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ORIGINAL ARTICLE

A. Borowski · U. Dirksen · L. Lixin · R. L. Shi
U. Göbel · E. M. Schneider

Structure and function of *ETAA16*: a novel cell surface antigen in Ewing's tumours

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Abstract Immunoscreening of an Ewing's family of tumour (EFT)-derived cDNA library using formerly described EFT-specific antibodies led to the isolation of a 3.5 kb cDNA, named Ewing's tumour-associated antigen 16 (*ETAA16*). The *ETAA16* cDNA shows no homology to any functionally characterised human gene. Only a bovine cDNA expressed in bovine testis and hepatocytes is functionally characterised as it encodes for a junction plaque associated protein and showed a homology of 69.9% at amino acid level to *ETAA16*. The human cDNA encodes for a 926 amino acid tumour antigen with a calculated molecular weight of 103 kDa. The epitope of the *ETAA16*-specific antibody, Ak16, covers the central region of the protein which is part of an extra cellular domain. The human *ETAA16* gene locus has been assigned to chromosome 2p13-15 by FISH analyses and is confirmed by the human genome sequencing project. As demonstrated by flow cytometry, the cell surface expression of *ETAA16* antigen is restricted to ET cell lines and not expressed on other small blue round cell tumours or other kind of tumour. RT-PCR analysis revealed a high expression of *ETAA16* in brain, liver and kidney while lung and heart were negative. Immunohistochemistry showed an intracellular expression of *ETAA16* in different kind of non-Ewing tumour tissues. These results suggest that *ETAA16* may function as a tumour-specific cell surface antigen in EFTs.

Keywords Ewing's family of tumour · Tumour-associated antigen · *ETAA16*

Abbreviations DAB: Diaminobenzidine · EFT: Ewing's family of tumours · ET: Ewing's tumour · ETAA16: Ewing's tumour-associated antigen 16 · ETS: E26 transformation specific · EST: Expressed sequence tag · EWS: Ewing's sarcoma · FISH: Fluorescence in situ hybridisation · FLI-1: Friend leukemia integration site-1 · MAB: Monoclonal antibody · MFI: Mean fluorescence intensity · ORF: Open reading frame · PALS: Periarteriolar lymphoid sheath · PNET: Primitive neuroectodermal tumour · UTR: Untranslated region

A. Borowski (✉)
Department of Anatomy, University of Bonn,
Nussallee 10, 53115 Bonn, Germany
E-mail: alexander.borowski@uni-bonn.de
Tel.: +49-228-739388
Fax: +49-228-737301

U. Dirksen · L. Lixin · R. L. Shi · U. Göbel · A. Borowski
Department of Paediatric Oncology,
Haematology and Immunology,
University of Dusseldorf, Moorenstrasse
5, 40225 Düsseldorf, Germany

E. M. Schneider
Section of Experimental Anaesthesiology,
Department of Clinical Anaesthesiology,
University of Ulm, Steinhoevelstrasse
9, 89075 Ulm, Germany

U. Dirksen
Child and Youth,
Department of Paediatric Haematlogy and Oncology,
University of Muenster, Albert Schweizerstr. 33,
48129 Muenster, Germany

Introduction

The identification of tumour-specific antigens (TSA) is of crucial interest in cancer research. TSA are helpful in differential diagnosis, may serve as a prognostic factor or even be used as an immune therapeutic target. Some TSA are exclusively expressed on malignant cells, while other tumour antigens represent over-expressed cellular antigens with an abnormal tissue distribution pattern [21, 25, 33].

Ewing's family of tumours (EFT) defines a group of rare malignancies in childhood characterised by a lack of distinguishing morphological features and the presence of balanced chromosomal translocations (see [10, 13] for a review). The chromosomal translocations fuse the RNA-binding protein *EWS* to members of the family of ETS transcription factors. In 85% of cases, the resulting

fusion protein is composed of the N-terminal, protein interacting part of *EWS* and of the C-terminal part of *Fli*-1 which carries the ets-DNA binding domain leading to the expression of the chimeric transcription factor *EWS*/*Fli*-1. The translocation products have transforming (in vitro) and tumourigenic (in vivo) activity (see [3] for a review). Additionally, the chimeric transcription factor leads to a *de novo* expression of potential tumour-associated antigens [2, 5, 11, 20, 36, 37], which is shown for *EWS*/*Fli*-1 but could be expected for the other EFT- specific chimeric transcription factors.

The ontogenetical origin of EFT is not well-characterised but it is generally considered to be of either mesenchymal or neural crest origin [6, 9, 22] and microarray analysis of EFT transcripts revealed an expression pattern correlated with fetale as well as brain and endothelial-expressed genes [31]. EFT are rare tumours of the bone and in 25 % the tumour has metastasised at the time of diagnosis. These patients have a poor prognosis with a 5-year survival of approximately 25%. In relapse situations, the prognosis may be even worse [19, 23]. Prior to the establishment of new therapeutic or prognostic features a well-founded knowledge on the tumour's biology is required.

In 1994, a panel of 16 anti-EFT monoclonal antibodies has been described [28]. We identified the corresponding antigen to the antibody 16, which has been shown to be highly specifically expressed on the cell surface of EFT and most of the analysed ET cell lines [28]. The antigen was named Ewing's tumour-associated antigen 16 (*ETAA16*). Here, we present our data of this novel EFT cell-surface associated antigen which displays only minor similarities to known human genes and shows no distinct, characterised protein domain.

Methods

Cell lines and cell cultures

The ET-specific antibodies are described [28]. Briefly, the monoclonal antibodies were generated by immunisation of Balb/c mice with fresh tumour material or the ET cell line VH-64 or in a mixture of both. After three immunisations, spleen cells were fused to NS-1 myeloma cells and hybridoma supernatant was screened by indirect immunofluorescence. Positive supernatants were further tested against frozen sections of ET biopsies compared to lymph node sections. The monoclonal antibody AK16 (SN58 1857.15.30) produce an IgG2bκ isotype.

The ET cell line A673, RD-ES and SK-ES-1 were obtained from ATCC. The ET cell lines RM-82, VH-64, WE-68 as well as the cell line GG-62, derived from a melonoma of soft part, were a kind gift from F. van Valen (Laboratory for Experimental Orthopaedic Research, Department of Orthopaedic Surgery, University of Münster, Germany [34]). The ET cell lines STA-ET-1, STA-ET-2.1, STA-ET-2.2 and STA-ET-5 were kindly provided by P. F. Ambros (Children's Hospital, Vienna, Austria) [1]. The neuroblastoma, osteosarcoma and rhabdomysarcoma cells were kindly provided by D. Dilloo (Department of Paediatric Haematology, Oncology and Immunology, University of Düsseldorf, Germany). All cells were cultured in DMEM/high glucose containing 10% FCS, 2 mM Glutamax I and 50 µg/ml Gentamycin (Invitrogen). ET cell lines were grown on collagen-coated plastic flasks [4].

