongs (5). NCAM, also known as CD56, is a member of the immunoglobulin superfamily (6). The protein is highly conserved between species where it is expressed by a variety of cells in the nervous system, in muscle cells (7) and on natural killer cells (8). In addition NCAM is expressed on a number of tumor cells, including SCLC (5). The presence of NCAM on SCLC is in line with the neuronal characteristics of these tumor cells (1,9).

Since NCAM is also present on normal cells it is not a 'perfect' tumor marker. Nevertheless, antibodies to NCAM conjugated to cytotoxic drugs have been studied in clinical trials as therapeutic agents. Patients with SCLC were treated with the anti-NCAM immunotoxin huN901-DM1, revealing acceptable toxicity and signs of clinical response (5). However, a later phase II study was discontinued due to poor results and a fatal outcome in one patient (http://investor.immunogen.com/releasedetail.cfm?ReleaseID=804315).

We took a closer look at the presence of the NCAM isoforms in SCLC and other cell types to investigate whether specific isoforms would be usable biomarkers. The human genome contains a single gene *NCAM1* for NCAM, located in band q23 of chromosome 11 (10). This single gene encodes several isoforms via alternative splicing. The major isoforms are NCAM-120, NCAM-140 and NCAM-180, each named according to its apparent molecular mass. These three molecules share the same extracellular domain. NCAM-120 lacks the transmembrane domain, encoded by exon 16, present in NCAM-140 and NCAM-180 (6). The NCAM-180 has a cytoplasmic tail that is 272 amino acids longer than NCAM-140 in man (*Figure 1*). These amino acids are encoded by NCAM exon 18.

During the development of the brain, expression of NCAM-180 is restricted to neural cells (11,12). Results from NCAM knock-out mice demonstrate that NCAM is crucial for the normal development of the brain and neuronal plasticity in the adult brain (13). Searching the *H. sapiens* expressed sequence tag library of GenBank with the DNA sequence of human NCAM exon 18 yielded 7 hits with a query score of >50% (October 26, 2017), five from fetal brain, one from thalamus and one from teratocarcinoma.

In this paper we describe studies on the expression of NCAM-180 in a panel of cell lines, tumor tissues and controls. We found that NCAM exon 18 is expressed in SCLC cell lines and confirmed NCAM exon 18 expression in human SCLC tumor tissue biopsies. No expression was found in most other cancer cell lines and PBMC of healthy controls. Using a recombinant protein E18, induced and purified from *E. coli*, a monoclonal antibody specific for E18 was produced. This antibody was used to characterize the expression of NCAM-180 in SCLC cell lines. Finally, we studied the immunogenic potential of E18 in a mouse model.

Methods

Cell culture, RNA extraction and cDNA synthesis

A panel of 44 cell lines (*Table 1*; obtained from the American Type Culture Collection®, Manassas, USA) was used. Peripheral blood mononuclear cells (PBMC) of 10 healthy individuals were used as controls. Growing cells were harvested, cell pellets washed with phosphate buffered saline (PBS) and total RNA extracted using Trizol (Invitrogen, Paisley, UK) according to standard procedures. For each cell line 2 µg of RNA was reverse transcribed into cDNA using Omniscript reverse transcriptase (Qiagen). Briefly, the RNA was incubated for 60 min at 37 ℃ with reverse transcription reaction mixture containing 0.5 mM of each dNTP, 1 µM oligo-dT primer (Eurogentec SA, Seraing, Belgium), 10 units RNase inhibitor (Applied Biosystems, Foster City, CA, USA)

