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Analysis of the ETAA16 Expression

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CONTENTS

ACKNOWLEDGMENTS 1

ABSTRAKT 2

ABSTRACT 3

1 INTRODUCTION 4

1.1 HISTOLOGY 4

1.2 IMMUNOHISTOCHEMISTRY 5

1.3 MOLECULAR GENETICS 6

1.4 TREATMENT AND PROGNOSIS 7

1.5 AIMS OF THE STUDY 7

2 MATERIAL AND METHODS 8

2.1 CELL CULTURE AND HARVESTING CELLS 8

2.2 EXTRACTION, PURIFICATION RNA FROM CELL LINES 12

2.3 FIRST STRAND cDNA SYNTHESIS 14

2.4 PCR AMPLIFICATION 16

2.5 RESTRICTION ENZYME DIGESTION 19

2.6 IMMUNOFLUORESCENCE MEASUREMENTS 20

3 RESULTS 21

3.1 QUANTITY, PURITY AND INTEGRITY OF ISOLATED RNA 21

3.2 EXPRESSION ANALYSIS OF ETAA16 GENE BY RT-PCR 21

3.3 FLOWCYTOMETRIC ANALYSIS 29

4 DISCUSSION 33

5 REFERENCES 37

APPENDIXES

CURRICULUM VITAE 47

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Abstrakt

Tumor-assoziierte Antigene geben neue Einblicke und Möglichkeiten in der Klassifizierung von Tumoren mit wichtigen prognostischen und therapeutischen Ansätzen. 1994 generierte L. Shi 16 Ewing Tumor (ET) spezifische Antikörper , die mit unterschiedlicher Spezifität Oberflächenstrukturen auf den ET erkennen. Eines der ET-spezifischen Antigene konnte von A. Borowski et al aus einer cDNA Expressionsbibliothek, die von der ET Zelllinie VH-64 hergestellt worden ist, mit Hilfe eines Immunoscreens identifiziert werden. Die cDNA von dem Ak16-definierten Antigen, welche mit *ETAA16* für **E**wing **T**umor **a**ssoziiertes **A**ntigen **16** betitelt worden ist, stellte ein neues humanes Gen dar. Ziel dieser Studie war es, die Gewebe-spezifische Expression von *ETAA16* zu untersuchen. Die Sequenz der *ETAA16* cDNA wurde zur Generierung von spezifischen Primern verwendet, um mittels RT-PCR ein Expressionsprofil für *ETAA16* in verschiedenen Tumoren, normalen Geweben und Zelllinien zu erstellen. Es wurden 95 verschiedene RNA-Proben untersucht, dabei handelte es sich um RNA aus 14 ET Tumorbiopsien, 16 Tumorbiopsien von Tumoren, die immunhistopathologisch zu den kleinen-blauen rundzelligen Tumren gezählt werden, 6 RNAs von normalem Gewebe, 17 ET-Zelllinien und 48 nicht-ET Zelllinien. *ETAA16* Transkripte konnten in einem breiten Spektrum von Tumorbiopsien, Zelllinien und normalem Gewebe nachgewiesen werden. Aus diesem Grunde wurde die Oberflächenexpression von *ETAA16* mit seinem spezifischen Antikörper erneut mit Hilfe der Durchflußzytometrie auf den Zelllinien untersucht. Die Ergebnisse zeigten eine restringierte Oberflächenexpression von *ETAA16* auf die ET-Zelllinien, wie schon vorher beschrieben. Gleichzeitig konnte eine intrazelluläre Expression von *ETAA16* auch in nicht-ET Zellen nachgewiesen werden. Diese Ergebnisse zeigen, daß *ETAA16* in einem breiten Spektrum an Geweben transkribiert und translatiert wird, aber die Oberflächenexpression ET spezifisch ist. Die Oberflächenexpression von *ETAA16* scheint auf Ewing Tumore restringiert zu sein, was auf eine ET spezifische Prozessierung zurückgeführt werden könnte. Diese ET spezifische Prozessierung gilt es in weiteren Untersuchungen zu analysieren und zu charakterisieren, um diese für die Diagnostik und auch für mögliche Therapien zu nutzen.

Abstract

Tumour associated antigens may provide new insights and approaches in classification of tumours with important prognostic and therapeutic implications. In 1994 Shi et al created a panel of 16 anti-Ewing Tumour (ET) antibodies. The ET-specific staining pattern can be obtained from this panel of monoclonal antibodies. Alexander Borowski et al have created an ET-cDNA library and the cDNA of mAb16-defined antigen (*ETAA16*)was cloned and isolated. Aim of the present study was to investigate the *ETAA16* gene expression in different ET tumour cells and other tissues. RT-PCR assay was used to analyse expression of ETAA16 in 95 samples including 14 Ewing sarcoma samples, 16 tumour biopsies, 6 different human tissue, 17 cell lines and 48 non-ET paediatric small round cell tumours. The expression of *ETAA16* mRNA was detectable in a wide variety of tumour and normal cells. Thus, the surface expression of the mAb16-corresponing antigen was re-evaluated by flow cytometry. The results showed the surface expression of the *ETAA16* antigen was restricted to ET cells as described before. However intracellular staining with the mAb16 was positive in ET and non-ET cells. This work shows that *ETAA16* mRNA and the mAb16 –related antigen is distributed over different tissues, but the mAb16 related surface antigen is expressed on ET cells only. Thus, surface expression of the mAb16 related antigen is uniquely regulated in ET cells and may be regulated by an ET specific pathway.

1 Introduction

Ewing's tumour (ET), a rather common, highly malignant primary tumour of bone and soft tissues, includes several related clinicopathologic neoplastic entities, such as Ewing's sarcoma (ES), the peripheral neuroectodermal tumour (PNET) and the malignant small cell tumour of the thoracopulmonary region (Askin's tumour) [1 2 3 4 5 6 7]. These tumours are histologically composed of poorly differentiated, small, round blue cells on conventional histologic and electron microscopic criteria with the poorly differentiated ES at one end, and PNET at the other end[2]. According to immunohistochemical studies, ET have neural, neuroectodermal or mesenchymal like characters. A breakthrough in the diagnosis of ET was the identification of the tumour specific translocation t(11,22) (q24;q12).

ET affects primarily white and Hispanic young people and is extremely rare in individuals of African or Asian origin. The reason for this striking ethnic distribution is not known[8]. The tumour rarely occurs in children younger than 5 years of age or in adults older than 30 years. The peak incidence is between 10 and 15 years of age with a slight predisposition to males[9]. Tumours can develop in almost any bone and soft tissues and often present with pain and swelling. The most common primary sites are the pelvis, femur, tibia and fibula.

Before 1970 local therapy of the tumour by surgery, radiation or both, as used, was associated with an approximately 10% 5-year-event-free survival rate. Although the combination of multi-drug chemotherapy regimens, radiotherapy and surgery has increased initial response rates for many ET patients up to a 5-years event–free survival, the prognosis for disseminated ET is still poor[10 11 12 13].

1.1 Histology

Typical histologic features of Ewing's tumour are homogeneously rounded (about 8-12u, in size), small hyperchromatic nuclei, inconspicuous nucleoli, and scant cytoplasm, and moderate mitotic activity. In addition to this conventional type of Ewing's tumour, variants have been classed as large–cell or atypical ES,

consisting of large cells (15-20μ in size), exhibiting prominent nuclei with condensed chromatin one or more nucleoli, high mitotic activity. Cytoplasmic glycogen deposits can be visualised in approximately 80% of these tumours. In some rare cases, tumour cells show an incomplete rosette pattern. [14] [15] [16] These histologic features are shared with distinct tumours as rhabdomyosarcoma, neuroblastoma, lymphoma, small cell osteosarcoma. Diagnosis to differentiate ES/PNET from those SRCT is possible with novel techniques of immunohistochemistry.

1.2 Immunohistochemistry

More and more immunochemical characters of ET are explored, and these molecular markers are helpful in differential diagnosis of ET. Furthermore, this technique has provided new insights in the origin of ET[17] [18]. ET cells *in vitro* either spontaneously express or can be induced to express some neurological markers as NCAM (neural cell adhesion molecule), NSE (neuron-specific enolase), S_{100} (the gliofibrillar acid protein) or Leu-7 (an antibody binding with a myelin associated glycoprotein located in cells of the neural crest) [19] [20] [21] [22] [23]. Independent of neural differentiation, ET can also exhibit some epithelial (Cytokeratin positive) and mesenchymal (Vimentin positive) characters[24] [25] [26] [27]. ET were also found to express $P^{30/32}$ Mic2 (CD99), a cell-surface glycoprotein over expressed in ET and other tumours such as rhabdomyosarcoma, ependymomas or leiomyosarcoma [28] [29] . The biological function of this glycoprotein seems to be involved in cell adhesion processes [30].