Flow cytometry analysis and immunohistochemistry

Surface marker analysis was performed by flow cytometry on a FACScan (BD Biosciences, Germany). For data analysis, the Cell Quest software was used. The cell surface expression was done as described [4]. Briefly, the detached cells were incubated with 20 µl hybridoma supernatant in the case of Ak16 or up to 20 ng purified Ak16 purchased from Alexis. As positive control, the MHC class-I specific unlabelled antibody B9.12.1 were used (Beckman-Coulter, Germany) and the secondary FITC-labelled goat-anti-mouse serum were used as negative control (BD). Intracellular staining was performed following fixation with BFA [29].

Paraffin-embedded human tissue sections were immunostained as described [12]. Briefly, sections were rehydrated in a Xylene and a descending ethanol/PBS line. The sections were incubated for 20 min in 2x SSC at 80 °C [26]. After a PBS washing step, the sections were incubated in 1% H_2O_2 for 10 min and permeabilised with 0,5% Triton-X100 in PBS for 10 min. The sections were blocked in 2% NGS/PBS for 30 min. The Ak16 incubation has been done with 10 ng/ml (Alexis, Switzerland) in blocking buffer overnight at 4 °C. The sections were washed three times for 5 min in PBS. The first antibody binding were detected with the ABC-complex after the instructions of the manufacture (Vector Laboratories) and visualized with the chromogenic substrate DAB (0,5% in PBS, Sigma). The sections were counterstained with hematoxilin (Chroma) and mounted with Kaiser's glycerol gelatine (Merck)

Confocal microscopy

Immunohistochemical stainings were recorded by confocal imaging using a Leica TCS-NT equipped with an argon–krypton laser. FITC fluorescence was excited with 488 nm and emission wavelength was recorded > 515 nm. Software applied to specifically detect surface expression and simulation of 3D-imaging was done by the Metamorph software (Visitron Systems, Germany).

Construction of a cDNA expression library from an ET cell line

A cDNA library was constructed from the ET cell line VH-64 in the ZAP Express vector (Stratagene) carrying a

CMV promoter for additional eukaryotic expression. Briefly, 10 µg of poly A+ mRNA, prepared by acidic-phenol extraction [7] and Oligotex-beads enrichment (Qiagen, Germany) were used to prepare double-stranded cDNA. The ZAP Express cDNA library was packed with gigapack II gold (Stratagene). A primary cDNA library containing 5.5×10^5 pfu with less than 2% non-recombinant background plaques was obtained. Amplifications were performed as described [26]. Integrity of the cDNA expression library was proven by measuring the cDNA size of randomly picked clones and gave 7% of recombinant clones with < 1 kb cDNA size.

Construction of deletion clones

The 5′- and 3′-deletion clones were generated with the Exo Mung bean deletion kit (Stratagene) and were sequenced with standard T7 and T3 primer by Thermosequenase cycle sequencing, separated on an A.L.Fexpress, and analysed with the A.L.Fwin 1.1 software (Amersham Biosciences). Deletions clones E (Δ 1766) and G (delta 3151) were constructed using the restrictionendonuclease site *Hin* dIII and *Sma*I, respectively, using standardised subcloning procedures [26]. Bioinformatics for generating continuous sequences was done with the DNasis software (Hitachi Software, Germany).

Transient transfection for flow cytometry analysis

COS-7 cells were transfected with the relevant cDNA clones using 2 µg of ultra-pure plasmid DNA in a ratio of 1:3 with FuGENE6 (Roche). After transfection, the cells were incubated for 24 h, 48 h and 72 h and subjected to flow cytometry analysis of the expressed gene. For transfection efficiency, the reporter gene EGFP from pEGFP-1 (Clontech) was cloned into the pBK-CMV and transfected as described above.

Western blot analysis

Cells were solubilised in 2x sample buffer (62.5 mM Tris/HCl, pH 6.8, 2% SDS, 10 % Glycerol, 0.1% BPB, 300 mM ß-Mercapthopethanol) by heating at 95 °C for 5 min. Proteins were separated by SDS polyacrylamide gel electrophoresis and blotted onto PVDF membranes (Millipore). The membrane was blocked in TBST buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05% Tween20) containing 2% BSA for 3 h at room temperature. The membrane was incubated overnight using a 1:100 dilution of the Ak16 hybridoma supernatant. Subsequently, the membrane was washed in TBST and incubated with secondary alkaline phosphatase (AP) conjugated rabbit-anti-mouse-Ig (DAKO, Germany) at 1:1000 in blocking buffer. After two washing steps in TBST, alkaline phosphatase reactivity was visualized by adding NBT and BCIP in 100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 2 mM $MgCl_2$.

Northern blot analysis and RT-PCR

Total RNA of benign tissues were purchased from Clontech. Total RNA was isolated by the acid-phenol extraction method as described in [7]. Messenger RNA was purified from VH64 by oligo dT-coupled beads (Dynal Biotech, Germany). Total RNA (25 µg) and mRNA (5 µg) were separated on denaturating formaldehyde agarose gels, transferred to Hybond-N membranes (Amersham Biosciences) by capillary blotting and fixed by UV cross-linking for 2 min. Probes were derived from the cDNA clones and labelled by the random primer method (High Prime Kit, Roche) using ^{32}P-labelled oligonucleotides and hybridised at 42 °C in a formamid-based hybridisation buffer. The Northern blots were washed two times in 2× SSPE/0.1%SDS for 30 min at 42 °C [26]. Time of exposure of the film (Eastman Kodak, Germany) was done for 2–4 weeks at −70 °C with an intensifying screen. For RT-PCR analyses, 2 µg total RNA were reverse transcribed with first-strand cDNA synthesis kit (Amersham Biosciences).

To assess the *ETAA16* gene expression, the following primers were generated:

Primer	Sequence 5′–3′	Position 5′	Amplification (bp)
ETAA16-S_1	GGCAGCATTTCAGTGCAGACATC	2235	522
ETAA16-R_1	AAGATTGCTGTCCAACAGCTTCC	2735	
ETAA16-S_7	CTCCATTGACAAAGCAGTTAGG	478	510
ETAA16-R_7	AGCATTAAAGGCTGCTTCAG	978	
ETAA16-S_8	CATAGGCAATGAGTCGGCGAAG	138	522
ETAA16-R_8	TCCTTTTGCTACACTGGGAGTAC	637	
ETAA16-S_{12}	TGGTTCTTCCAGGAAGTTCAAG	2446	520
ETAA16-R_{12}	GTTATCAGCAGTCTTCGTGAA	2944	
GAPDH-S	CACCCATGGCAAATTCCATGGC	Ex3, 213	296
GAPDH-AS	GCATTGCTGATCTTGAGGCT	EX5/6, 487	

Polymerase chain reaction amplification reactions were done with the Taq polymerase after standard cycle conditions with an annealing temperature of 55 °C. Only the primer pair 8 needs Q-solution for amplification (Qiagen, Germany). Multiplex PCR analyses for the *ETAA16* transcript were performed using a combination of primers for the housekeeping gene *GAPDH* and the

different *ETAA16*-primer pairs. The cycle conditions and annealing temperature were the same as described above. The primer for *GAPDH* (50 nM) was half-concentrated compared to the ETAA16 Primer (100 nM). The estimated primers lead to amplification products of 269 bp for *GAPDH* and around 500 bp for *ETAA16*.