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   1 GCAAAGTAACAGTGTTTTTGCCTCATTTTA CAGATGAAGGGACTGAGGCAGGGTGATTAA ATCTGAAGCTCAAGGTCACACAGCTAGCAA
  91 GGGTTGGGCTGAGACTTATACATTTGAATC TGTTGGCTCTGGATGCCCACCGTGCCAGCC TTGGGTTGAGTCATAGCTGCTCTTCCGTGG
 181 GAGCCCCTCCCTGCAGCTGCCCTAGGGTCT AACCAGCAGTACTGTCTCCACAGGAAAGAT GAGTCCAAGGAGCCCATCGTGGAGGTTCGA
                                                             K  D    E  S  K  E  P  I  V  E  V  R
 271 ACGGAGGAGGAGAGGACCCCAAACCATGAT GGAGGGAAACACACAGAGCCCAACGAGACC ACGCCACTGACGGAGCCCGAGTACGTGGGC
      T  E  E  R  T  P  N  H  D    G  G  K  H  T  E  P  N  E
 361 TGGGAGGGGGCTGGCACCTGCTCCGTAGAGA CCCCCACACCCACCTCCCCACCCTACCCCC ACCTCCCGTGCCCCTACCCACAGCTCCCGG
 451 CCAAACAGTTCAGGCCAGGATTCCCAAACCC TTGGCCACAAGACCTGCTTGGCTGAGAAAT GCCTGACCTCAGGCATTTCTGGTTCTATTT
 541 CGGTTGGCTGTGGGTACCCTTAGAAGGCTCC TGTATAAGTAGGGATCCCTGGATCCCTAGC CCCTGTTCTCAAGTTATACACATATTCTGG
 631 AAATGGCTTAAGGTGGCAAAATCTCACTGA AAGGCTTAGCAGAGTCTGCTTAGGATTTCT TGAAATTCTAGGGTTTTGTGTTTTCCCCCT
 721 GAAAGGATAAGGGAAGCTGGGGACCTGGTA AAGTTCTCTGCCCCTCCTCTGTTCAGAAAC CTCGGGGACCCACGGAGCCCCAGACCAGTT
 811 GTGGTTGTAATGCCTGCCTAGCTGTCAAGG ATCTTATGGGCTTAAAGGGAATGGAGTTTC AGGGAACCACAAAATCTGTGACTGGAAGAA
 901 CCCACAGCTCCTATGAGCCATTCTGAGTAC TCGTGATCTGCCTTATTCGTGACTTTTCCC ATCGGTGCCCTGGGGAGCCCTGCAGGACAG
 991 CTGGGCCCAGTTCCTCTGGGACCTGGGAAG CCTAGAGCAGGTGGATGCTGAACAGAAGCC TGCCCCTCCTTGAATGGAGATCCAGGACAG
1081 GGATCAGGAAGCCCTAGGCAGGTCTGCAAG GACTTCAGGGAAGGGAGCTCTATGCAGAG AGCTGGAGAGACATCAGCAGGGAAGGGCTA
1171 GCAGGTCCCTTTTGAGACCGCCACGTTCTC TGTCGTCTCCTGTGTCCTCTTCTGCCCACC TGGGCGTGTGCTTCCTCTCAGCATCCTTCT
1261 CTCTCTGTGGGCCTGTGTCCGTGCCTGTGC CTAACTGCATGCCCCCACCCCCATGGTCCC TGTCCTGGGCTTCAGCCCTCCTCCTCGCTT
1351 CTCTGTCACGTCTCAGCTTCATTGTCTATT CTCTTCTACTTCCTGTCCTCCCACAGGCTG CCTGCCGACACTACGGCCACTGTCGAGGAC
                                                             L    P  A  D  T  T  A  T  V  E  D
1441 ATGCTGCCTTCTGTCACCACCGTCACCACT AACTCTGACACTATCACCGAAACCTTTGCC ACTGCTCAGAACAGCCCCACCAGTGAGACC
      M  L  P  S  V  T  T  V  T  T    N  S  D  T  I  T  E  T  F  A    T  A  Q  N  S  P  T  S  E  T
1531 ACCACCCTGACCTCCAGTATTGCCCCGCCG GCCACGGCCACGCCTGACTCAAACTCTGTA CCGGCTGGCCAGGCCACCCCTTCCAAGGGG
      T  T  L  T  S  S  I  A  P  P    A  T  A  T  P  D  S  N  S  V    P  A  G  Q  A  T  P  S  K  G
1621 CCCAGCGCCTCTGCCCCCTCCCCGGCCCCA GCTTCAGCCCCCAAGGTCGCCCCCCTCGTT GACCTGAGCGACACCCCGACCTCAACCCCT
      P  S  A  S  A  P  S  P  A  P    A  S  A  P  K  V  A  P  L  V    D  L  S  D  T  P  T  S  T  P
1711 GCCGCTAGCAATTTGTCTTCTAGTGTCCTG GCTAACCAAGGGGCTGTCCTCAGCCCAAGC GCCCCTGCTGGTGTCGGGGAGGCCTCTAAG
      A  A  S  N  L  S  S  S  V  L    A  N  Q  G  A  V  L  S  P  S    A  P  A  G  V  G  E  A  S  K
1801 GCTCCTCCGGCCAGCAAGCCCACCCCTGCA CCAGTCCCCACCCCGACTGGGGCAGCCAGT CCTCTAGCAGCAGCGGCTGCCCCTGCCACA
      A  P  P  A  S  K  P  T  P  A    P  V  P  T  P  T  G  A  A  S    P  L  A  A  A  A  P  A  T
1891 GAAGCCCCTCAGGCCAAGCAGGAGGCTCCC AGCACGAAGGGCCCGGACCCGGAGCCCACC GACCCCGGAGCCGCGAAGAGCCCGGCCGAG
      E  A  P  Q  A  K  Q  E  A  P    S  T  K  G  P  D  P  E  P  T    Q  P  G  A  A  K  S  P  A  E
1981 GCAGCCACAGCCCTTGCTAGCCCGAAGAGC GAGGCTGCCTCCGTCAGCACCACAAACCCT TCCCAGGGCGAGGACTTTAAAATGGACGAA
      A  A  T  A  L  A  S  P  K  S    E  A  A  S  V  S  T  T  N  P    S  Q  G  E  D  F  K  M  D  E
2071 GGGAACTTCAAGACCCCAGATATTGACCTT GCAAAGGATGTTTTTGCAGCCCTGGGCTCT CCTGCTCCCGCCGCTGGGGCCAGTGGACAA
      G  N  F  K  T  P  D  I  D  L    A  K  D  V  F  A  A  L  G  S    P  A  P  A  A  G  A  S  G  Q
2161 GCCCCTGAGCTTGCTCCTTCCACTGCAGAC AGCTCTGTTTCGCCTGCGCCAGCAAAGACC GAGTACGGCCTCTCTTTGTCTGCTGTCATC
      A  P  E  L  A  P  S  T  A  D    S  S  V  S  P  A  P  A  K  T    E
2251 GGGTTGTGTCCTGTGGTGGTGTGCATTTGT GCTGTGCTGTGTCCTCCTTGTTAGATGTGT CTTCAGCCTCATCCAGGGCTTCTCTGAGGC
2341 GACCGTCAACCAAGGGGCTGGGATAAGGCA GAGAGAGCAGGACAGGCAGCTGGGCCCAG GAGCAGCCGCCTGCTGGCTAATTAGGCCAT
2431 GTTAATTATGTTTTATCCTTATCATCCTAG CAGCGGCTTCTGTTTTCCCTGGGGGAGCCC TTGCAGTCAGGCCACTGATGAGAATAGCTT
2521 GGGAAGCAGGCTAAGAAGGCCAAGGTGCCC TTATCAGCCACCCTGGGGCCCCCAGTCGGT GTGTTATTCAGTGCCAGGCTGAGTCCCTAC
2611 CAGAAAGGCACTGATGTGCAGGAAGAAAAG AGGTAGGCCCCAGGCCCGTGGCAGGGGAGG GTGCAGTTCAGAGGTCCCCTCACACTGTGG

2701 CTCAGATGCTCACCCTCCCCTCCCAACCCC GGCCCCCAGCCCACTCTTGCTTCTCCTGCT CTTAGAGTGGCTGGGAAGACTCGGGGCTCC
2791 CCAGCATCAAATGGCTGCTGGGTCTCAAAG TTCAAGAGAAAGGCCTTAACCTTTCAGGTT TGTAAGTGTCTTGCCAAAATTCAGTGGCAC
2881 CAGCTGAGGCTGGGGTCTGTGCCTGTCCCC ATGCATCCTCAGACCCGACTCCCTTTCCTG GAACTGATGGAGGCAGAGTCAGACCTAGAT
2971 TTTTATAGAGGCTCCTATGGCTGCTCCCCA GATAAGCTGCCATGCTGCCACCATGGGCTG CAAGGGGCTCTCGCCAGGAGCCCTGAATCT
3061 CAGCATCGGGAGGGAAAAGGACTCTGATAG CCCTGCCCACTCAGCTGCCCTCAACTCTCG CATAGCCACCAGGCCCACTCTAGTACCCCA
3151 CCAGGCTGCCATGAAAAGCTGCCTCCATCA GTGTTTTCTCACCCTACCCAGGCAGGCCCT AGAGGCATCATCCTGCAACAGCACAGGGTG
3241 GGGTGGGGAAGGGACAGGCAAGAGTGGGTT GCAAAGGGGCCCCTGAAATCAGTGCTGGGA GCCCAGCCCAGCCCAGCAGGCTGCATTTAC
3331 ATCCAGCTGAGCTGTTGCCACTGCAGCTGG TCAGGGTGAGGAGGAGCTGCCTCTGCTGCC CCCTTCCACCACCCCAGGTAAAAACACAGC
3421 CTCTGGCATCAACTTAGGGCTGGAAGAGGA AAGGACTCGTTCTTCTGACTCAGTGCTTCT CAACTATGGCTGCACATTAGAGTCACTTGG
3511 GTGCTTTTAAAAGTCACTGTGTCCGGACCA ATTAAATCAGGATTTCCGCGAACACAGCTC GGGCTTGGGTGATTTTTAGTGCTCCCAGGT
3601 GATTTGACGGGCAGAGGTTGGAGCCGGGTG CTGTCACTGGAGAACCCCTCCTCCTCCCTG CTGTCCCCAAGGCCTGGATGCAGGGGCGAG
3691 ATGTCTGCAGACCGTGGTCTCAGTGGTTCT GTTTTTCCTGATTCTCTGCAGGAAGGGCCCC GTAGAAGCAAAGCCAGAGTGCCAGGAGACA
                                                             K  G  P    V  E  A  K  P  E  C  Q  E  T
3781 GAAACGAAGCCAGCGCCAGCCGAAGTCAAG ACGGTCCCCAATGACGCCACACAGACAAAG GAGAACGAGAGCAAAGCATGATGGGTGAAG
      E  T  K  P  A  P  A  E  V  K    T  V  P  N  D  A  T  Q  T  K    E  N  E  S  K  A
3871 AGAACCGAGCAAAGATCAAAATAAAAAGTG ACACAGCAGCTTCACCAGAGCATTTCCAAC ACCACAGACACACACACGCACGCACACACA
3961 CAAACACACACATGCACACACACACATCTCAT TTCTCTAGT
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Figure 1 Sequence of the region of the human *NCAM1* gene encoding exons 17, 18 and 19. The protein sequences present in the NCAM 180 kDa splice variant are indicated. Bold nucleotides indicate the position of the PCR primers used: Forward exon 17 (5'CAAACCATGATGGAGGGAAA3'), forward exon 18 (5'CCACCGTCACCACTAACTCTGACACTATCAC3'), reverse exon 18 (3'GT TGGGAAGGGTCCCGCTCCTGAAATT5') and reverse exon 19 (3'CCTCTTGCTCTCGTTTCGTA5').