A reliable marker, identifying ET would not only be helpful in the differential diagnosis of ET but might also be useful in tumour immunotherapy. Thus, many scientists make effort to investigate the specific function and value of other antibodies against the ET. In order to identify novel ET-antigens, 16 immuno-monoclonal antibodies have been produced against ET. 9 antibodies precipitate antigens with the same molecular mass of 80kDa (mAb1, 2, 3, 4, 5, 10, 11, 12, 14). mAb7 and mAb8 appear to detect an identical antigen with molecular mass of 46kD. mAb9 is reactive to Mic2. Antigens defined by mAb13,15,16 have

higher-molecular mass of 140kD[31]. Recently, the cDNA of the antigen defined by mAb16 referred as *ETAA16* was cloned and sequenced [32].

1.3 Molecular genetics

An important advance in the diagnosis of ET has been the identification of consistent chromosomal translocations associated with unique tumour types[33]. These translocations represent an exchange of genetic material between two chromosomes to create novel fusion genes. ET shows a specific chromosomal translocation t(11;22)(q24;q12). This translocation fuses the DNA binding domain of an *ETS* family member and the N-terminal of *EWS* gene. In approximately 85% *EWS* is fused to the *ETS* member *FLI-1*. The exact biological functions of wild-type *EWS* remains largely unknown, increasing evidence suggests that it is involved in mRNA transcription. The *FLI-1* gene is a member of the *ETS* (avian erythroblastosis virus transforming sequence) family of DNA-binding transcription factors that are implicated in the control of cellular proliferation, development and tumourigenesis [34 35 36]. Besides the most common *EWS/FLI-1* translocation, *EWS* is fused to other *ETS* domains as *ERG* t(21q22) [37 38], *ETV-1* t(7q22), *E1AF* t(17q12) or *FEV* t(2q33) [39 40 41]. The functional consequences of these less frequent variant translocations are thought to be analogous to the *EWS/FLI-1* rearrangement, but subtle differences are likely to exit. In all cases the resultant chimerical protein creates a novel protein with an unique function might play a critical role in tumour biology.

Studies on the biological function of the EWS-fusion-products identified them as transcriptional activators[42 43 44 45]. The EWS/FLI-1 protein can bind the same DNA sequences as native *FLI-1*. However, EWS/*FLI-1* is a stronger transcriptional activator than wild type *FLI-1*[46]. Analysis of differently expressed RNA in *FLI-1* or *EWS/FLI-1* transduced NIH3T3 cells, several interesting genes were identified. Some of these genes are implicated to play a role in control of cell growth, differentiation and oncogenesis[47]. Inactivation of EWS/*FLI-1* by antisense oligonucleotides can inhibit growth of ET cells *in vitro* and *in vivo*[48 49]. In addition, antisense inhibition of *EWS/FLI-1* results in increased susceptibility to chemotherapy-induced apoptosis in ET cell lines. This suggests that the

fusion proteins may exert inhibition of apoptosis, which would normally occur in these cells[50]. Thus *EWS/ETS* translocations seem to have important functions in tumour biology. The characterisation may lead to new diagnostic and therapeutic approaches in ET.

1.4 Treatment and Prognosis

In the last 20 years, the systemic chemotherapy combined with effective local treatment, consisting of radiotherapy, surgery, or both has become standard therapy of ET[51]. The conventional induction chemotherapy consisted of VACA (vincristine, adriamycin, cyclophosphamide, and actinomycin D) in CESS81, VAIA (ifosfamide instead of cyclophosphamide) in CESS86 and EVAIA (additional etoposide) in EICESS92 has been used in the treatment of patients with localised ET[52]. However, conventional treatment is not successful in patients with primary multifocal bone disease and patients with early (< 2 years after diagnosis) or multifocal relapse. The introduction of a high-dose therapy (hyperfractionated total body irradiation, melphalan, etoposide+/-carboplatin) and autologous hematopoetic stem-cell transplantation[53 54 55] and subsequent administration of recombinant interleukin 2 (IL2) revealed initially promising results. The development of novel risk-adapted therapeutic strategies may improve the outcome of patients with disseminated disease or early relapse.

1.5 Aims of the study

In order to learn more about the specific biology of ET cells and to identify putative new immunological targets, Shi et al have produced 16 monoclonal antibodies against ES by immunising BALBC/6 mice with the ES cell line VH-64 and fresh tumour[31]. Specificity has been tested by immunohistochemistry. The cDNA of mAb16 defined antigen (*ETAA16*) has been cloned. *ETAA16* gene defines a novel human gene with no homology with the known human gene sequences[32]. Investigation of the putative *ETAA16* gene and the corresponding antigen could open new advantages for ET diagnosis and treatment.

Aim of this work was:

1) To investigate whether *ETAA16* gene expression is restricted to ET family.

2) To investigate whether the *ETAA16* antibody specifically binds to ET-cells.

2 Material and Methods

2.1 Cell culture and harvesting cells

2.1.1 Materials

RPMI-1640 medium, Eagle medium.

DMEM medium: Dulbeccos' Modified Eagle Medium.

FBS: Fetal bovine serum.

PBS: Phosphate Buffered Saline (Ph7.5).

0.02% EDTA: 0.02% Ethylene diaminetetraacetic disodium salt (EDTA) dissolved in PBS.

0.2% Trypsin: 0.2% Trypsin dissolved in PBS.

0.05%/0.02% Trypsin in PBS.

NEAA: non-essential amino acid.

Nap: sodium pyruvate.

L-Glutamine, Sodium bicarbonate.

The above reagents were purchased from Biochrom (Berlin, Germany).

Dimethysulfhoxide (DMSO), Penicillin, Streptomycin (Sigma, Deisenhofen, Germany). 25cm^2 tissue culture plastic flask (T25), 2ml plastic ampules (Costar). Collagen S (Boehringer, Mannheim, Germany).

Growth medium 1: RPMI-1640 supplemented with 10% FCS, 2mM L-glutamine.

Growth medium 2: Dulbecco's modified Eagle Media supplemented with 10% FCS, 2mM L-glutamine, 100U/ml penicillin, and 100μg/ml streptomycin.

Table 1. Cell lines

Cell lines	Origin	Organism	Growth Properties	Cell Culture
Ewing's Tumour				
A673	ATCC	Human	Adherent in collagene S treated culture flasks	1
CADO-ES1	DMSZ	Human		1
ES-MA	Kindly provided by M. E. Schneider Institute of Anesthesiology, University of Ulm Germany	Human		1
ET-1	Kindly provided by P.F. Ambros Children's Hospital, Vienna, Austria	Human		1
ET-2.1		Human		1
ET-2.2		Human		1
ET-5		Human		1
GG-62	Gift from F. van Valen Westphalian Wilhelms University of Münster, Germany	Human		1
RM-82		Human		1
TC-83		Human		1
VH-64		Human		1
NT-68		Human		1
WE-68		Human		1
RD-ES	ATCC	Human		1
SK-ES-1	ATCC	Human		1
Neuroblastoma				
Be(2)C	ECACC	Human	Adherent	2
IMR-32	ATCC	Human	Adherent	2
Kelly	ATCC	Human	Adherent	1
ROONEY	ATCC	Human	Adherent	1
SK-N-SH	ATCC	Human	Adherent	1
Osteosarcoma				
HOS	ATCC	Human	Adherent	2
KHOS	ATCC	Human	Adherent	2
MG-63	ATCC	Human	Adherent	2
ROS	ATCC	Rat	Adherent	2
SAOS-2	ATCC	Human	Adherent	2
TM791	Kindly provided by Dr. Dilloo Heinrich Heine University	Human	Adherent	2