FISH analysis

Metaphases from normal human lymphocytes were hybridised with the clone, 6.1.1, labelled with biotin-16-dUTP by nick-translation [8]. The biotin-labelled DNA probe was visualised with avidin-DCS-FITC (Vector Laboratory). Signal amplification was achieved by additional incubation with biotinylated goat-anti-avidin (Dianova, Germany) and avidin-DCS-FITC. DAPI served as chromosomal counterstain. Images for FITC and DAPI were captured for both fluorescent dyes, digitalised and merged by false colour assignment.

Results

Expression cloning of ETAA16 using EFT-specific antibodies

In order to identify putative antigens recognised by the 16th anti- EFT mAb (Ak16) [28], an expression cloning strategy was applied. A ZAP Express vector-based cDNA expression library was constructed from the ET cell line VH-64 and screened with a pool of all 16 EFT-specific antibodies. The antibody 16 reacted with four clones (Fig. 1a: 3.3.1, 6.1.1, 8.1.1., 9.2.2), which were specific for the same 3.45 kb cDNA named *ETAA16*. The clone 3.3.1 carried a homologue, but shorter cDNA insert of 3.35 kb, lacking 150 bp at the 3′-end and 54 bp at the 5′-end (Fig. 1a; Accession AJ242682.2).

The Northern blot hybridization using *ETAA16*-specific probe showed a low-abundant transcript with the expected size of about 3.5 kb in (Fig. 1b). The result

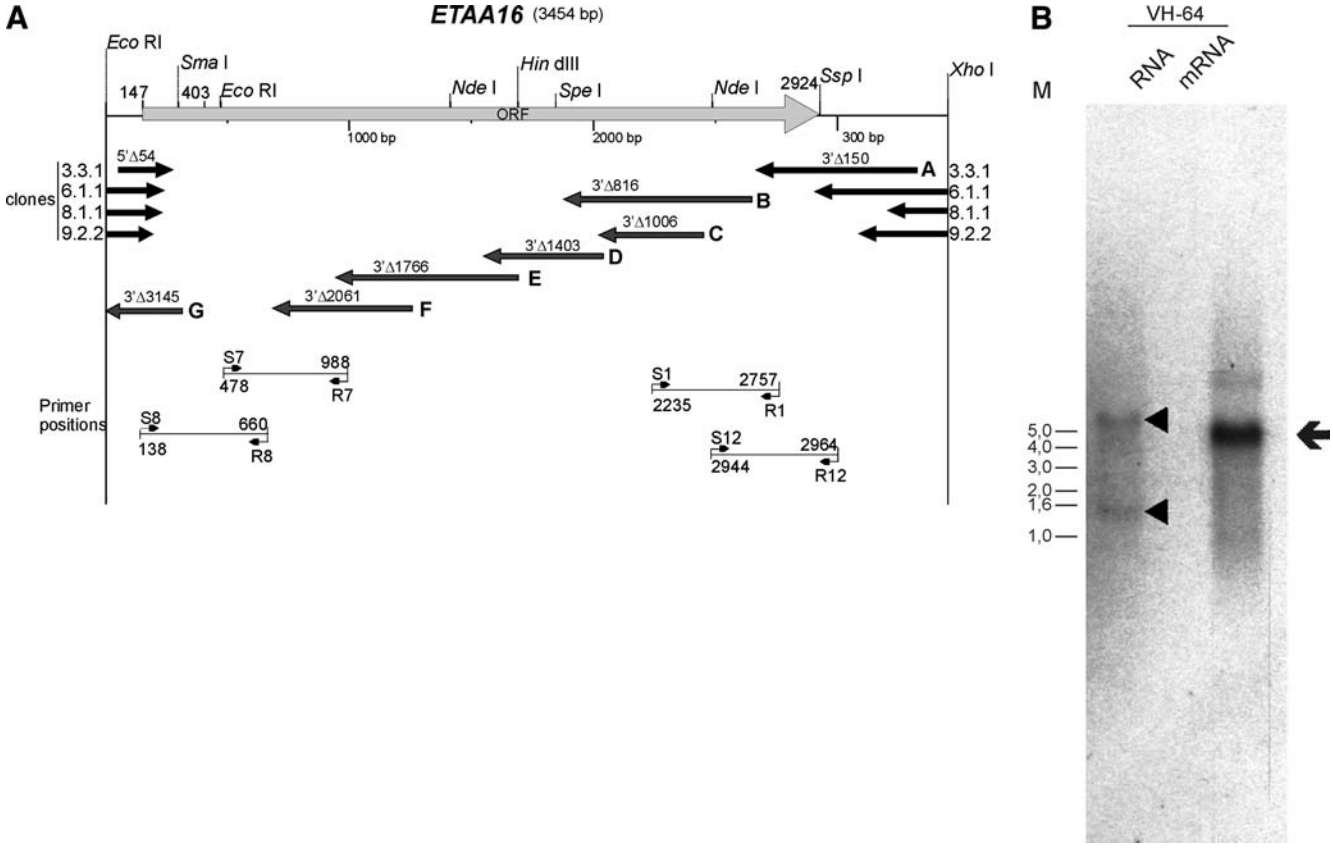

Fig. 1 **a** Schematic view of *ETAA16* cDNA. The 5′- and 3′-sequences of the four independent *ETAA16* clones (3.3.1, 6.1.1, 8.1.1, 9.2.2 *black arrows*, 5′ and 3′ starting region) revealed that all clones are homologous but clone 3.3.1 shows deletions at the 5′-(54 bp) and at the 3′-end (150 bp). The whole sequence of *ETAA16* was decoded by different 5′- and 3′- (*grey arrow bars*) deletion clones of the parental clone 6.1.1. The ORF lies between nucleotides 147 and 2925 nt (broad light *grey arrow* bar). Major restriction enzyme recognition sites are indicated. The primers which were used for RT-PCR analysis are indicated. **b** Northern Blot of *ETAA16*. Northern Blot analysis was performed using a [^{32}P]-labelled *Nde*I/*Ssp*I 642 bp fragment of *ETAA16* derived from the 3′-end of the ORF (**a**). The probe was hybridised to VH-64 total RNA (25 µg) and mRNA (5 µg), which were separated on a 1% formaldehyde agarose gel. Cross-reactivity of the *ETAA16* probe with ribosomal RNAs in the total RNA fraction are indicated by an *arrow head*, *ETAA16*-specific mRNA in the poly A+ fraction by an *arrow*