Table 1 Overview of cell lines tested by reverse transcriptase PCR for the expression of NCAM1 exon 18

Cell line	Cancer type	NCAM 140	NCAM 180
HS-10A	SCLC (carcinoma)	++	++
H69	SCLC (classic)	++	++
H82	SCLC (variant)	+	++
GLC-1	SCLC (variant)	+	++
GLC-1 M13	NSCLC (classic)	+	++
H1437	NSCLC adenocarcinoma	–	–
H520	NSCLC	+	–
H1299	NSCLC (from lymph node metastasis)	–	–
H727	NSCLC	+/–	–
MR65	NSCLC	–	–
A549	Non-small cell bronchoepithelial carcinoma	–	–
H1792	Lung adenocarcinoma (from metastatic site: pleural effusion)	–	–
H460	Large cell lung cancer (from metastatic site: pleural effusion)	++	+
H720	Atypical lung carcinoid	+	–
PC3	Prostate adenocarcinoma (from bone metastasis)	–	–
MDA-MB-435S	human breast carcinoma (from metastatic site: pleural effusion)	–	–
JAR	Placenta choriocarcinoma (often metastatic)	++	+
A375	Skin malignant melanoma	++	–
A431	Skin squamous carcinoma	–	–
SUM 159PT	Anaplastic carcinoma	–	–
HT29	Epithelial colon adenocarcinoma	–	–
Colo205	Colon adenocarcinoma	–	–
HCT 116	Colon carcinoma	–	–
MCF7	Breast adenocarcinoma	–	–
MCF7-10A	Nontumorigenic breast epithelial cells (very low ER)	–	–
MDA-MB-239	Breast carcinoma	–	–
SUM 149PT	Breast cancer intraductal carcinoma	–	–
A2780	Human ovary carcinoma	–	–
Hela	Cervix cancer	–	–
LnCap	Human prostate carcinoma	–	–
DU145	Human prostate cancer	–	–
SJSA	Osteosarcoma	++	+
HL60	Promyelocytic leukemia	–	–
Jurkat	T cell lymphoma	–	–
Molt-4	Human acute lymphoblastic leukemia	–	–

Table 1 (*continued*)

Table 1 (*continued*)

Cell line	Cancer type	NCAM 140	NCAM 180
K562	Lymphoblast chronic myeloid leukemia	–	–
SH-SYSY	Human neuroblastoma	++	+
CCI	Astrocytoma	+	+/–
U87MG	Brain glioblastoma-astrocytoma	+	–
CM	Insulinom	–	–
Bon-1	human endocrine pancreatic tumor cell line	+/–	–
QGP	human pancreatic islet culture	+	–
PBMC_1	PBMCs of healthy control	+	–
PBMC_2	PBMCs of healthy control	+	–
PBMC_3	PBMCs of healthy control	+	–
PBMC_4	PBMCs of healthy control	+	–
PBMC_5	PBMCs of healthy control	+	–
PBMC_6	PBMCs of healthy control	+	–
PBMC_7	PBMCs of healthy control	+	–
PBMC_8	PBMCs of healthy control	+	–
PBMC_9	PBMCs of healthy control	+	–
PBMC_10	PBMCs of healthy control	+	–
NHK-10	Normal human keratinocytes	–	–
CH-ME-3	Human foetal microglial cell	–	–

+/–, + and ++: PCR product, weak to strong signal; –: no product. NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; PBMC, peripheral blood mononuclear cell; NCAM, Neural Cell Adhesion Molecule.

and 4 units Omniscript reverse transcriptase (Roche, Applied science, Indianapolis, USA) in 20 µL total reaction volume. Human tumor biopsies: we used 10 sections (10 µm thickness) of the frozen biopsies for RNA extraction and subsequent cDNA synthesis. Frozen sections were homogenized using a Qiashredder homogenizer and total RNA was subsequently extracted using the RNeasy Micro kit (Qiagen). cDNA synthesis was performed using the First strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany). Quality control of the newly synthesized cDNA was done using a human β-actin-based PCR amplification reaction.

Differential expression PCR analysis of NCAM splice variants

PCR amplification was performed according to standard procedures. Briefly, 0.2 µL cDNA was added to an amplification mixture (20 µL) containing 2 µL 10× PCR gold buffer (Applied Biosystems Foster City, CA, USA), 1.6 µL MgCl2 (25 mM), 0.2 µL dNTP (10 mM, Promega, Madison USA), 0.1 µL forward primer (0.5 µM), 0.1 µL reverse primer (0.5 µM) and 0.2 µL *Taq* polymerase (AmpliTaq Gold, 250 Units, Applied Biosystems). Nucleotide sequences of forward and reverse primers for the two NCAM splice variants studied are shown in *Figure 1*. As a control primers for the β-actin mRNA (forward, 5'GCCAACCGCGAGAAGATGAC3'; reverse, 5'GATGGGCACAGTGTGGGTGAC3') were used. PCR reactions were performed in a T3 Thermocycler (Biometra, Westburg, Leusden, The Netherlands) and conditions were as follows: 30 s at 94 ℃, 30 s at 60 ℃, 1 min at 72 ℃ for 35 cycles, initiated by 12 min at 94 ℃ and terminated by 10 min at 72 ℃. PCR products were loaded on 2% agarose gels. Detection was performed using Gelstar (Gelstar Nuclei acid gel stain, Cambrex, Rockland, ME, USA).

Production of recombinant E18 protein

His-tagged E18 protein was produced in *E. coli* BL21(DE3) pLysS using the pRSET based high-level recombinant protein expression system (Invitrogen Ltd, Paisley PA4 9RF, UK). For the isolation of the protein, bacterial pellets were lysed in 5 mL lysis buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8.0) with lysozyme (1 mg/mL), DNase (5 µg/mL) and protease inhibitors (HALT^{TM} Protease inhibitors EDTA-free, Thermo Fisher Scientific Inc., Waltham, MA, USA). The recombinant protein was purified on a Ni^{2+}-NTA agarose column (Qiagen, Venlo, The Netherlands). The isolated protein was eluted by imidazole (300 mM) and renatured by stepwise dialysis against PBS (4M, 2M, 1M, 0.5M urea, 1× PBS).

Production of E18-specific monoclonal antibodies

After medical ethical approval of the study protocol, Balb/c mice were immunized with purified His-tagged E18 protein from *E. coli*. We used subcutaneous immunization with 10 µg of protein in PBS. Mice were immunized 3 times with 3 weeks intervals. A final boost was given 3 days before the animals were sacrificed. Complete spleens were squeezed through sterile 70 µm nylon filters in culture medium and used to prepare hybridoma cells by standard procedures. Hybridoma clones were isolated and the secreted monoclonal antibodies were tested by ELISA. Positive clones were subcloned and then grown in a 'miniPerm' bioreactor according to standard procedures (14). The IgM monoclonal antibodies from the supernatant of the miniPerm culture were first isolated on a ConA affinity column (PBS, pH 7.4) and subsequently eluted using 0.5 M α-methyl mannoside in PBS of pH 7.4. The eluted fraction of the ConA column was subsequently purified on a Superose-6 gel filtration column and filtered through a 5-µm filter.