Cell lines	Origin	Organism	Growth Properties	Cell Culture
U2-OS	Kindly provided by M.E Schneider Instituted of Anesthesiology, University of Ulm Germany	Human	Adherent	2
Rhabdomyosarcoma				
SJRH30	ATCC	Human	Adherent	1
RD	ATCC	Human	Adherent	1
		Leukemia		
Acute Megakaryocytic Leukemia				
CMK	ATCC	Human	Suspension, sometimes adherent loosely	1
Chronic Myeloid Leukemia				
MEG01, Megakaryoblast	ECACC	Human	Adherent loosely	2
AML				
KG1a	ATCC	Human	Adherent	2
ALL				
AD	Kindly provided by Dr. Dilloo Heinrich Heine University	Human	Adherent	2
RS				
T-Cell				
Jurkat	ATCC	Human	Suspension	1
H9K	ATCC	Human	Suspension	1
B-Cell				
Daudi	ATCC	Human	Suspension	1
Raji	ATCC	Human	Suspension	1
HEL	ACC11	Human	Suspension	1
HL60	ATCC	Human	Suspension	1
Histiocytic Lymphoma				
U937	Kindly provided by M.E Schneider Institute of Anesthesiology, University of Ulm	Human	Suspension	2
Choriocarcinoma				
JAR	ATCC	Human	Adherent	1
Hepatocarcinoma				
HepG2	ATCC	Human	Adherent	2

Cell lines	Origin	Organism	Growth Properties	Cell Culture
PNET, Brain				
PFSK-1	ATCC	Human	Adherent	1
Cervixcarcinoma				
Hela	ATCC	Human	Adherent	1
Teratocarcinoma				
PA-1	ATCC	Human, mouse	Adherent	2
Fibroblast				
HFFF13	ECACC	Human	Adherent	2
HFFF15	ECACC	Human	Adherent	2
HFFF32	ECACC	Human	Adherent	2
NIH3T3	ATCC	Mouse	Adherent	1
N50	Established in our lab (KMT Lab) Heinrich-Heine University Germany	Human	Adherent	1
H.D.F	Kindly provided by W. Frings Institute of Dermatological University of Münster Germany	Human	Adherent	1
Epithelial				
UBC	Kindly provided by M. E. Schneider Institute of Anesthesiology, University of Ulm Germany	Human	Adherent	1
Endothelial				
HUVEC	Kindly provided by Dr. Stoldt Heinrich Heine University Germany	Human	Adherent	1
Embryonal Kidney, E1A transformed				
293T	ATCC	Human	Adherent	1
FNX-A	ATCC	Human	Adherent	1
FNX-E	ATCC	Human	Adherent	1

2.1.2 Methods

2.1.2.1 Cell Culture

The cell lines were obtained as described in Table 1. As the majority of cell lines were frozen in liquid nitrogen, cells were carefully thawed prior to cell culture. Briefly, cells were thawed quickly in a 37°C water-bath. Subsequently, the cell suspensions were transferred to a 5ml centrifuge tube and diluted in 5 folds pre-warmed growth medium. Small sample was removed to determine the cell count. Cells were then centrifuged at 225g for 5min. Supernatant was discarded and the cells were resuspended in the medium adjusting to $0.5\text{-}1.0\times10^6$ cells per millilitre (ml) in growth medium.

Since the majority of Ewing's tumour cell lines has the tendency to grow as loosely adherent cells or as tightly packed floating aggregates, plastic tissue culture flasks for this cell line were coated with collagens ($5\mu g/cm^2$) before cells were seeded in. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.1.2.2 Harvesting Cells

For harvesting cells growing in suspension, cells were centrifuged for 5min at 225g and the supernatant was discarded. To harvest cells growing in monolayer, cells were detached with 1ml 0.02%EDTA per cm^2 for 5min to 15min. Cell suspensions were picked up in 5ml culture medium, centrifuged and then adjusted to 5×10^6 cells /ml. For RNA isolation, the harvested cells were washed two times in ice-cold PBS and frozen in liquid nitrogen. Flow cytometry analysis was performed immediately after cell culture.

2.2 Extraction, purification RNA from cell lines

2.2.1 Materials

RNeasy Mini Kits, including Rneasy Mini Spin Columns, collection tubes (1.5ml, 2.0ml), Buffer RLT, Buffer RW1, Buffer RPE, Rnase free water, 14.5M ß-mercaptoethanol (ß-ME) (all reagents by Qiagen, Hilden, Germany), ethanol

(96-100%) (Merck, Haar, Germany), UV-Vis Spectrophotometer (Beckman Unterschleissheim Germany).

2.2.2 Methods

RNA was isolated according to the manufacturer instructions. Briefly, the working solution of Buffer RLT was prepared by adding 10μl ß-ME per 1 ml of Buffer RLT, and buffer RPE was prepared by diluting stock solution with 4 volumes of ethanol (96-100%) before extraction and purification RNA from the cell line.

5×10^6 cells were resuspended in 350μl or 700μl Buffer RLT. Lysate was pipetted directly onto a QIA-shredder column sitting in the 2ml collection tube, and centrifuged for 2min at 8000g to homogenize the cells. 350μl of 70% ethanol was added to the homogenized lysate and mixed thoroughly by pipetting. The 700μl sample, including any precipitate having formed, was added to a RNeazy mini spin column sitting in a 2ml collection tube, and centrifuged for 15s at 8000g. In order to wash the sample, the 700μl of buffer RW1 was pipetted onto the RNeasy column, and centrifuged for 15s at 8000g .To exclude possible contamination of DNA, 10μl DNaseI stock solution was added to 70μl Buffer RDD and mixed by gently inverting the tube. The DNaseI working solution (80μl) was pipetted directly onto the spin-column membrane and incubated at the room temperature for 15min to digest the DNA.

For the next washing step, 700μl Buffer RW1 was added into the spin column, centrifuged for 15s at 8000g. Afterwards, the spin column was placed in a new 2ml collection tube. 500μl of buffer RPE was pipetted into the spin column, and centrifuged for 15s at 8000g repetitively.

The RNeasy spin column was placed in a new 2ml collection tube and centrifuged at full speed for 1min to eliminate any risk of possible Buffer RPE carryover.

To elute RNA from the column, the RNeasy column was transferred into a new 1.5 ml collection tube, and 50μl of RNase-free water was pipetted directly onto the RNeasy membrane by centrifuging for 1min at 8000g.

Quantification of total RNA was carried out by measuring the absorbance (A) at 260nm in a spectrophotometer. Because purine and pyrimidine bases have absorption maximum in the region of 260nm(A260), it is possible to obtain an approximate estimation of total RNA by the formula: 40×A260×dilution factor (μg/ml). Based on the spectral properties, the purity of RNA can be evaluated by the ratio between the absorbance values at 260 and 280 nm, because protein has an absorption maximum at 280nm, the value should be always above 1.8.

2.3 First Strand cDNA Synthesis

2.3.1 Material

First-Strand cDNA Synthesis Kit contains Bulk First-strand cDNA Reaction Mixes: Cloned, FPL pure TM Murine Reverse Transcriptase, RNAguard TM (porcine). Rnase/Dnase- Free BSA, dATP, dCTP, dGTP and dTTP in aqueous buffer. DTT Solution: 200mM. NotI-d(T) 18 primer: 5`-d(AAC TGG AAG AAT TCG CGG CCG CAG GAA T18) in aqueous solution at 5μg/μl. Rnase-Free Water: Treated with diethyl pyrocarbonate (DEPC). All the reagents were obtained from Amersham Pharmacia Biotech (Freiburg, Germany).

Table 2. RNA samples of cell lines

Cell lines	Origin	Cell type
VMCUB1	Gift of the department of Urology, Heinrich Heine University Düsseldorf Germany	Bladder carcinoma
VMCUB2		Bladder carcinoma
HT1376		Bladder carcinoma
639V		Bladder carcinoma
647V		Bladder carcinoma
5637		Bladder carcinoma
RT4		Bladder carcinoma
T24		Bladder carcinoma
TCC-SUP		Bladder carcinoma
Z82		Bladder carcinoma
BFTC905		Bladder carcinoma
BFTC909		Bladder carcinoma
SKRC48		Kidneycell carcinoma
SKRC58		Kidneycell carcinoma
KTCTLB		Kidneycell carcinoma
KTCKL26A		Kidneycell carcinoma
Jara1		Teratocarcinoma
Jara2		Teratocarcinoma

Some RNA samples were extracted from the cell lines as described in method 2.2. Some RNA samples were kindly given directly and were illustrated in Table 2 and Table3.