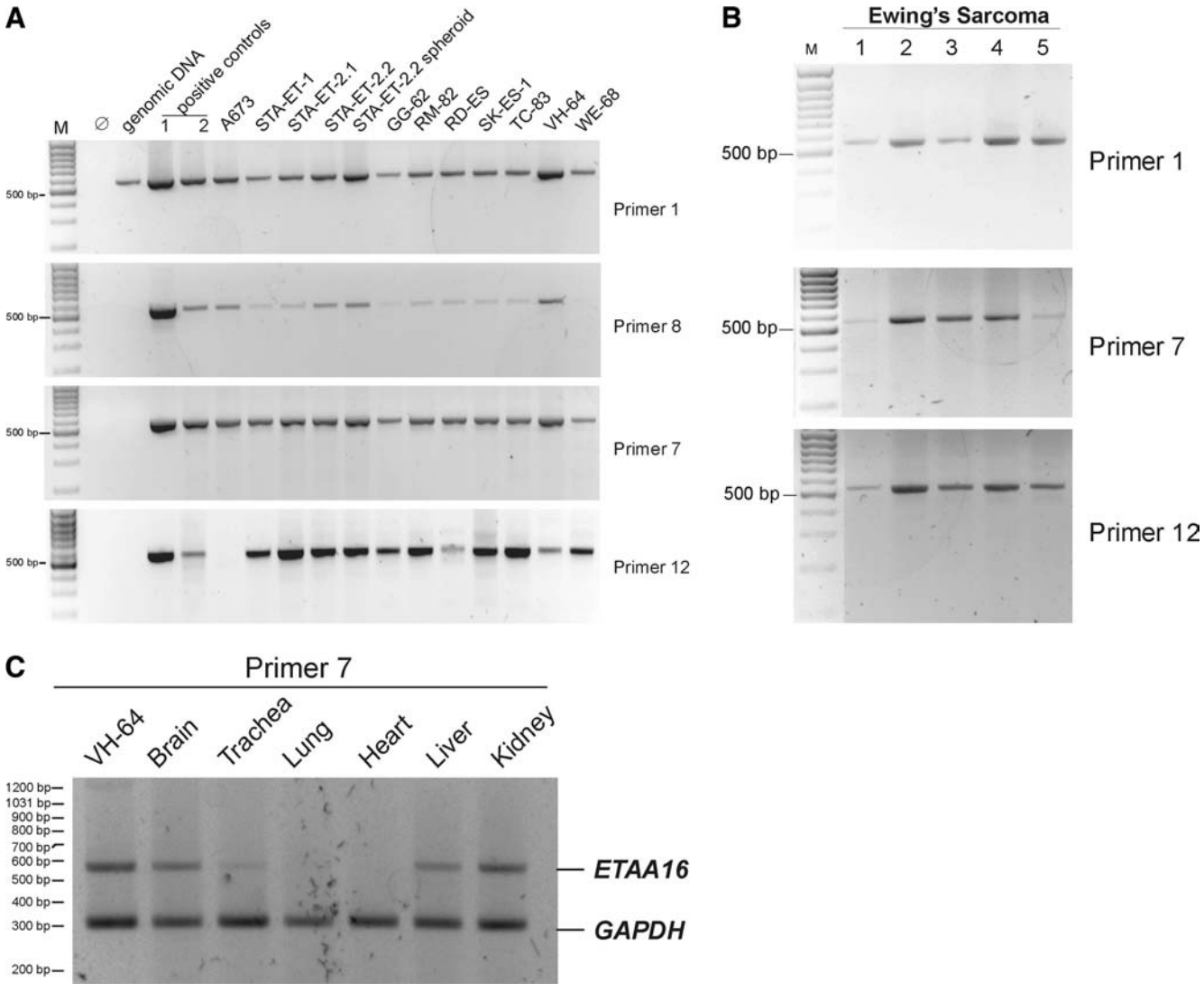

Fig. 2 RT-PCR analysis of *ETAA16*. *ETAA16* transcription was analysed using four specific primer pairs (Fig. 1a, Material and methods) and amplified from oligo (dT) primed cDNA obtained from 13 ET cell lines (**a**) (the cell line GG-62 were formerly described as atypical Ewing's Tumour [34], but is now redefined as a malignant melanoma of soft parts [27]. **b** Five EFT tumour biopsies. **c** Six different benign tissues were analysed in multiplex RT-PCR. As control, the housekeeping gene *GAPDH* was amplified and the *ETAA16* transcript was detected with primer 7

was proven by hybridisation with a 5′ probe of the *ETAA16* open reading frame (ORF, data not shown).

Expression pattern of ETAA16

Expression pattern for *ETAA16* was assessed in ET cell lines and EFT tumour biopsies by RT-PCR with four specific primer pairs from the 5′-part (primer pair 8 and primer pair 7) and from the 3′-ORF (primer pair 1) and the 3′-ORF to the 3′-UTR (primer pair 12) of *ETAA16*. The primers amplified a specific product of around 500 bp (Fig. 1a). All tested ET cell lines and all tested EFT biopsies expressed the *ETAA16* transcript (Fig. 2a, b).

In order to prove the EFT-specificity of the *ETAA16* monoclonal antibody Ak16 [28], different cell lines of small-blue-round cell tumours were tested for their *ETAA16* cell surface expression (Table 1). Eight of 12 ET cell lines expressed *ETAA16*. Lacking *ETAA16* cell surface expression is not affected by the chimeric transcription factor as the *ETAA16* negative ET cell lines SK-ES-1 (*EWS*/*Fli-1* Type II), RM-82 and CADO-ES1 (*EWS*/*ERG*) expressing different types of the chimeric transcription factor. Furthermore the neuroblastoma, osteosarcoma and rhabdomyosarcoma and a lot of cell lines derived from different kinds of tumours were negative for *ETAA16* cell surface expression (Table 1). Only the CML cell line MEG-01 shows a weak cell surface expression of *ETAA16* (Table 1) and is also positive for the ET antigen Mic2/CD99 as well as another antigen of the ET-specific antibody 15 (data not shown [28]). Multiplex RT-PCR comparing *GAPDH* versus *ETAA16* expression analysis of benign tissues revealed a specific expression of *ETAA16* in brain, liver and kidney while

Table 1 Cell surface expression of *ETAA16* in small-blue-round cell tumour cell lines detected by the corresponding antibody Ak16 [28]

Cell line	Ak16 (MFI)
Ewing's tumour	
A673	5.553,32
CADO-ES1	–
STA-ET-1	21.528,16
STA-ET-2.1	8.869,52
STA-ET-2.2	12.017,26
STA-ET-5	–
RD-ES	18.217,12
RM-82	–
SK-ES-1	–
TC-83	5.455,60
VH-64	10.294,99
WE-68	14.929,86
Neuroblastoma	
Be(2)c	–
IMR32	–
Kelly	–
Rooney	–
SK-N-SH	–
Osteosarcoma	
KHOS	–
SA-OS	–
MG-63	–
U2-OS	–
ZK-58	–
Rhabdomyosarcoma	
RD	–
SJRH30	–
Malignant melanoma of soft parts	
GG-62	–
Primary skin fibroblasts	
HFFF	–
Embryonic kidney	
293	–
Acute megakaryocytic leukemia	
CMK	–
Chronic myeloid leukemia	
K562	–
MEG-01	461,72
Acute myeloid leukemia	
HL-60	–
KG1a	–
Acute lymphatic leukemia	
AD	–
RS	–
T-cell leukemia	
Jurkat	–
Burkit lymphoma	
Daudi	–
Raji	–
Erythroleukemia	
HEL	–
Histiocytic lymphoma	
U937	–
Choriocarcinoma	
JAR	–
Hepatocarcinoma	
HepG2	–
PNET, brain	
PFSK-1	–
Glioblastoma	
U-87	–
Cervixcarcinoma	
HeLa	–
Teratocarcinoma	
PA-1	–
Bladder carcinoma	
5637	–
HT1376	–
SD	–
T24	–

Cells were stained indirectly using the non-conjugated murine Ak16 and a secondary FITC-labelled goat-anti-mouse antibody. The mean fluorescence intensity (MFI) is indicated for all cells where specific binding of Ak16 was seen. Cells which did not show an AK16-binding compared to control-staining using the secondary antibody are indicated as negative (–)

trachea, lung and heart were negative. The results are shown for primer 7 (Fig. 2c) and confirmed with the *ETAA16* specific primer 12 (data not shown).