ELISA to determine E18-specific monoclonal antibody titers

To test the antigen specificity of the monoclonal antibodies ELISA plates were coated with 100 µL of a 10 µg/mL His-tagged E18 protein solution in 50 mM NaHCO₃, pH 9.5. After overnight coating at 4 ℃, plates were washed three times with washing buffer [PBS with 0.05% (v/v) Tween-20] and subsequently blocked for 2 h at 37 ℃ with blocking buffer (2% non-fat dry milk, Nutricia Protifar Plus) in washing buffer. The purified E18 monoclonal antibody was serially diluted in blocking buffer (0–130 ng/well) and incubated for 2 h at 37 ℃. Plates were washed three times

and bound antibodies detected using a polyclonal rabbit anti-mouse immunoglobulin conjugated with horseradish peroxidase (Dakocytomation, Denmark) (1:1,000 in blocking buffer). The conjugate was incubated for 1 h at 37 ℃. Then the plates were washed and the substrate 3,3',5,5'-tetramethylbenzidine (Calbiochem) added for detection. The substrate was incubated for 15 minutes at 37 ℃, the enzymatic reaction stopped with 0.5M H₂SO₄, and staining measured at 450 nm using a 96 multiwell plate based reading platform (Multiscan spectrum, Thermolab systems, Finland). To check for the presence of antibodies against the His-tag of the protein as used for the immunization, a control ELISA was performed with irrelevant His-tagged proteins produced and purified like the E18 protein.

Immunocytochemistry for E18 in SCLC cell lines

Antigen specificity of E18 antibodies was tested using immuno-cytochemistry. Therefore NCI-H69 and NCI-H82 cells were collected by centrifugation from the cell culture medium. Cell pellets were washed, resuspended in PBS and subsequently spotted on 18-wells, 4 mm diagnostic microscope slides (Cell-line, Brunschweig, Germany). The negative control cell line HCT116 was trypsinized, seeded in culture medium and grown overnight in a 12 well plate with a 18 mm glass slide on the bottom of the well. Before immunostaining, cells were permeabilized and fixed for 30 sec in methanol/acetone at –20 ℃ and incubated for 1h at room temperature with the primary antibodies against E18 or NCAM (RNL-1, 1 mg/mL, 1:100 diluted in PBS with 0.1% BSA; Nordic-Mubio, Susteren, The Netherlands). Then, cells were washed in PBS, and incubated for 1h at room temperature with the fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (diluted 1:100 in PBS with 0.1% BSA). Stained cells were washed and stepwise de-hydrated in alcohol, cells were mounted in glycerol and viewed through a fluorescent microscope (Axioskop, Zeiss, Germany). Stained cells were also visualized with a Zeiss confocal imaging system [Stack scan mode, scaling: X: 0.1 µm, Y: 0.1 µm, stack size: X: 50.19 µm, Y: 32.43 µm, Scan zoom 2.8, Plan-apochromat objective (63×/1.40 Oil DIC M27, excitation at 488 nm].

Flow cytometry of NCI-H82 cells stained with the E18-specific monoclonal antibody

NCI-H82 cells, expressing NCAM-180, were stained in suspension with the purified E18 monoclonal antibody.

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The cells were harvested by centrifugation and washed twice with PBS. Half of the cells were permeabilized for 15 min with ice-cold methanol. Both, permeabilized and non-permeabilized cells were resuspended in buffer (PBS containing 0.1% NaN3 and 0.1% BSA). Staining was done for permeabilized and non-permeabilized cells (5×10^5 cells in 100 µL buffer) using the purified E18 monoclonal antibody (1.3 mg/mL, 200 µL, 1:400 diluted in PBS with 1% BSA). Permeabilized and non-permeabilized cells were stained for 1 h at 4 ℃, washed twice with buffer, resuspended in 100 µL buffer and incubated for 1 h with 5 µL undiluted rabbit anti-mouse FITC (Dako, F0313). An incubation with secondary antibody alone was used as a control. Cells were washed twice and resuspended in 200 µL buffer. Fluorescence was measured using Cellquest software on a FACSCalibur (BD, FACSCalibur).

Immunisation and antigen specific cytotoxic T cells (CTL) measurement

Mice (Balb/c) were immunized with E18 protein and the E18-encoding DNA in the pCI mammalian expression vector (Promega). Immunization was subcutaneously with protein [12 µg protein in Abisco-100 (Isconova Sweden) or 10 µg protein nonformulated] and intramuscularly with DNA (2 times 50 µg DNA). Immunization was performed at the day 0 and day 21. Immunized animals were sacrificed at day 31. Mice spleens were isolated and squeezed to prepare a single cell suspension. Spleen cell suspensions were seeded for *in vitro* restimulation at a cell density of 1.5×10^6 cells/well (100 µL). *In vitro* restimulation was performed in four replicate samples for each spleen suspension. Restimulation was performed with a 9-mer peptides covering the E18 protein sequence (Pepscan, Lelystad, The Netherlands). A total of 67 peptides (9-mers, 5 amino acids overlap) were divided over 3 mixtures each containing 22 peptides (0.2 µg of each peptide/well). Control restimulation was performed with medium (negative control) and PMA/ionomycin (0.05 µg/mL/0.5 µg/mL) (positive control) and an irrelevant peptide mixture (overlapping peptides from the NCAM 7pi8 region, 17 peptides, 0.2 µg of each peptide/well). For all restimulations intracellular transport of cytokines was inhibited by adding 5 µg/mL Brefeldin A. Restimulation was performed for 4 hours at 37 ℃ in 5% CO_2. Cell surface CD8 was stained using the FITC-labeled rat anti-mouse CD8 (BD Biosciences, Breda, The Netherlands). Cells were fixed and permeabilized according to standard procedures and subsequently stained for the intra-cellular cytokine

IFN-γ using the APC-conjugated rat anti-mouse IFN-γ (BD, Biosciences). The proportion of IFN-γ secreting CD8+ positive cells as measured by FACS represents the number of antigen specific CD8+ T cells (CTL).
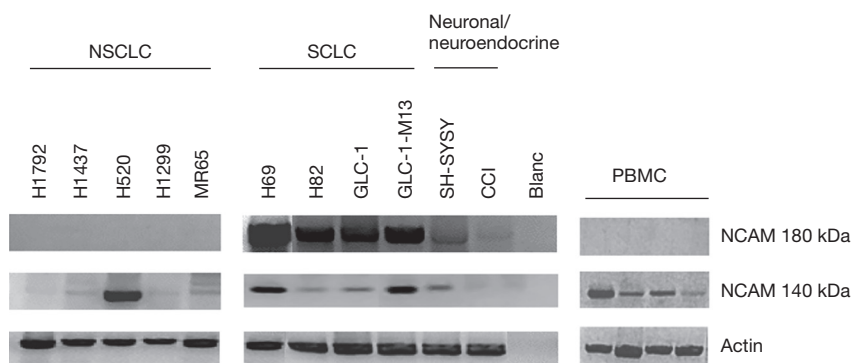
Results

Identification of coding regions in the NCAM1 gene and development of assays to detect NCAM isoforms

The NCAM protein sequence is present in GenBank under Accession number EAW67201. It consists of 1,119 amino acids and has, without posttranslational modifications, a calculated molecular weight of 119,447. The sequence of a complete transcript of the human *NCAM1* gene, containing all 19 exons was not present in the databases. The sequences were reconstructed starting with the DNA sequence containing the NCAM region determined by Hattori *et al.* (Homo sapiens genomic DNA, chromosome 11q, clone:RP11-839D17, complete sequence, GenBank: AP000880.4). The region containing nucleotides 60,000–64,000 of the sequence was translated into protein. Exons 17, 18 and 19 were identified in the same reading frame (*Figure 1*). The contiguous sequence of the exon 17–19 translation products was identical to the corresponding part of EAW67201. The exon 18 encoded part of NCAM is 272 amino acids long and has a molecular weight of 26,134.