Table 3. Biopsy and normal tissue RNA

Biopsy	Origin	Cell type
2345A	Kindly provided by B. Dockhorn-Dworniczak, Institute of Pathology Westf. Wilhems University Münster Germany	Ewing Tumour
2327E		Ewing Tumour
1984B		Ewing Tumour
2226D		Ewing Tumour
2385A		Ewing Tumour
2175A		Neuroblastoma
1470A		Neuroblastoma
2122A		Neuroblastoma
0943II		Neuroblastoma
0977III		Neuroblastoma
0699I		Neuroblastoma
2206(2)		Rhabdomyosarcoma
1242		Rhabdomyosarcoma
12551		Rhabdomyosarcoma
2129		Rhabdomyosarcoma
873		Rhabdomyosarcoma
Brain	Were purchased from Clontech Laboratory, Heidelberg, Germany	nomal cell
Trachea		nomal cell
Lung		nomal cell
Skin		nomal cell
Liver		nomal cell
Kidney		nomal cell

2.3.2 Methods

First-strand cDNA Synthesis carried out using a cDNA synthesis kit (Phamacia Biotec, Freiburg, Germany) was in a standard 33 μl reaction containing 1 μg total RNA and 0.2 μg NotI-d$(T)_{18}$ primer. Prior to cDNA synthesis, 20μl RNA solution containing 1μg total RNA was denatured at 65°C for 10 minutes, then chilled on ice. Subsequently, 11μl Bulk First-Strand cDNA Reaction Mix, 1μl DTT Solution and 1μl NotI-d(T) 18 (1:25) were added to the solution and mixed thoroughly. The reverse transcription reactions were carried out for 1 hour at 37°C and cooled to 4°C or stored at -20°C until use.

2.4 PCR Amplification

2.4.1 Materials

250 units Taq DNA Polymease, 10×PCR Buffer, 5×Q solution, 25mM $MgCl_2$, dNTP Mix were obtained from Qiagen (Hilden, Germany). cDNA, described as 2.3. Thermocycler (Biometra, Göttingen, Germany). 1×TBE buffer: 90 mM Tris; 90 mM boric acid and 1mM EDTA. Agarose gel (Biozyme, South Wales, United Kingdom).

2.4.2 Methods

2.4.2.1 Primers:

The sequences of the *ETAA16* cDNA were obtained and used to design the primer pairs[32]. As the structure of genomic DNA is still unknown, four pairs of specific primers were designed using the program Genefisher (http://bibiserv. Techfak.Uni-bielefeld.de/genefisher) which were displayed in potential region of UTR, ORF and transmembrane domain to ensure the target gene was amplified. *GAPDH* specific primers were used to check the integrating of the cDNA. Additional, *EWS/ FLI-1* primers were used as a second control. Primer sequences (5`-3`), position and PCR product sizes were designed as follows:

***ETAA16* Primers**

Primer	Sequence5'-3'	Position	Ampl.(bp)
ETAA16-S_1	GGCAGCATTTCAGTGCAGACATC	2235	522
ETAA16-R_1	AAGATTGCTGTCCAACAGCTTCC	2735	522
ETAA16-S_7	CTCCATTGACAAAGCAGTTAGG	478	510
ETAA16-R_7	AGCATTAAAGGCTGCTTCAG	978	510
ETAA16-S_8	CATAGGCAATGAGTCGGCGAAG	138	522
ETAA16-R_8	TCCTTTTGCTACACTGGGAGTAC	637	522
ETAA16-S_{12}	TGGTTCTTCCAGGAAGTTCAAG	2446	520
ETAA16-R_{12}	GTTATCAGCAGTCTTCGTGAA	2944	520

The site of *ETAA16* primers are shown in Figure1 (see result).

***GAPDH* Primer**

Primer	Sequence 5'-3'	Position	Ampl.(bp)
GAPDH-S	CACCCATGGCAAATTCCATGGC	Ex3,213	296
GAPDH-R	GCATTGCTGATGATCTTGAGGCT	Ex5/6, 487	296

***EWS/FLI-1* Primer**

Primer	Sequence 5'-3'	Position	Ampl.(bp)
EWS/FLI-1 -S	TCCTACAGCCAAGCTCCAAGTC	*EWS* Ex9	Variable
EWS/FLI-1 -R	GAATTGCCACAGCTGGATCTGC	*FLI-1* Ex4	

2.4.2.2 PCR Reactions

In order to determine the frequency and specificity of *ETAA16* gene, the four pairs of primers specific to the *ETAA16* were used to amplify the *ETAA16* gene.

For primer 1, 7, 12, the PCR master mix was same. Each amplification reaction was performed in 25μl volumes containing 1 μl of template (cDNA), 0.2 mM dNTP, 1.5 mM $MgCL_2$, 1×PCR buffer, 1U Taq DNA Polymerase, 0.2 mM of each 5\` and 3\` primer pairs (the primers varied with primer 1, 7, 12 in each experiment). For primer pair 8, the amplification reaction failed to yield PCR product using the same PCR master mix as that of previously described. Thus, Q-solution was added to the PCR master mix. Each reaction was carried out in 25 μl volumes containing 1 μl of template (cDNA), 0.2mM dNTP, 1.5mM $MgCL_2$, 1×PCR buffer, 1U Taq DNA Polymerase, 0.2mM of each 5\` and 3\` primer 8, 1×Q solution. Probes were denaturated by 94°C for 4 minutes. Followed by 30 cycles of PCR: each cycle consisted of denaturation at 94°C for 30 second, annealing at 55°C for 30 second, elongation at 72°C for 45 second and the final extension at 72°C for 10 minutes.

In order to check the integrity cDNA and the quality of PCR amplification, the "house keeping„ gene, glycerol aldehyde-3-phosphate dehydrogenase *(GAPDH)* which is ubiquitously transcribed in cells was chosen as a control. For the *GAPDH* PCR, the same master mix was used as that of *ETAA16*. However, using *GAPDH* primers the conditions of the PCR-reaction was as follows: an initial denaturation step was carried out for 4 minutes at 94°C, 25 cycles were

performed with the following parameters, denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds and elongation step at 72°C for 30 seconds, the final step was extension for 4 minutes.

To identify ET cells and to test whether *ETAA16* expression might be related to *EWS/FLI-1* expression, RT-PCR analysis was performed amplifying the t(11:22) translocation (*EWS/FLI-1* gene) believed to be specific for Ewing's family of tumours. The PCR master mix was same with that of *ETAA16* but the primer sets were changed to *EWS/FLI-1* primers. Following an initial denaturation step of 4 minutes at 94°C, each cycle consisted of denaturation at 94°C for 30 seconds, primer annealing at 65°C for 30 seconds, and primer extension at 72°C for 45 seconds for a total of 30 cycles. The final elongation step was extended for another four minutes.

Using a housekeeping gene as internal control by coamplification the *ETAA16* gene with *GAPDH* gene in a multiplex PCR allows the control of the quality of the PCR reaction itself. Thus, we established an *ETAA16/GAPDH* multiplex PCR. When using *ETAA16* primers and *GAPDH* primers in equal concentration, we found *GAPDH* to be preferentially amplified. Thus, *GAPDH* primers were serially diluted and the amount of *ETAA16* primers was kept constant. Multiplex PCR was performed in 25 μl volumes, each reaction contained 1 μl of template (cDNA), 0.2mM dNTP, 1.5mM $MgCL_2$, 1×PCR buffer, 1U Taq DNA Polymerase, 0.2mM one set of *ETAA16* primers, to each tube, a 1:2 serial dilution (ranging from 1:6 to 1:1) of *GAPDH* primers were added and then amplifications were carried out for 30 cycles. Following an initial denaturation step of 4 minutes at 94°C, each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 45 seconds. The final extension was carried out for another 4 minutes.

Within each PCR reaction, four additional controls were used to prove the integrating and specificity of the PCR reaction. They are illustrated in Table 4.

Table 4. Four additional controls used in each PCR reaction

Symbol	Type of control	Template
ϕ	Negative	No cDNA
G	Genomic	Genomic DNA of VH64
K	Positive	Clone 6.1.1. of cDNA expression library which was cut by *Bam* HI /*Xho* I
C	Positive	cDNA of VH64

Note:

ϕ: the negative control, the PCR result should always be negative.

G: the genomic control, the PCR result should be negative. If the result is positive, the total RNA extracted from cells should be exclude the DNA contamination by DNAase digestion, thus the none-target gene PCR product can be avoided.

K,C: The PCR result should always be positive.

A-10μl aliquot of each amplification reaction was analyzed by electrophoresis in a 1.5% agarose gel (ultra pure), containing 0.2μg/ml of ethidium bromide. Gels were analyzed using Fluorescence I Imaging System (Bio-Rad, München, Germany).

2.5 Restriction Enzyme Digestion

In order to analyze whether the designed *ETAA16* primers amplify the correct sequence, some PCR products of different cell lines with different tissue origin were digest by restriction enzymes.