Confirming the expression analysis at transcriptional level, several non-transformed human tissue sections were tested in immunohistochemical analysis with the Ak16. As positive controls, cryosections of three independent Ewing's Tumour biopsies were positively stained with Ak16 as well as Mic2 (data not shown). Liver and kidney showed an Ak16 specific strong intracellular signal (Fig. 7a, b) while sections from a human lung biopsy were negative (not shown). Interestingly, the staining of the kidney was restricted to the epithelial of the proximale tubuli while the distale tubuli and the malpighian corpuscles were negative (Fig. 7b). In all tested tissues, vessels were also negative. In the marginal sinus of the spleen, subsets of lymphocytes in the B-cell corona were stained positive with the Ak16. The corresponding antigen were also expressed in intracellular compartments (Fig. 7c). Analysis of the Ak16 binding to the adrenal gland tissue showed a strong signal in the zona glomerulosa while zona fasciculata and reticularis exhibited a weak signal. The adrenal medulla as well as the capsule were negative for Ak16 staining (Fig 7d). The staining of different parts of brain sections revealed a weak astrocyte staining and a possible ependyma staining (data not shown).

Primary structure of ETAA16

With an intent to learn more about the putative *ETAA16* protein, computational analyses of the *ETAA16* sequence were performed. The analyses identified an ORF of nearly 2.7 kb (Fig. 1a, Accession AJ242682.2) and two distinct start codons in the same reading frame. The first start codon at position 145 is not in good confidence to the Kozak sequence, which should have a purine (adenine) at position −3 and a pyrimidine (guanine) at position +4 [16]. In contrast, the second start codon at position 403 is in a good context to the Kozak sequence ANNatgG. Given the first start codon to be the functional start codon, it

Table 2 Homologies of *ETAA16* to full-length cDNA sequences

Accession[a]	Clone	Origin	Size (bp)	Homology	Score
AK025155	FLJ21502	Human colon cDNA[b]	2833	99%	5477
AK026300	FLJ22647	Human small intestine cDNA[b]	1757	99%	3342
Y08459		B. taurus cytoplasmic protein associated with junctional plaques of Sertoli cells and hepatocytes	3148	84%	1017
AK019965	5730466H23	Mus musculus 8 days embryo whole body cDNA[c]	2637	84%	321
AK018594	9130006G04	Mus musculus male cecum cDNA[c]	2158	80%	131

[a]EMBL gene bank accession numbers[b]NEDO cDNA sequencing project (http://www.nedo.go.jp/bio-e/)[c]RIKEN cDNA sequencing project (http://www.riken.go.jp/index.html)

would encode for a protein of 926-amino acid and a predicted molecular mass of 103 kDa. The 3′-UTR encodes for three polyadenylation sites (pos. 3057, 3296, 3408 Accession AJ242682.2). The G/C content of the 5′-UTR is 60.4 % and of the 3′-UTR is 27.4 %. The ORF shows a G/C content of 37.1 %.

Using the alignment programs FastA and BLAST, *ETAA16* revealed no homology to any known human cDNA (EMBL accession AJ242682.2). The NEDO sequencing project identified two human homologous sequences from a human cDNA expression library of colon AK025155, with a 99% homology of a 2.9-kb and of small intestine, AK026300 with a 99% homology of 1.4 kb. Both are homologue to the 3‘-end of *ETAA16* as is expected for EST’s. To date, these cDNAs are not particularly characterized and there is no idea of the corresponding protein. The only functional characterised homologous cDNA show 84% homology at nucleic acid level and 69.9% homology at amino acid level and was isolated from a bovine expression library. The cDNA encodes for a junction plaque-associated protein in Sertoli cells and hepatocytes (Table 2, Fig. 3). The RIKEN cDNA project revealed two homologous mouse cDNAs species of 84% (AK019965, whole body cDNA of an 8-day-old embryo) and 80% (AK018594, male mouse caecum cDNA) homology, respectively (Table 2, Fig. 3). The cDNAs from human (AJ242682.2), bovine (Y08459) and mouse (AK019965) share the same ORF which is in good context to the first start codon for *ETAA16*.

Some homologous human and murine ESTs have been identified which are not restricted to a tissue-specific expression (Table 3, http://www.meb.uni-bonn.de/anatomie/cellbio/research/recpubs_e.htm). Six human EST sequences are homologous to the 5′-end of *ETAA16* (Table 2: BM905777, BM043722, AW978869, AW188186, AW418715, AA829856). The longest homologous EST (BM905777: 80–3276 bp), has been isolated from a leiomyosarcoma-specific cDNA library which seems to have some common features with EFT [14]. Other homologues EST’s were isolated from a prostate carcinoma cell line (BM043722) and human tonsillar cells enriched for germinal centre B cells ($CD20^+$, IgD^- ; AA829856). Three ESTs (AW978869, AW188186, AW418715) are of unknown tissue origin. More homologous EST are depicted under supplemental data (http://www.meb.uni-bonn.de/anatomie/cellbio/re-

Fig. 3 Alignment of the homologous human (AJ242682), bovine (Y08459) and mouse (AK019965)-deduced protein sequence to *ETAA16*. Similar amino acids are displayed with a *black background* while the same chemical properties are signed with a *grey background* (BLOSUM45)