PCR primers were designed for differential expression PCR studies of exon 18. The positions of the primers are indicated in *Figure 1*. The primer sequences annealing within exon 18 result in a PCR amplicon of 604 bp upon expression of NCAM-180 and no PCR amplicon when the NCAM-140 kDa is expressed. A second PCR was done to study the expression of NCAM-140. The forward primer in exon 17 and a reverse primer in exon 19 result in a 180 bp PCR amplicon for the NCAM-140 splice variant. The expression of NCAM exon 18, was confirmed using the exon 17 forward primer with the exon 18 reverse primer, yielding a 715 bp amplicon for NCI-H82 cells. This PCR excludes that PCR amplicons as found in the PCR done with a forward and reverse primer within the exon 18 are due to a contamination of the cDNA sample with genomic DNA. When exon 18 is expressed, a 715 bp PCR product will be formed. Actin control PCRs were used throughout.

Expression of the exon 18 in SCLC cell lines

RT-PCR was performed to study expression of NCAM

Figure 2 Expression of NCAM 140 and 180 kDa variants in cancer cell lines and peripheral blood monocytes of healthy donors. Forward and reverse exon 18 primers were used to detect NCAM 180 expression, resulting in a 604 bp amplicon. Forward exon 17 and exon 19 reverse primers were used to detect NCAM-140 expression. The presence of a 180 bp amplicon was indicative for NCAM-140 expression. As a control to exclude contamination with genomic DNA a PCR with forward exon 17 primer and reverse exon 18 primer was carried out on cell line H82, resulting in a 715 bp amplicon (data not shown). NSCLC, non-small cell lung cancer; PBMC, peripheral blood mononuclear cell; NCAM, Neural Cell Adhesion Molecule.

exon 18. Results are summarized in *Table 1* and *Figure 2*. NCAM-180 expression was found in 1/9 NSCLC and 2/6 neuronal and neuroendocrine tumors. None of the leukemia cell lines, and among the non-lung cancer cell lines only the placenta choriocarcinoma (JAR) and the osteosarcoma (SJSA) expressed NCAM-180. Our data show a significant expression of exon 18 for all (5/5) SCLC cell lines studied (4 of these are shown in *Figure 2*). Exon 18 was also expressed in cell lines SH-SYSY and CCI (*Figure 2*). In none of the PBMC of healthy controls (0/10) exon 18 expression was found. We sequenced the expressed exon 18 in SCLC cell lines to check for potential sequence mutations compared to the sequence indicated in *Figure 1*. A completely identical nucleotide sequence (815 bp) was found for the SCLC cell lines.

NCAM-180 expression in SCLC tumors

To determine whether the results obtained in SCLC cell lines are a reflection of the *in vivo* situation SCLC tumor biopsies were investigated. RNA was extracted from 10 sections (10 μm) for each of the 10 frozen SCLC tumor biopsies. cDNA was synthesized and differential expression PCR performed as described before. We also amplified exon 18 both using the forward-reverse primer combination as well as the NCAM exon 17 forward primer combined with the exon 18 reverse primer. We used the cDNA of the NCI-H82 cell line as a positive control and RNA extracted from a squamous cell carcinoma biopsy as

a negative control. Our PCR data show exon 18 expression in 8/10 (80%) of the SCLC tumors studied (*Figure 3*). The signals varied, from strong (S_9) to medium (S_1, S_2, S_3, and S_{10}). For the samples S_4-S_8, a nested PCR was done. In this case, cDNA was preamplified using the NCAM exon 16 forward and exon 19 reverse primer, followed by further amplification with exon 17 forward and exon 18 reverse primers. This resulted in a signal in S_4, S_6 S_7 but not in in S_5 and S_8. The differences in expression levels in the different SCLC tumor samples are difficult to interpret. No normalization was made for the size of the tumor nor the ratio of tumor versus normal tissue taken during biopsy sampling. Moreover, the amplification reactions were not quantitative. Nevertheless, our results clearly show the expression of NCAM exon 18 in human SCLC tumors. These data confirm the differential expression studies for *in vitro* cultured SCLC cell lines and indicate that E18 is a SCLC-specific tumor antigen.

Production and purification of recombinant E18 in E. coli

To facilitate further studies we produced recombinant E18 in *E. coli*. The E18 coding region was PCR-amplified from cDNA of NCI-H69 cells. The recombinant protein, including the His-tag is 314 amino acids, and the predicted molecular mass is 30.6 kDa (*Figure S1*). The purified protein was analyzed by SDS-PAGE, and the sequence was analyzed using liquid chromatography electrospray ionization tandem mass spectrometry. SDS-PAGE showed

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Nested PCR
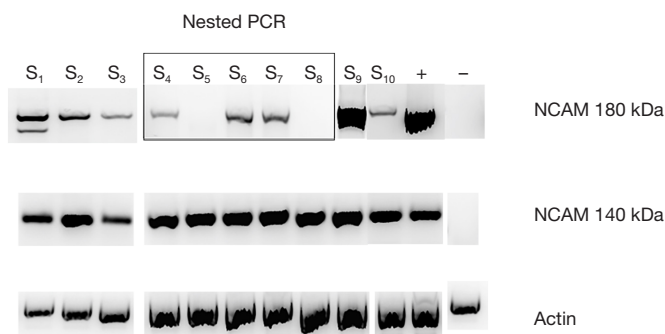
Figure 3 Detection of NCAM-180 and NCAM-140 expression in SCLC tumors. Forward exon 17 primer and revers exon 18 primer were used to detect NCAM-180 expression. Samples S4 to S8 were preamplified with 16F (5'GTGGCCTGTTCATGTGCATT3'), located in the coding region of exon 16 and exon 19 reverse primer. NCAM, Neural Cell Adhesion Molecule; SCLC, small cell lung cancer.

that the protein is partially present as a dimer. Sequence analysis however confirmed the E18 sequence of the protein. Why the protein formed dimers is not clear.

Monoclonal antibody production

Balb/c mice were immunized with a truncated E18 protein (*Figure S1*). The hybridoma clone with the highest specificity was selected (MUMi-21B2. As shown in Supplementary *Figure S2*, panel A, the selected hybridoma clone produced antibodies binding to the E18 recombinant protein. No binding was found when ELISA plates were coated with His-tagged NCAM exon 8–10 or NCAM exon 15–17 proteins produced and purified from *E. coli* or other unrelated proteins. Thus the monoclonal antibody is specific for the E18 and is not cross reactive with the His-tag as expressed at the N-terminus of the purified proteins or with *E. coli* proteins potentially left as contaminants in the purified protein as used for immunization. Isotyping showed that the monoclonal antibody was of the IgM type. Sensitivity of the MUMi-21B2 monoclonal antibody was confirmed in a dose-response ELISA. Plates were coated with serially diluted recombinant protein. Serially diluted coating was confirmed using the anti-His polyclonal antibody detection (bars in *Figure S1*). As shown in the same Figure (bars in *Figure S2*, panel B), the purified monoclonal antibody specifically binds the serially diluted recombinant protein as coated to the plates. The data show that the optimal antibody concentration is about 4 ng antibody/ well, and that the sensitivity is 10–20 ng/mL in the ELISA as used. Thus, the MUMi-21B2 monoclonal antibody is specific for the E18 protein with a sensitivity of 10–20 ng/

mL. The monoclonal antibody recognizes an epitope in the recombinant protein produced in *E. coli*. In this host no posttranslational modification occurs. To determine whether the same epitope also is available in *in vivo* expressed eukaryotic proteins we performed immunocytochemistry to test the purified MUMi-21B2 monoclonal antibody for binding to E18 present in cultured SCLC cell lines.