2.5.1 Material

Substrate: *ETAA16* PCR products from HFFF, UBC, U2OS, VH-64, SA-OS, A673, N50 cell lines. Restriction enzymes were *FokI* and *XmnI* (NEB, Frankfurt, Germany). The PCR products of primer 1 and primer 7 were restricted by *FokI* at the site of 172bp, 326bp. The PCR products of primer 12 were restricted by *XmnI* at the site of 160bp, 340bp.

2.5.2 Method

A 10 μl aliquot of amplification reaction product was incubated with 10U restriction enzyme in 1× enzyme buffer at 37°C for 1h. The reaction product of digestion was analysed as previously described by 1.5% agarose gels containing 0.2μg/ml of ethidium bromide.

2.6 Immunofluorescence Measurements

2.6.1 Materials

Cell lines used in this experiment are described in 2.1.The primary antibodies were listed in Table 5. PI (propidium iodide) (Merk, Haar, Germany). FBS Buffer (PBS plus 0.1% BSA,0.1% NaN_3). Goat anti-(mouse)-IgG-FITC was purchased from Miles (Frankfurt, Germany).

Table 5. Primary antibodies used in Immunofluorescence Measurements

Primary antibodies	Antigen	Purchaser	Concentration	Ref.
B9.12.1	HLA Class I associated with β_2 microglobulin	Immunotech, Marseille, France	Hybridoma supernant 50μl/5×10^5 cells	[56]
AK15	Antigen 15 of ES	Kindly provided by M. E. Schneider Institute of Anesthesiology, University of Ulm	Hybridoma supernant 10μl/5×10^5 cells	[31]
AK16	Antigen 16 of ES		Hybridoma supernant 10μl/5×10^5 cells	[31]
12E7	MIC2	DAKO, Carpinteria, USA	1:50 20 μl/ cells	[57] [58]

2.6.2 Methods

Adherent cells were detached with 1ml 0.02 % EDTA pe cells r cm^2 for 5min to 15min. After recovering the EDTA, the flask was gently tapped to loosen the cells. 3ml growth medium was added to re-suspend the cells. Cell suspension was transferred into a centrifuge tube, centrifuged at 225g for 5min, the supernatant was discarded completely. Subsequently, 5×10^5 cells were resuspended and washed with 3 ml FACS Buffer (PBS 2%FCS + 0.1% NaN_3) at 4°C. Then the cell pellets were incubated with primary antibody on ice in the

dark for 30min. After the incubation was washed twice with 3ml FACS Buffer at 4°C, 20µl 1:20 goat-anti-mouse-IgG-FITC (Santa Cruz, Heidelberg) was added and incubated for 30 min. After two further washings, 200µl PI (1µg/ml) was added to exclude dead cells. For each sample, 10000 cells were recorded in a FACScan flowcytometer, equipped with FACScan research version 2.1 for data analysis.

Results are presented as mean fluorescence intensity (MFI), calculated by the following formula: Percent positive cells multiplied with the mean channel of the positive cells. The MFI of the isotype-specific negative control is subtracted from each MFI value of each individual sample[59].

3 Results

3.1 Quantity, Purity and Integrity of isolated RNA

High-quality total RNA was isolated from cell lines according to the manufacture's instruction. The quantity of the RNA was determined by measuring the absorbance at 260nm(A_{260}). The purity of RNA was calculated by the ratio between the absorbance values at 260nm and 280nm. The ratio was constantly above 1.8.

3.2 Expression analysis of ETAA16 gene by RT-PCR

Recently, the human cDNA encoding the *ETAA16* antigen has been isolated and sequenced [32]. The antibody, which is directed against *ETAA16* antigen has been reported to be relative specific for ES in immunohistochemical studies as described previously [31]. In order to investigate the molecular specificity of *ETAA16*, a RT-PCR reaction was developed.

Primers were designed with the program Genefisher (uttp://bibiserv.techfak. uni-bielefeld.de/genefisher) corresponding to the *ETAA16* cDNA sequence. As the genomic DNA structure of *ETAA16* is still unknown, the four pairs of primers were designed to cover defined regions of the known cDNA. The primers should cover the potential exon-junctions, because these potential exon-junctions are useful structures to amplify the cDNA and not the genomic DNA (Figure 1).

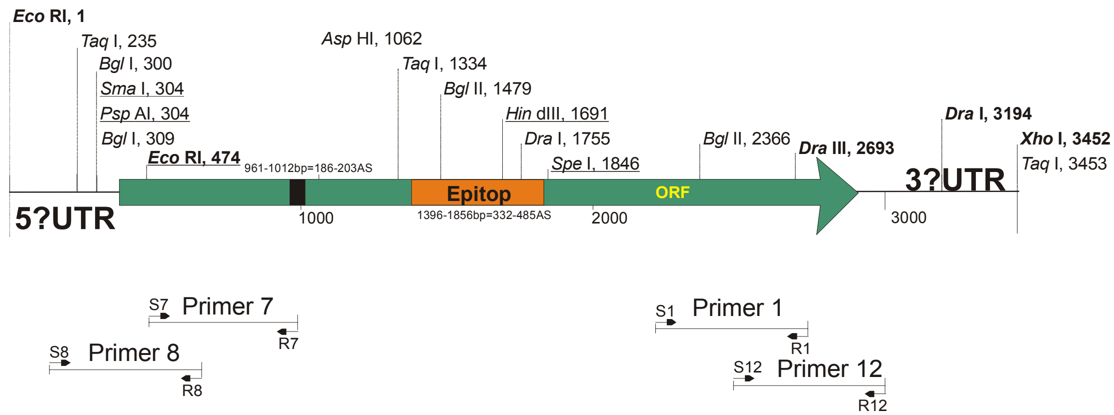

Figure 1. The site of the *ETAA16* primers in cDNA: Primer 7,8 were placed in potential transmembrane domain region, the site of S_7, R_7 o is 478bp to 978bp and the site of S_8, R_8 is 138bp to 637bp. Primer 1, 12 were placed in potential Opening Reading frame (ORF), the site of S_1, R_1 is 2235bp to 2735bp and the site of S_{12}, R_{12} is 2446bp to 2944bp.

The RT-PCR analysis of the *ETAA16* gene was evaluated in 14 samples. The samples obtained from cell line ET-2.2 exhibits two types of growth patterns (adherent and suspension). Therefore, according to the growth patterns ET-2.2 was divided into two samples. As a control for the integrity of the isolated RNA and successful reverse transcription, Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) which is ubiquitously expressed was amplified.

The *EWS/FLI-1* gene, t (11;22)(q24, q12) which was specific to the Ewing's family was also amplified to analyse the presence of the fusion gene in cell lines.

Amplification of *ETAA16* using primer1, primer7, primer 12 showed positive signals in all samples. The amplification product of primer 8 was significantly weaker than those of the other primers, but was still detectable in 13 of 14 samples, except WE-68. As shown in a representative gel in Figure 2. The integrity of the cDNA was checked with the amplification of *GAPDH* transcript and all of these 14 samples were positive. In the same way, the *EWS/FLI-1* translocation was detectable in 12 of 14 samples, except GG-62, RM-82 as described in literature[32].