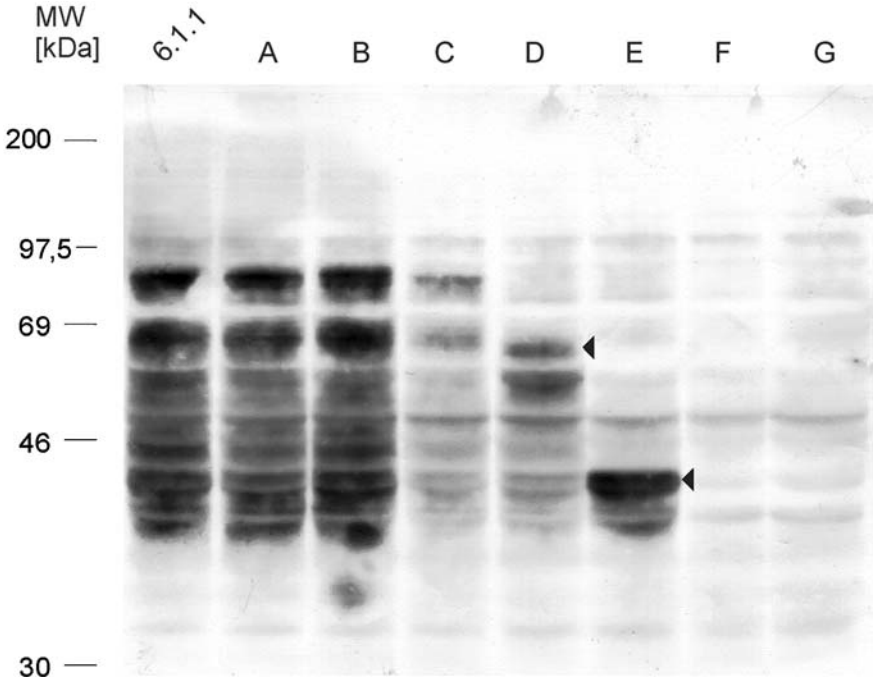

Fig. 4 Epitope mapping of *ETAA16* by Western Blot. Prokaryotic expression of clone 6.1.1 (full-length) and 3′-truncated *ETAA16* clones were expressed in Escherichia coli. The full length and truncated clones were detected by the EFT-specific Ak16 after blotting on PVDF membranes. The full-length clones (A, B) gave rise to a band with a MW of ˜90 kDa, and clones D, E resulted in consecutively smaller protein bands (*arrowheads*). The Clones F and G were found to lack the Ak16 binding epitope so that a fragment of 460 bp (1396 to 1856) codes for the epitope which leads to an 153 aa peptide (416–569 aa) epitope

search/recpubs_e.htm). Thirty-one of 50 EST are isolated from different stages of embryonic development (data not shown).

Epitope mapping of the ETAA16-specific antibody Ak16

To characterise the Ak16-recognising epitope of *ETAA16*, full length and 3′-deletion clones representing C-terminal polypeptide truncations were generated (Fig. 1a). The binding of Ak16 was analysed by Western blot technique (Fig. 4). Binding of Ak16 to protein extracts of cells expressing the full length cDNA clone 6.1.1 showed a 95 kDa protein. The 3′-deletion clones A–C display truncated cDNA fragments, but the *ETAA16* protein was not shortened. The 3′-deletion clones D and E display truncated *ETAA16*, 55 kDA and 43 kDA respectively, but retained binding to Ak16. The cDNA deletion clones F and G which lacked a fragment of 2061 and 3145 bp at the 3′-end, did not bind Ak16. Thus, the minimal cDNA fragment 1396–1856 bp of *ETAA16*, corresponding to aa 416–569, is necessary for the immune recognition by Ak16.

Chromosomal localization

Fluorescence in situ hybridisation on metaphase chromosomes was performed using clone 6.1.1 (Fig. 5).

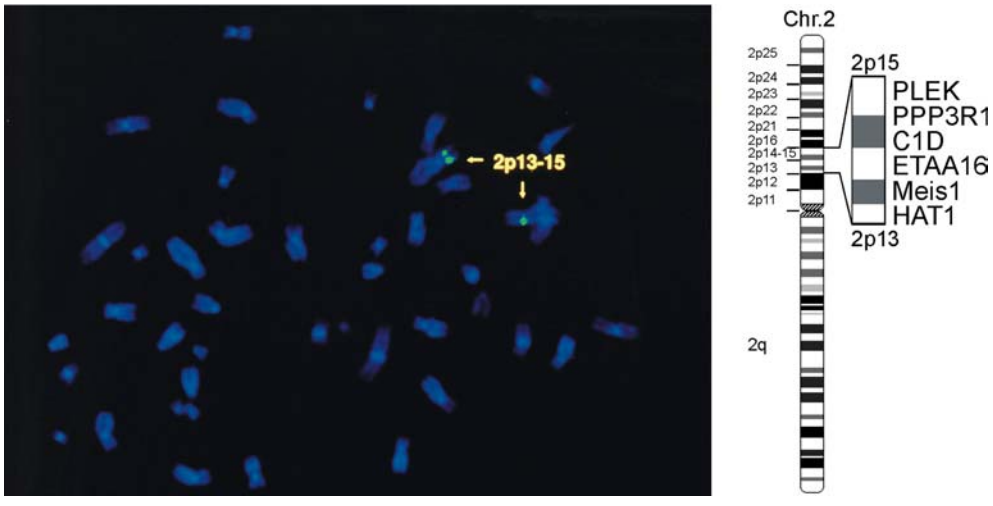

Fig. 5 Chromosomal localisation of ETAA16. In situ hybridization of ETAA16 specific cDNA sub clone fragments of the original clone 6.1.1. cDNA fragments were labelled with biotin, and were detected by avidin-FITC after hybridisation (*arrows* at chromosome 2p13–15). Schematic view to the bands p13–15 of chromosome 2 with *ETAA16* adjacent, functional identified genes