NCAM 180 staining in SCLC

The SCLC cell lines NCI-H69 and NCI-H82 showed clear expression of exon 18 in the RT-PCR. We performed immunocytochemistry on these cell lines with the MUMi-21B2 antibody. The cell lines were spotted on glass slides, fixed and permeabilized with ice-cold methanol and acetone and probed with the MUMi-21B2 monoclonal antibody. Staining with the RNL-1 monoclonal antibody, a commercially available NCAM-specific antibody, was used as a positive control. RNL-1 recognizes an extracellular epitope of the NCAM protein. The MUMi-21B2 antibody recognizes the cytoplasmic E18 part of the NCAM protein and only stains cells expressing the NCAM-180. *Figure 4A*, shows a strong reaction of the MUMi-21B2 monoclonal antibody, comparable to staining with antibody RNL-1 (*Figure 4*, panel B). No staining is observed in the HCT116 cell line, derived from colon carcinoma. The specific membrane bound localization of E18 is better shown in confocal images. *Figure 4C* shows NCI-H69 cells stained with the MUMi-21B2 monoclonal antibody. Going step-by-step through the depth of the stained cells, we observed a strong binding of the antibody at the cell-cell inter-phase of adherent cells.
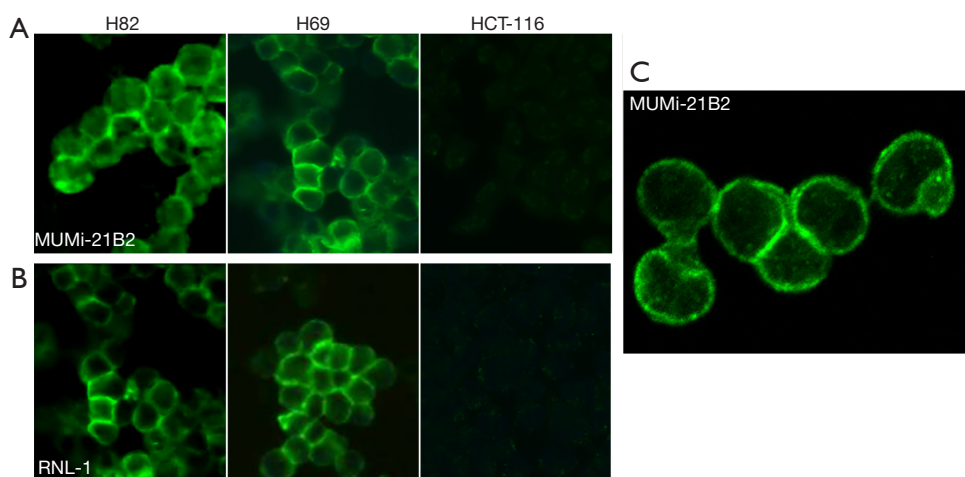
Figure 4 Localisation of E18 in SCLC cell lines. (A) Immunostaining of SCLC cell lines H82 and H69 with monoclonal antibody MUMi-21B. Cell line HCT116 (*Table 1*) was used as a negative control; (B) antibody RNL-1 against the extracellular domain of NCAM was used as a positive control; (C) confocal microscopy shows that the epitope recognized by MUMi-21B is located close to the cell membrane. NCAM, Neural Cell Adhesion Molecule; SCLC, small cell lung cancer.

Cytoplasmic expression of E18

To confirm the intracellular location of E18 we used NCI-H82 cells. Binding of the monoclonal antibody MUMi-21B2 was evaluated for both permeabilized and non-permeabilized cells and fluorescence intensity was measured by flow cytometry. Our data clearly show that the MUMi-21B2 antibody binds to a cytoplasmic part of the NCAM protein. No binding of the antibody was found when non-permeabilized NCI-H82 cells were stained. As shown in *Figure S3*, panel A, staining of non-permeabilized NCI-H82 cells, results in a fluorescence intensity peak comparable to the fluorescence of staining with a secondary antibody alone (*Figure S3*, panel B). After permeabilization on the other hand, a shift of the peak to higher fluorescence intensity is found (*Figure S3*, panel A). This higher intensity is comparable to the intensity of the peak as measured for the RNL-1 antibody staining of NCI-H82 cells. For RNL-1 however, no difference in fluorescence is found for permeabilized versus non-permeabilized cells, because the RNL-1 monoclonal antibody binds to an extracellular NCAM epitope and the binding does not depend on the permeabilization of the NCI-H82 cells (*Figure S3*, panel C). These data demonstrate that MUMi-21B2 antibody specifically binds to a cytoplasmic part of the NCAM protein.
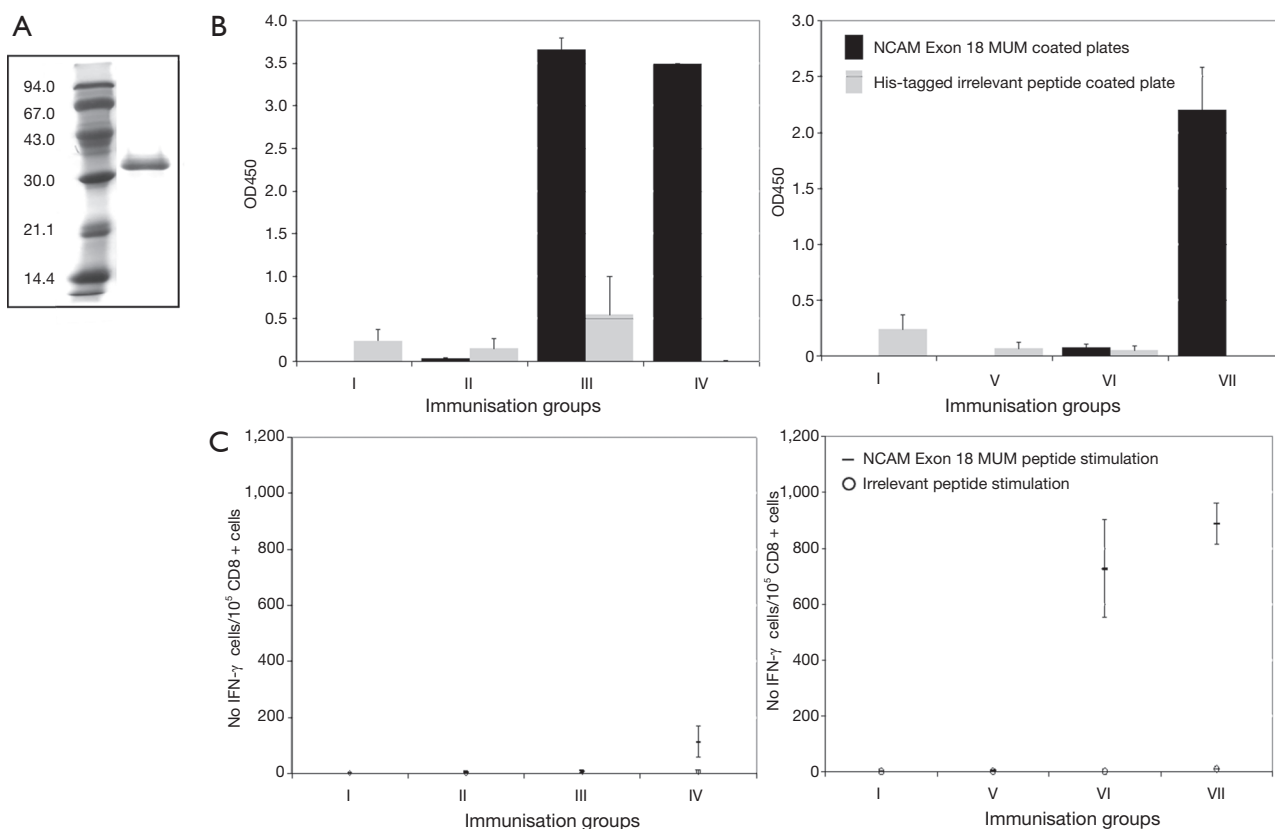
Antibody response in E18- immunized mice