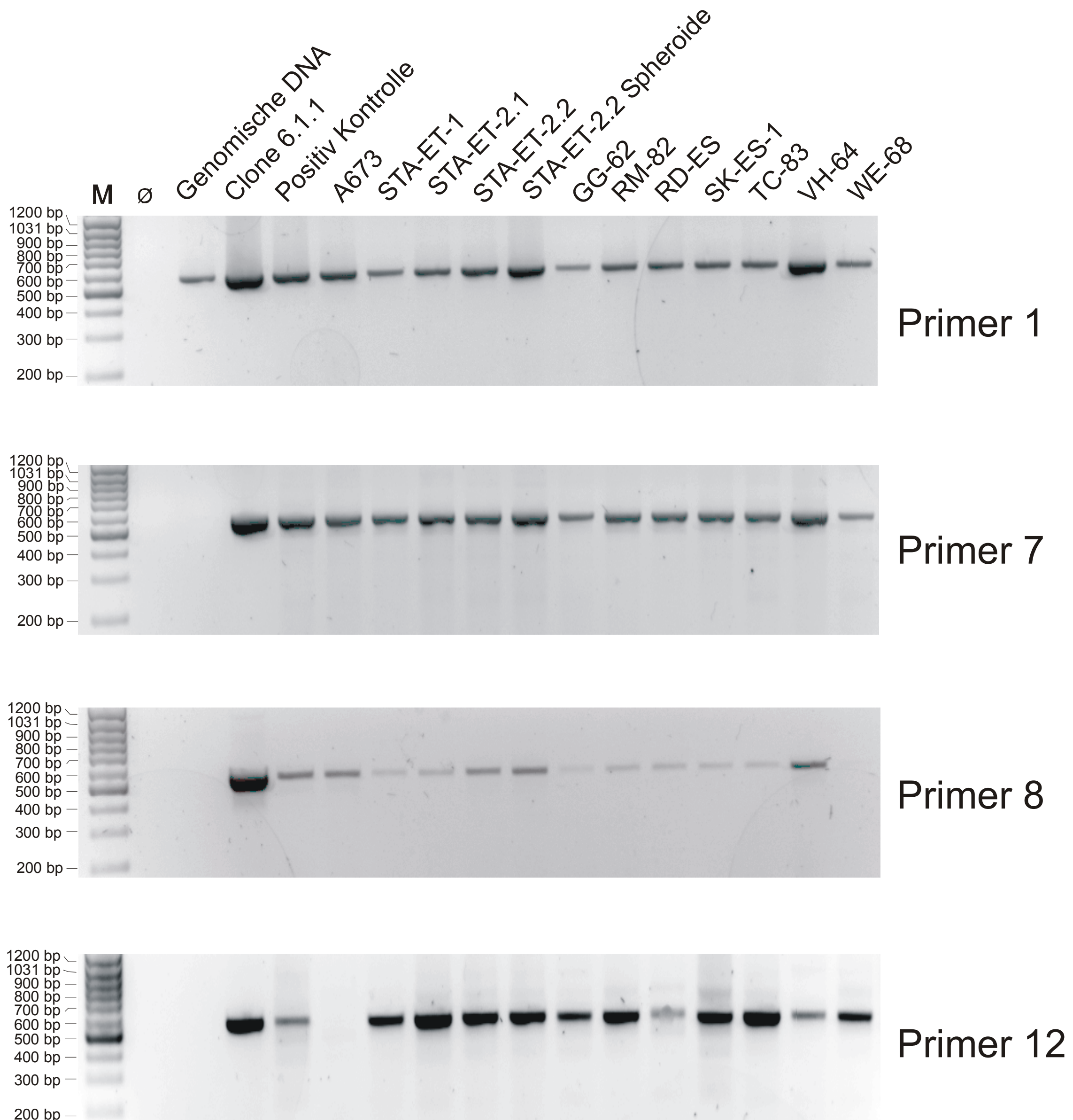

Figure 2. PCR products of *ETAA16* specific amplifications in 14 samples run on a 1.5% agarose gel and stained with ethidium bromide. A-D: Expression of *ETAA16* in Ewing's tumour permanent cell lines with primer 1, 7, 8, 12 respectively, the amplification product is 500bp. M, size markers; Ø, water-negative PCR control; G, genomic control; P, positive control; A673, ET-1, ET-2.1, ET-2.2 (divided into ET-2.2 adherent, ET-2.2 suspension), GG-62, RM-82, SK-ES-1, TC-83, VH-64, WE-68, were Ewing's tumour permanent cell lines.

As an established cell line does not represent the entire nature of the corresponding cell *in vivo*, the transcripts of *ETAA16* were tested on DNA of 5 Ewing's tumour biopsies. Neuroblastoma and rhabdomysarcoma share histological features with Ewing's tumour. They also present as soft tissue lesions with undifferentiated microscopic appearance of uniform small blue round cells. To evaluate whether the *ETAA16* gene transcript is specific to ET,

the amplification of *ETAA16* gene was performed in DNA of 5 Ewing's tumour biopsies, 6 neuroblastomas and 5 rhabdomysarcomas. The results showed that *ETAA16* PCR product with primer1, primer7, primer12 was detected in all of the 5 Ewing's tumour biopsies, and also in the 6 neuroblastomas and 2 cases the 5 rhabdomysarcomas (Figure 3).

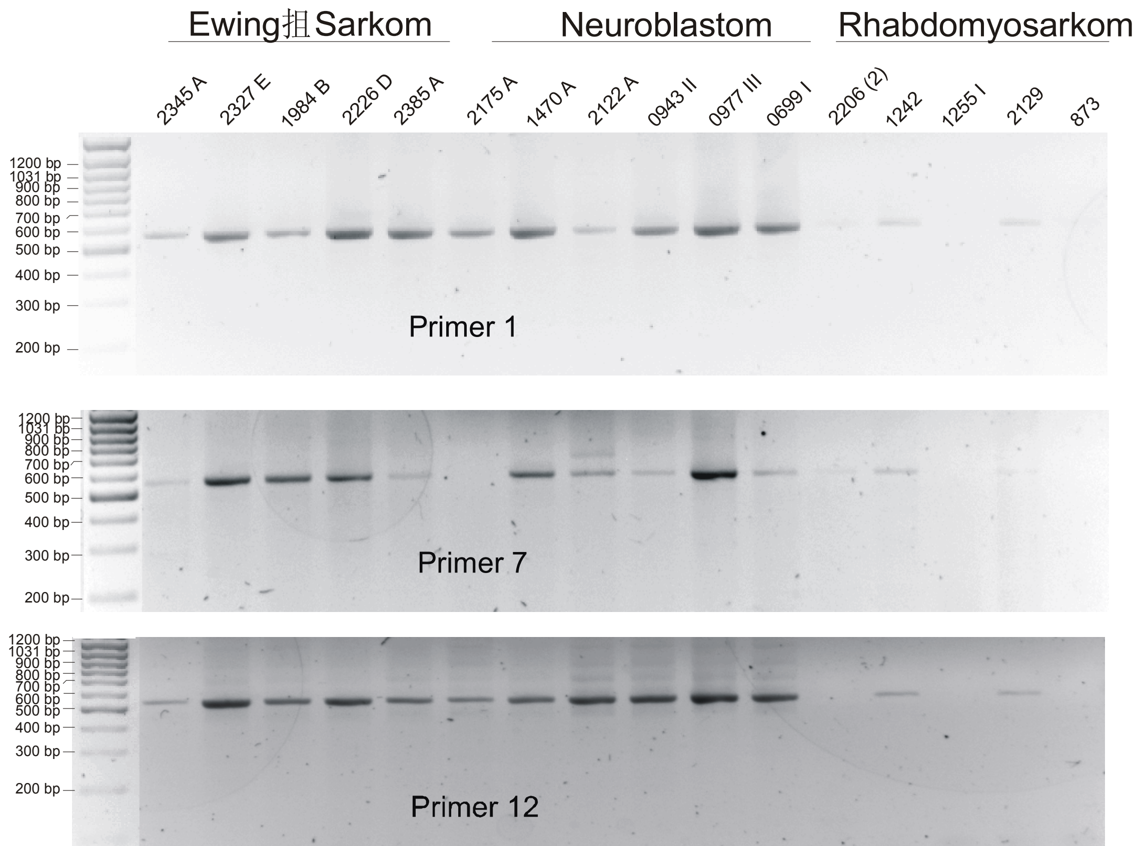

Figure 3. PCR products of biopsies (500 bp) with primer1, 7, 12. M, size makers; Ø, water-negative PCR control; G, genomic control; P, positive control; Lane 2345A, 2327E, 1984B, 2226D, 2385A were biopsies of Ewing's tumour; Lane 2175A, 1470A, 2122A, 0943II, 0977III, 0699I were biopsies of neuroblastoma; Lane 2206(2), 1242, 12505I, 2129, 873 were biopsies of rhabdomyosarcoma.

As the *ETAA16* RNA expression was not ET-specific, now, we investigate the expression pattern of *ETAA16* in distinct kinds of tissues as brain, trachea, lung, heart, liver and kidney. Positive results were detectable with primer 1, 7, 12 in brain, liver, and kidney. To confirm the transcripts of the *ETAA16* RNA in normal human tissues, a multiplex PCR was developed as described in Materials and Methods. The results of coamplification were identical to that of the single RNAamplification, as shown in Figure 4.