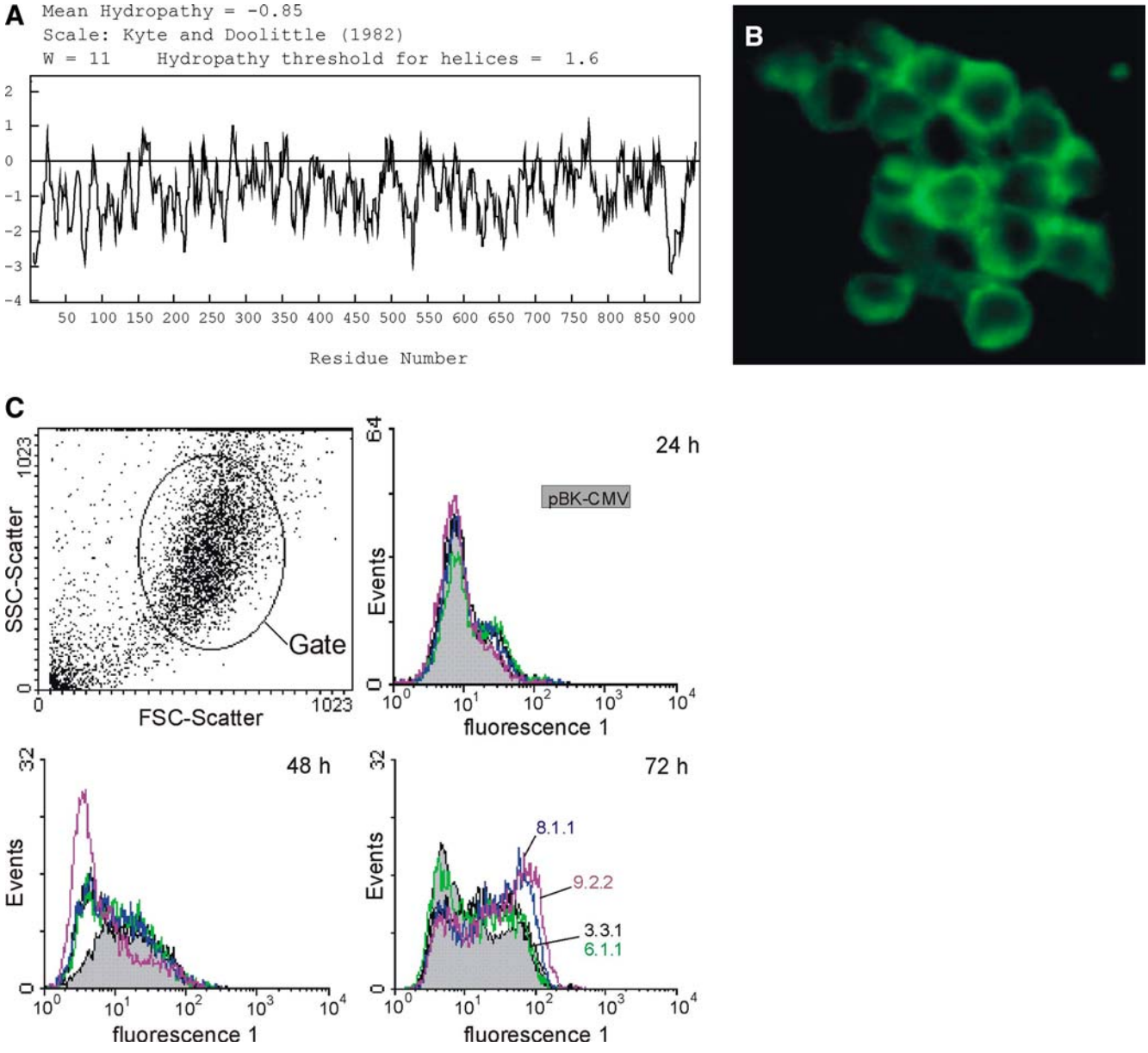

Fig. 6 Physical properties of *ETAA16* related to cellular expression (**a**) Surface staining of ET cell line VH-64 with Ak16 and FITC-labelled anti-mouse IgG. Examination was done by confocal laserscan microscopy following surface rendering by the Metamorph software (Leica TCS NT; excitation at 488 nm, emission wavelength > 515 nm). (**b**) Hydropathy plot of the deduced protein sequence of *ETAA16* [17] (**c**) Flow cytometry analyses of intracellular *ETAA16* following transient transfection of *ETAA16* specific clones under the control of the CMV-promoter into COS-7 cells. Cells were fixed and stained with Ak16 and goat-α-mouse-FITC. Intact cells were gated in the forward/sideward plot. The *histograms* show the gated COS-7 cells determined after 24 h, 48 h and 72 h of transfection. The fluorescence of the Ak16 stained cells are shown as *overlays in contrast to the grey* under laid vector control pBK-CMV

Accordingly, *ETAA16* was assigned to chromosome 2p13–15, which corresponds to the most recent report on gene mapping by the International Human Genome Sequencing Consortium [18]. *ETAA16* is located in the vicinity of PPP3R1, the gene is coding for calcineurin B [32] and close to MEIS1 (myeloid ecotropic viral integration site 1) [30].

Ectopic transient expression of ETAA16

The predicted *ETAA16* protein sequence lacks significant hydrophobic regions, which usually indicate putative transmembrane domains (Fig. 6a) [17]. This result was not expected as *ETAA16* is clearly expressed on the cell surface of EFT cells and ET cell lines as demonstrated by immunofluorescence flow cytometry (Table 1) and immunohistochemistry (Fig. 6b, [28]). However, transient expression of any of the four independently derived *ETAA16* cDNA clones driven by the CMV promoter in COS-7 cells failed to induce surface expression (data not shown), although, transfected

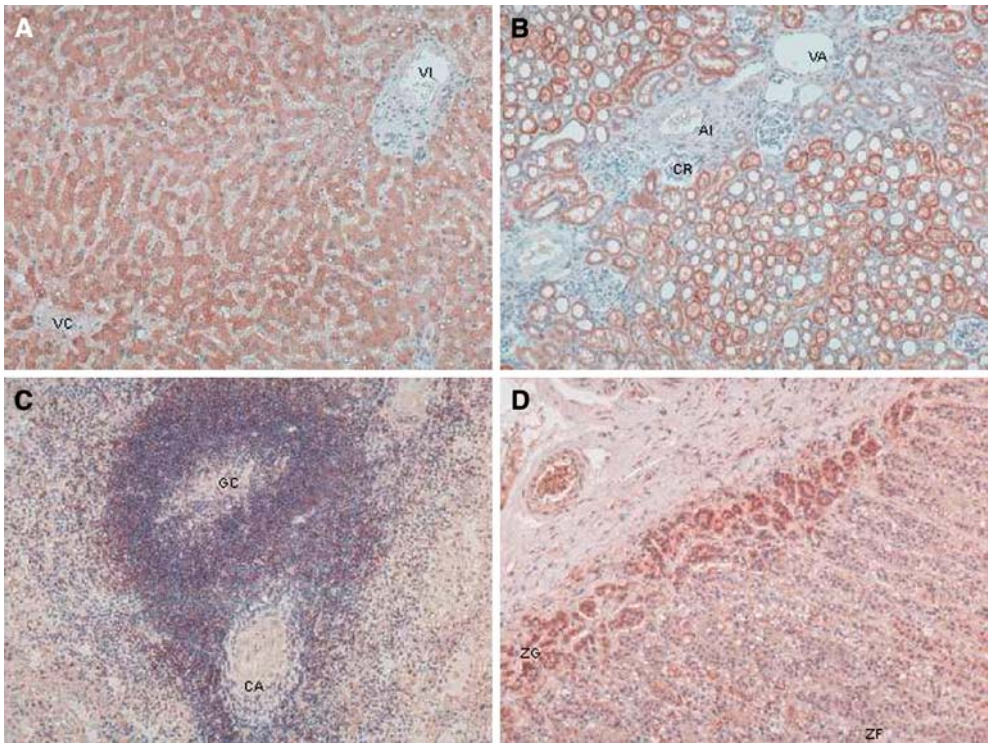

Fig. 7 Immunohistochemical analysis with Ak16 staining of human paraffin embedded tissue sections with the ET-specific antibody Ak16. The antibody binding were detected by a peroxidase-conjugated goat-anti-mouse (H + L) and visualised with DAB (*brown*). The section was counterstained with hematoxilin which stains the nucleus (*blue*). **a** liver, **b** kidney: cortex, **c** spleen: periarteriolar lymphoid sheath (PALS), **d** adrenal gland: cortex. *VC* vena centralis, *VI* vena interlobularis, *CR* corpusculum renalis, *AI* arteria interlobulares, *VA* vena, *GC* germinal center, *CA* center arterioles, *ZG* zona glomerulosa, *ZF* zona fasciculata

COS-7 turned unequivocally positive for the cytoplasmic expression of *ETAA16* 72 h after transfection (Fig. 6c).