Balb/c mice were immunized with E18 protein and plasmid

DNA. SDS-PAGE analysis of the antigen revealed a pure protein, without dimers, with the expected molecular mass (32 kDa) (*Figure 5A*). The His-tagged recombinant protein antigen was inoculated either as suspension in PBS or formulated in Abisco-100. DNA immunization was performed by intramuscular injection of the mammalian expression vector pCI containing the exon 18 encoding DNA. Mice were immunized at day 0 and boosted on day 21. Read-out was performed on day 31 after the first immunization. Animals immunized with E18 protein both in PBS or formulated in Abisco-100 developed anti-E18 specific serum antibody titers as shown in *Figure 5B* group III and IV (dark bars). No binding of serum antibodies was shown when ELISA was performed with plates coated with His-tagged irrelevant protein that was also produced in *E. coli* [*Figure 5B* group III and IV (grey bars)]. The data show that no anti-histidine antibodies or no antibodies against *E coli*-linked protein were produced in the serum of the immunized animals. Immunization with plasmids encoding the exon 18 did not induce serum antibody titers, but when DNA-immunized mice were boosted with formulated E18 protein a clear induction of anti E18 antibody titers was found (*Figure 5B* group VI and VII). We conclude that immunization of mice with E18 protein induces a significant humoral immune response.

CTL response in E18-immunized mice

Balb/c mice were immunized as described. Mouse spleens

Figure 5 Antibody and CTL responses in E18-immunized mice. (A) showing an SDS PAGE of the E18 protein preparation used in the experiments; (B) depicts the antibody titers resulting from immunization and (C) gives the data for CTL responses. Mice were primed on day 0 and boosted on day 21 with (I) PBS/PBS; (II) adjuvant/adjuvant; (III) E18 in adjuvant/E18 in adjuvant; (IV) E18/E18; (V) empty vector pCI/empty vector pCI; (VI) pCI with E18 coding sequence/pCI with E18 coding sequence; (VII) pCI with E18 coding sequence/E18 in adjuvant. CTL, cytotoxic T cell.

were isolated and restimulated in vivo with overlapping nonamer peptides covering the amino acid sequence of E18 protein. A total of 67 overlapping peptides divided over 3 pools was used. We calculated the mean number of IFN-γ secreting cells per 10^5 CD8+ cells (*Figure 5C* group I-VII, 2 mice in each group). Our data show no or a low number of IFN-γ secreting CD8+ cells in the groups immunized with E18 (*Figure 5C* groups III and IV). For the DNA-immunized mice on the other hand intramuscular injection of the exon 18 encoding DNA clearly induced a significant CTL response (*Figure 5C* groups VI and VII). We found 727 IFN-γ secreting cells on a total of 10^5 CD8+ T cells (0.7%). There was a slight increase in the number of IFN-γ secreting CD8+ T cells for mice immunized with DNA and boosted with protein in Abisco-100 (887 IFN-γ secreting cells on 10^5 CD8+ cells, 0.9%). For all spleens as a negative

control, an *in vitro* restimulation with an irrelevant peptide pool consisting of 17 overlapping peptides (9-mers, 5 amino acids overlap) covering a 76 amino acid sequence of the exon 7pi8 region of the NCAM protein was performed. For the recombinant protein as well as for the DNA-immunized animals no IFN-γ secretion of CD8+ T cells was found. Since T cells from immunized animals could not be restimulated with the irrelevant peptide pool, we conclude that immunization specifically induces E18-specific T cells, thereby confirming the specificity of the E18 immunization. We conclude that immunization with E18 and exon 18 encoding DNA induces an E18-specific T cell response. E18 protein immunization only resulted in a limited number of IFN-γ secreting T cells, whereas immunization with exon 18 encoding DNA induced a significant cellular E18-specific immune response.

Discussion

The results of this study show that the expression of NCAM exon 18 can be used as a tumor marker for SCLC. To determine the specificity of this marker more precisely, screening of larger panels of SCLC tissue compared to tissues of other tumor types have to be carried out. We have developed the tools to make these studies possible.

The precise biological consequences of expression of protein E18 are not clear. E18 could be a driver of cell proliferation an activity required during, but not after, embryonic development. Schor *et al.* (15) identified epigenetic modulation resulting in alternative splicing of NCAM and expression of E18 in neuroblastoma cells. A similar modulation could be the result of exposure to tobacco smoke. Ditlevsen *et al.* (16) reviewed the data on intracellular signaling by NCAM. They conclude that NCAM-180 can mediate neurite outgrowth. But other explanations are possible where expression of E18 is a result rather than the cause of carcinogenesis.

But even without understanding why E18 is expressed in SCLC and not in other tumor cells and normal cells, this finding can be used in the diagnosis and treatment of SCLC.

First the level of E18 circulating in the body can be used to follow the tumor load. This means that the effect of chemotherapy can be monitored. This could lead to a more refined chemotherapy that can be stopped when the required effect has been reached. After that regular monitoring should detect a relapse before clinical signs arise. At this point renewed or alternative treatment would be possible. There is a number of alternative treatments, but due to the rapid progression of the disease these are difficult to test in clinical trials (17-19). Real time monitoring of the tumor load in patients would enable personal medication. The recently developed possibilities to take frequent blood samples from patients and to analyze E18 or mRNA for E18 in these samples makes this approach possible (20,21).

Second, the presence of E18 in tumor cells offers a target for immunotherapy. Although E18 is intracellular, it will be processed by proteasomes and the resulting peptides will be presented on the cell surface. In this way CTLs can recognize the tumor cells and kill them. Our preliminary experiments in mice have shown that this approach has potential.

Acknowledgements

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: SCLC tissue sections came from archival material at the Department of Genetics and Cell Biology. The animal experiments were carried out at CIRES and were approved by the Bezirksregierung Arnsberg under number 50.8735.1.

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MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPSSRSAAGTMG
LPADTTATVEDMLPSVTTVT TNSDTITETFATAQNSPTSE TTTLTSSIAPPATATPDSNS
VPAGGQATPSKGPSASAPSPA PASAPKVAPLVDLSDTPTST PAASNLSSSVLANQGAVLSP
SAPAGVGEASKAPPASKPTP APVPTPTGAASPLAAAAPA TEAPQAKQEAPSTGPDPEP
TQPGAAKSPAEAATALASPK SEAASVSTTNPSQGEDFKMD EGNFKTPDIDLAKDVFAALG
SPAPAAGASGQAPELAPSTA DSSVSPAPAKTE

E18 protein with <u>amino acids encoded by multiple cloning site pRSET</u>
Total 314 amino acids. Molecular weight 30 633.