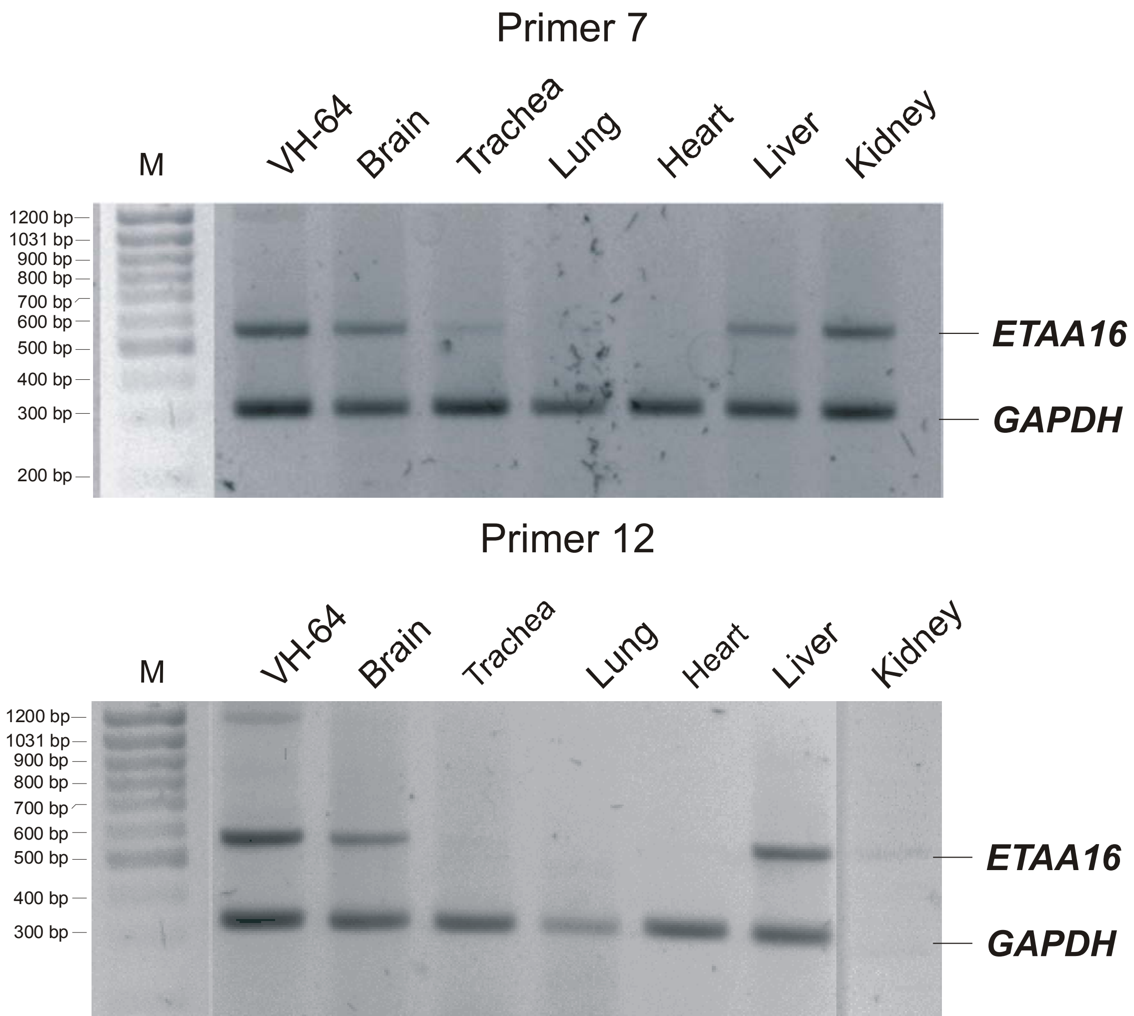

Figure 4. Products obtained after multiplex amplification of *GAPDH* gene (350bp) and *ETAA16* gene (500bp). M, size makers; φ, water-negative control; G, genomic control; P, positive control. Coamplification products of *GAPDH* (350bp) and *ETAA16* gene (500bp) were detected in brain, kidney and liver. Trachea, lung and heart show only *GAPDH* (350bp) transcripts.

Since the *ETAA16* RNA was detected in different cells, more cell lines including 48 other paediatric malignancies and 17 normal cell lines were employed to further investigate the expression pattern of *ETAA16*. The results are summarized in Table 6.

Table 6. *GAPDH, EWS/FLI-1, ETAA16* PCR product of 48 tumour cell lines and 17 normal cell lines.

Cell lines	GAPDH	EWIS/FLI-1	Primer 1	Primer 7	Primer 8	Primer 12
Neuroblastoma						
Be(2)C	+	-	+	+	+/-	+
IMR-32	+	-	+	+	+	+

Cell lines	GAPDH	EWIS/FLI-1	Primer 1	Primer 7	Primer 8	Primer 12
Kelly	+	-	+	+	+	+
ROONEY	+	-	+	+		-
SK-N-SH	+	-	+	+	+	+
Osteosarcoma						
HOS	+		+	+/-	-	+
KHOS	+		+	+/-	-	+/-
MG-63	+		+	+		+
ROS (rat)	+		-	-		-
SAOS-2	+		+	+		+
TM791	+		+	+	-	+
U2-OS	+		+	+	-	+
Rhabdomyosarcoma						
RD	+	-	+	+		+
SJRH30(rat)	+	-	+	+		+
Leukaemia						
Acute Megakaryocytic Leukaemia						
CMK			+	+		+
Chronic Myeloid Leukaemia						
K562	+	-	+	+		+
MEG01, Megakaryoblast	+	-	+	+		+
AML						
KG1a	+	-	+	+	+	+
Monomac6	+	-	+/-	+	+/-	+/-
ALL						
AD	+		+	+	+	+
RS	+	-		+		+
T Cell phenotype Leukemia						
Jurkat	+	-		+		+
H9K	+	-	+	+	+/-	+
B-Cell						
Daudi	+	-				
Raji	+	-		+		+
HEL	+	-		+		+
HL60	+	-		+		+
Histocytic Lymphoma						
U937	+	-	+	+	-	
Choriocarcinoma						
JAR	+	-	+	+	+	+
Hepatocarcinoma						

Cell lines	GAPDH	EWIS/FLI-1	Primer 1	Primer 7	Primer 8	Primer 12
HuH7	+	-	+	+	-	+
HepG2	+	-	+	+	-	+
PNET						
ZK58	+	-	+	+/-	-	-
PFSK-1	+	-	+	+	-	+/-
Glioblastoma						
T98	+	-	+	+	+	+/-
U-87	+	-	-	+	-	+
Cervixcarcionoma						
HeLa	+	-	+	+	+	
Teratocarcinoma						
Jara1	+	-	+	+	+	+
Jara2	+	-	+	+	+	+
PA-1	+	-	+	+	+	+
Kidneycarcinoma						
KTCKL26A	+	-	+	+	+	+
KTCTLB	+	-	+	+	+	+
SKRC48	+	-	+	+	+	+/-
SKRC58	+	-	+	+	+	+
Bladdercarcinoma						
639V	+	-	+	+	+	+
647V	+	-	+	+	+	+
BFTC905	+	-	+	+	+/-	+/-
BFTC909	+	-	+	+	+/-	+/-
HT1376	+	-	+	+	+	+
RT4	+	-	+	-	-	+
T24	+	-	+	+	-	+/-
TCC-SUP	+	-	+	+	+/-	+/-
VMCUB1	+	-	+	+	+	+
VMCUB2	+	-	+	+	+	+
SD	+	-	+	+	+	+
Z82	+	-	+/-	+/-	+/-	+/-
Fibroblast						
HFFF13	+			+		+
HFFF15	+		+	+		+
HFFF32	+		+	+		-
NIH3T3 (rat)+A27	+		-	+		-
NIH3T3/ GP+envA			-	+		-

Cell lines	GAPDH	EWIS/FLI-1	Primer 1	Primer 7	Primer 8	Primer 12
M-12						
N50	+	-	+	+	+	+
Primary skin fibroblasts	+	-	+	+	+	+
Keratinocyte	+	-	+/-	-	+	+
Epithelial						
UBC	+	-		+		+
Endothelia						
HUVEC	+	-	+	+/-	+/-	-
Embryonal Kidney Cell, E1A transformed						
293T	+	-	+	+	+	+
FNX-A	+	-	+	+	+	+
FNX-E	+	-	+	+	+	+

As shown in Table 6, the *GAPDH* PCR product was positive in all tested cell lines. No transcripts of *EWS/FLI-1* were found in these none ET samples, which confirmed the diagnosis of the SRCT included in this study. For amplification of *ETAA16*, primer 1, primer 7, primer 12 detectable amplification product (500bp) was evident in most samples, except H9/PMA (a T-cell line was stimulated by PMA). ROS (an osterosarcoma cell line derived from rat). Primer 8 amplification product (500bp) can be detected in few cell lines.