Discussion

One of the goals in tumour immunology is the identification of tumour antigens which might be a target for immunotherapeutic agents and/or provide further insight into the tumour biology. We describe the finding of a novel EFT-associated antigen detected by screening of an EFT-specific cDNA expression library. The antigen is recognised by the EFT antibody 16 [28] and is named *ETAA16*.

The 3.45 kb transcript of *ETAA16* and surface expression of the *ETAA16* antigen was detected in almost all analysed EFT biopsies and 8 of 12 ET cell lines derived from osseous Ewing's sarcomas and peripheral neuroectodermal tumours. The expression of *ETAA16* is not influenced by the *EWS*/*ets* fusion type as some of ET cell lines expressing the fusion protein but lack the cell surface expression of *ETAA16* (Table 1). Other small, blue, round cell tumours and various human cell lines did not show an *ETAA16* surface expression (Table 1).

Table 3 Homologies of *ETAA16* to human EST sequences

Accession	Clone	Origin	Position in *ETAA16* (bp)	Homology	Score[a]
BM905777	IMAGE:5556133 (5′)	Uterus: leiomyosacoma	80–3276	97%	1467
BQ018004	IMAGE:5879583 (5′)	Fibrosarcoma cell line HT-1088	2377–3087	98%	1316
BG571053	IMAGE:4714045 (5′)	Placenta	2609–3276	98%	1237
BM043722	IMAGE:5446350 (5′)	Prostate, carcinoma cell line	35–713	97%	1199
BF383104	IMAGE:4050818 (5′)	Brain, primitive neuroectoderm	2085–2795	96%	1120
BF082070	RC3-BN0034-130900-115-e02	Normal breast, adult	2187–2743	99%	1051
BF115150	IMAGE:3135156 (5′)	Kidney	2747–3288	98%	1019
AV719015	GLCBLH06 (5′)	Corresponding non cancerous liver tissue, adult	2695–3295	95%	1003
AW978869	EST390978	Not specified	15–367	99%	692
AW188186	IMAGE:2664794 (5′)	Not specified	74–367	100%	583
AW418715	IMAGE:2909650 (5′)	Not specified	74–367	99%	575
AA829856	IMAGE:1369572 (5′)	Germinal center B cell	74–367	98%	543
AA808357	IMAGE:1334423 (5′)	Germinal center B cell	2943–3284	94%	537

Further EST are given under supplemental data

However, at transcriptional level, RNA from brain, liver and kidney tissues are positive for *ETAA16* (Fig. 2c).

In concordance to RT-PCR data, immunohistochemical analysis shows a positive staining of several non-EFT tissues (Fig. 7). The Ak16 signal was detected mainly in cytoplasmic compartments in these tissues. This corresponds to our flow cytometry data which showed a negative cell surface *ETAA16* staining on equal cell types (Table 1) and transient expression data also revealed the possibility that *ETAA16* is not transported to the cell surface in non-EFT cells (Fig. 6c). The detection of *ETAA16* in the immunohistochemistry provided further information: the expression in kidney sections is restricted to the epithelia of the proximale tubuli in the cortex renalis (Fig. 7b). Subtypes of lymphocytes in the marginal sinus of the spleen binding Ak16 (Fig. 7c). This confirms the bioinformatical data as two EST homologues to *ETAA16* were identified in $CD20^+$, IgD^- germinal center B cells (Table 3, accession: AA829856, AA808357). The cortex of adrenal gland displayed a strong staining of the zona glomerulosa, which is of mesenchymal origin while the adrenal medulla (neural crest origin) was negative (Fig. 7d). Furthermore, RT-PCR analysis of *ETAA16* expression was positive in the RNA extract of whole brain (Fig. 1d), but immunohistochemical data showed a weak staining of astrocytes, only. These results indicate that *ETAA16* is not expressed in neuronal cells but in cells of mesenchymal origin which give some evidence to the mesenchymal origin of EFT's and not the neuronal one [6, 22].

Homologies to human EST sequences of leiomyosarcoma and prostate cancer as well as germinal centre B-cells reflect also tissues of mesodermal origin (Table 3). However, the histogenesis of Ewing's tumours is still a matter of debate. Similar to neural crest progenitors EFT express catechol acetyl transferase and neuron-specific enolase [6]. Lacking differentiation markers in vivo, ET cell lines tend to express a variety of neuronal markers in vitro [22]. EFT are generally poorly differentiated, probably as a consequence of early genetic alteration in form of the *EWS*/*ets*, in most cases *EWS*/*Fli-1*, rearrangement. *Fli*-1 is expressed in mesenchymal cells of the neural crest and mesodermal tissues [24, 35] and several authors suggest that EFT evolve from these mesenchymal cells. This hypothesis is strengthened by our alignment and immunohistochemical data and it looks like that *ETAA16* is expressed in a mesenchymal background. The mouse and various human EST derived from fetal tissues indicating that *ETAA16* may belong to the ontogenetically early genes which has to be approved in further experiments. Unique to the EFT's, in contrast to the tissue with a mesenchymal origin, is the cell surface expression of *ETAA16*. Epitope mapping identified the aa 416–569 as extra cellular, AK16 binding part in EFT (Fig. 4).

To find out whether *ETAA16* holds an active function in EFT, computational comparison of *ETAA16* with genes in the databases was performed. Unfortunately, these analyses gave no hints for a putative functional role of *ETAA16*. Thus, we focused on detailed analysis of the cDNA structure. Two distinct start codons were detected, one at position 145 and a second in the same reading frame at position 403. The second carries an optimal initiation sequence as described by Kozak [15] which supports the fidelity of initiation at the ribosome. In contrast, the first one did not carry any of the optimal initiation sequences [16]. Two heterologous proteins from *M. musculus* and *B. taurus* which are coded by two 84% homologues cDNAs show an equal start codon at position 145, which represents the corresponding initiations site of translation (Fig. 3). Similar unfavourable initiation sites have been identified in sequences of potent regulatory proteins such as cytokines or growth factors, which may be harmful if overexpressed [16]. Thus, the start codon at position 145 seems to be more likely relevant for the translation of *ETAA16*, especially as the full-length clones express a nearly 95 kDa protein after prokaryotic expression (Fig. 4), which nearly correlates with the deduced MW from 103 kDa of this translated ORF.

Two human homologous sequences (AK025155, with 5′-deletion of 621 bp, AK026300 with a 5′-deletion of 1697 bp) with 99% homology at the 3′ end of *ETAA16* have been identified from a human cDNA expression library of colon and of small intestine, respectively (NEDO sequencing project, Table 2). However, the products of these genes are not known and so there are no new insights in the putative function of the *ETAA16* protein. Only the 84% homologous, bovine cDNA implies a possible intracellular function for *ETAA16* in that —by homology (Fig. 3)—it could play a role in the formation and function of junction plaques (Table 2: Y08459).

Surface expression of *ETAA16* seems to be restricted to EFT cells and represents a highly selective and unique feature of the EFT cells. The mechanism which leads to the selective cell surface expression has to be evaluated in further experiments. *ETAA16* is a specific cell surface antigen for EFT and might serve as an additional marker for EFT diagnosis. Additional data will be needed to find out a possible prognostic or even therapeutic relevance of the Ak16.

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