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKD
RWGSELEICSWYHGIRFPE
AASNLSSSVLANQGAVLSPSAPAGVGEASKAPPASKPTPAPVPTPTGAASPLAAAAPATEA PQAKQ
EAPSTKGPDPEPTQPGAAKSPAEAATALASPKSEAASVSTTNPSQGEDFKMDEGNFK
AAAITS

Truncated E18 expressed in *E. coli* with <u>amino acids encoded by multiple cloning site pRSET</u>
Total 181 amino acids. Molecular weight 18 345. Italics indicate identical sequences.

Figure S1 Amino acid sequences of the E18 protein expressed in *E. coli* and of the shortened version used to produce the monoclonal antibody against E18.
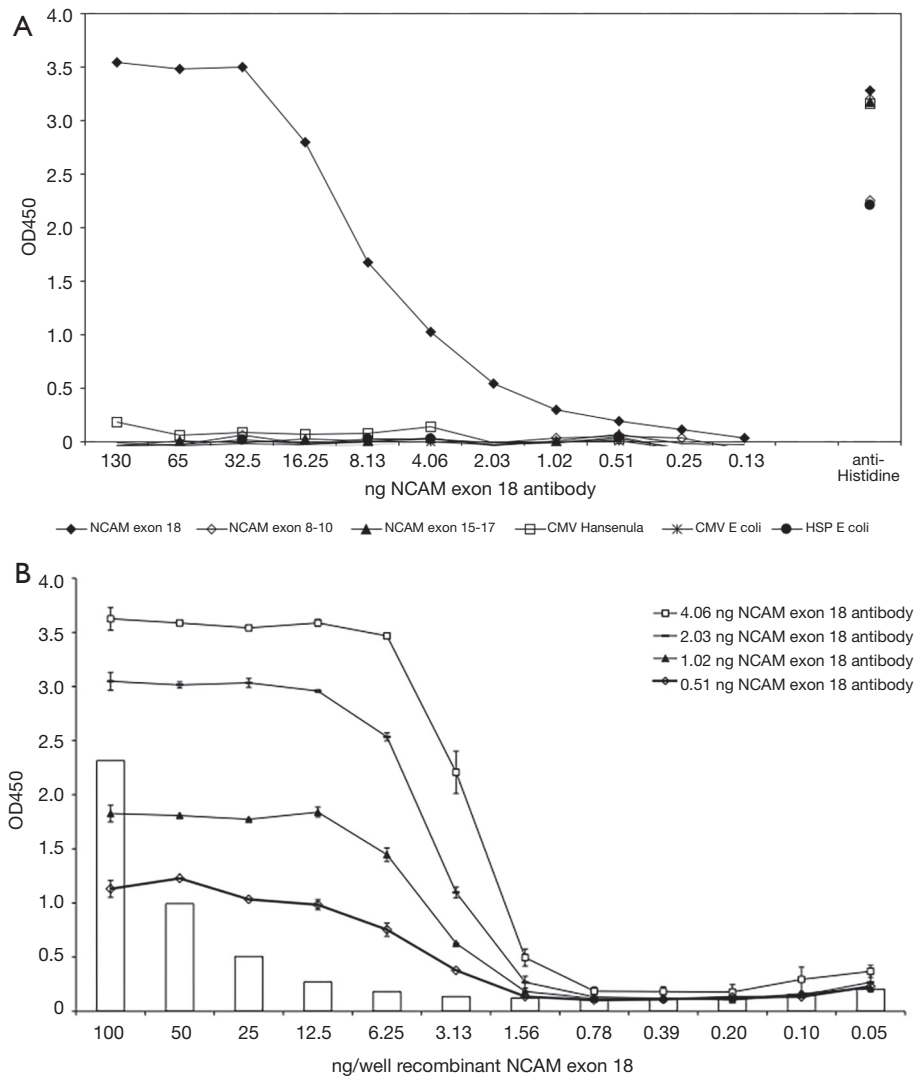
Figure S2 Specificity and sensitivity of monoclonal antibody MUMi-21B2. (A) Binding of monoclonal antibody to E18. Microtiter wells were coated with 1 μg recombinant antigen and the antibody was diluted as indicated. Negative control antigens are indicated. An antibody against this tag measured the signal caused by the His-tag; (B) various combinations of coated E18 proteins and dilution of the monoclonal antibodies were used to determine the sensitivity of the assay.
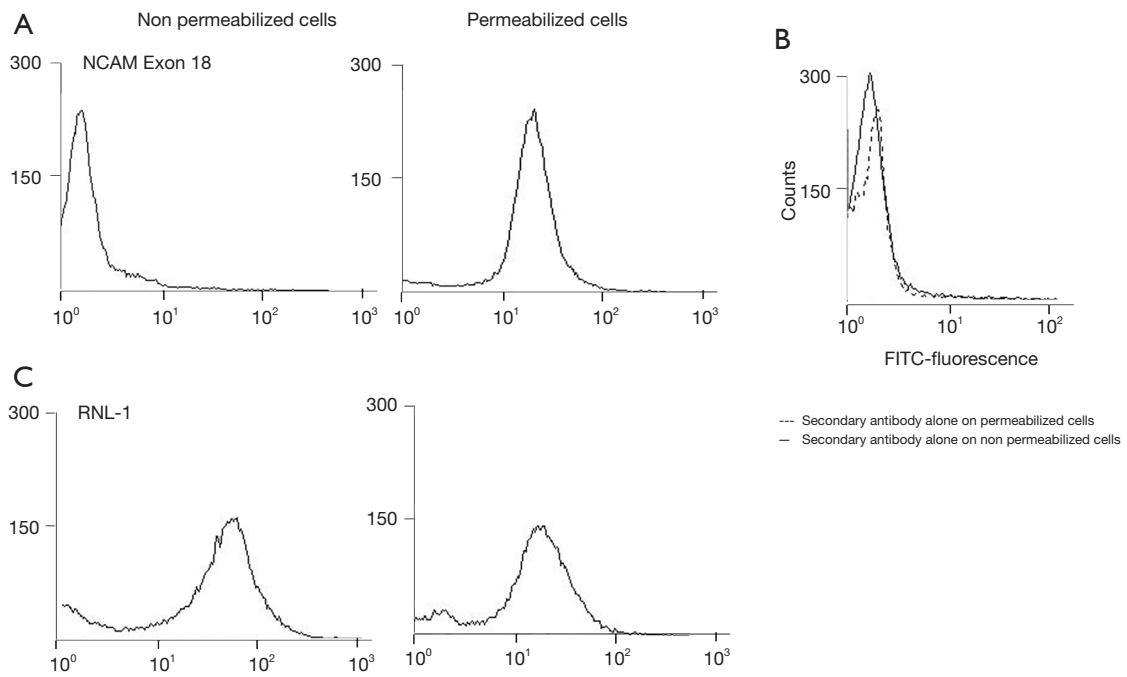
Figure S3 Evidence for the intracellular location of the E18 region of NCAM recognized by MUMi-21B obtained by flow cytometry of intact and permeabilized cells. (A) The exon 18 encoded epitope is only recognized by monoclonal antibody MUMi-21B in permeabilized cells. NCAM, Neural Cell Adhesion Molecule; (B) negative control in the absence of MUMi-21B; (C) positive control using a monoclonal antibody to an extracellular domain of NCAM.