As some cell lines had the heterogeneity amplification products of *ETAA16* RNA with Primer 1, Primer 7, Primer 12, the results were confirmed by multi-plex PCR. Data is not shown.

In order to analyse whether the designed *ETAA16* primers amplify the correct sequence, restriction enzyme digest assay was performed in some PCR products of different cell lines with different tissue origin. The amplification product of primer 1 was digested by restriction enzyme *Fok* I, and gave the estimated 104bp, 340bp fragments. The amplification product of primer 7 was digested with *Rsa* I and leads to the estimated 160, 340 bp fragments. The amplification product of primer 12 was digested by restriction enzyme *Hinc* II and also gave the estimated 273bp, 227bp fragments. Thus, the primers amplified the correct *ETAA16* RNA product. Data are not shown.

3.3 *Flowcytometric analysis*

The *ETAA16* antibody was initially described to bind an ET-specific antigen as demonstrated by immunohistochemical analysis. The results of the molecular expression of the *ETAA16* antigen, however, showed that the RNA was ubiquitously exited in cells of SRCT. To see the accurate surface expression of the *ETAA16* RNA, cells were analysed by FACScan flow cytometry. Staining of cells was calculated in mean fluorescence intensity. According to previous studies on the ET antibodies created by Shi, the antibody 15 and 16 showed a similar molecular weight and a very similar binding pattern in the immunohistochemistry. Therefore we included the antibody 15 in our flow cytometry studye. As a positive control, the mAb B9.12.1 recognising a monomorphic determent of HLA-class I molecules which is expressed on human cell surface was used. Furthermore the MIC-2 antigen ,known to be overexpressed on the surface of ET tumours was also employed to compare the expression patterns of *ETAA-16*. The results of representative cells surface staining with mAb16 are shown as a histogramm overlay in Figure 5.

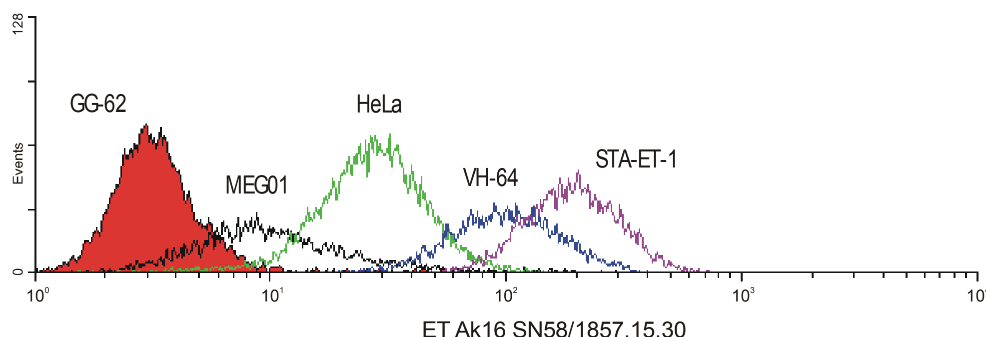

Figure 5. *ETAA16* surface staining of different cell lines with its specific monoclonal mAb16. The *EWS/Fli-1* negative ET cell line GG-62 is negative for mAb16. MEG01 and Hela, these two non-ET cell lines show comparatively weak positive for mAb16. The *EWS/Fli-1* positive ET cell lines VH-64 and STA-ET-1 show strong positive for mAb16. Antibody binding was evaluated by flowcytometry.

The results of all investigated cell lines for the cell surface expression of ETAA16 are summarised in Table 7.

Table 7. Surface staining of monomorphic HLA class I antigen(B9.12.1), MIC-2 antigen (12E7), antigen 15 (AK15 SN58/162.28.11.37)and antigen 16(AK16 SN58/1857.15.30)on different cell lines.

	Mean Fluorescence intensity			
Cellline	**B9.12.1**	**12E7**	**AK15 SN58/162.28.11.37**	**AK16 SN58/1857.15.30**
Ewing's tumour				
A-673	14.754,65	4.062,34	2.552,48	5.553,32
CADO-ES1	105.294,40	7.013,64	20,31	152,86
ET-1	8.598,33	2.916,56	13.238,90	21.528,16
ET-2.1	6.760,76	2.910,00	6.145,65	8.869,52
ET-2.2	11.256,28	5.154,15	6.389,78	12017.26
ET-5	311,81	310,04	39,33	76,85
GG-62	24.875,40	1.454,02	0,36	1.64
RD-ES	12.738,70	3.877,23	377,78	790.55
RM-82	16.032,77	1.898,92	3,50	8.31
SK-ES-1	3.193,50	1.938,26	8,44	49.59
TC-83	31.387,22	961,37	3.770,82	5455.60
VH-64	2.745,72	2.492,82	5.659,40	10294.99
WE-68	71.482,79	3.422,20	5.545,51	14929.86
Neuroblastoma				
Be(2)C	26.019,51	938,69	21,78	99.46
IMR-32	35,70	37,75	70,65	155.21
Kelly	1,28	-0,82	2,18	17,87
ROONEY	18.732,39	137,87	-1,01	32.25
SK-N-SH	313,20	-8,53	-17,51	-31.00
Osterosarcoma				
KHOS	17.404,69	2,53	4,72	13.62
MG-63	31.178,99	705,93	17,25	129.13
ROS (rat)	1,21	0,45	0,42	13.59
SAOS-2	22.541,05	21,32	4,98	32.95
Rhabdomysarcoma				
RD	3.873,53	4,86	-1,66	7.00
SJRH30(rat)	1,93	4,35	0,84	6.04
Acute megacaryoctic leukemia				
CMK	25.825,58	203,94	0,19	0.74
Chronic myeloid Leukemia				
K562	248,12	390,40	144,33	35.52
MEG01, Megacaryoblast	18.496,20	1.373,92	580,94	461.72
AML				
KG1a	50.161,81	209,70	108,61	100.26
ALL				

	Mean Fluorescence intensity			
Cellline	**B9.12.1**	**12E7**	**AK15 SN58/162.28.11.37**	**AK16 SN58/1857.15.30**
AD	67.361,69	-8,23	-12,13	-11.93
RS	70.482,48	141,68	0,14	4.26
T Cell Phenotype Leukemia				
Jurkat	40.503,95	862,69	3,50	2.28
B-Cell				
Daudi	213,00	2,62	3,51	26.67
Raji	49.971,23	50,04	22,44	27.98
HEL	19.329,74	711,99	364,85	100.51
HL60	16.580,54	14,95	8,08	3.65
JAR	0,34	0,17	4,37	1.93
Hepatocarcinoma				
HepG2	14.642,05	0,70	-1,12	126,21
PNET				
PFSK-1	842,22	7,91	3,60	8.44
zk-58	13.028,20	9,09	7,45	39,57
Glioblastoma				
3.3.1.1.1.1.1.1	41.422,35	5,38	-1,53	1,91
U-87	0,40	2,02	4,86 P30/32 mic2	49.38
Cervixcarcionoma				
HeLa	14.562,23	7,85	1.204,15	2670.34
Teratokarcinoma				
PA-1	3.668,67	18,68	0,37	7.20
Bladdercarcinoma				
HT1376	35.284,98	743,32	-9,29	642,24
SD	22.835,83	1,13	-0,14	1,19
T24	7.588,43	35,57	-2,27	64,70
VMCUB1	2.684,39	8,63	0,43	22,02
VMCUB2	5.706,59	227,22	1,20	61,47
Fibroblaste				
HFFF15	57.929,01	155,90	24,58	19.72
NIH3T3(rat)	0,40	0,86	1,95	13,83
Primary Haut fibroblast	46.341,54	178,10	-3,65	20.92
Epithelial				
UBC	41.004,01	5,10	2,02	6,25
Embryonal kidney Cell, E1A transformed				
293T	2.019,86	-2,90	63,55	9.31
FNX-A	9.093,68	413,29	19,83	123.04
FNX-E	5.373,39	1.057,78	8,30	-5.36

The results in Table 7 showed: HLA class I was detected on all cell lines except of IMR-32, Kelly, ROS, SJRH30, JAR, NIH 3T3. Staining of the ET cell lines A673, ET-1, ET-2.1, ET-2.2, RD-ES, TC-83, VH-64, WE-68 revealed a strongly positive signal for mAb16 and mAb15. Three of the ET cell lines: CADO-ES1, ET-5 and SK-ES-1 were comparatively weak positive and the *EWS/Fli-1* negative cell lines GG-62 and RM-82 were negative for these two antibodies. It is important to note that many tumours with SRCT-like features and, thus, often considered in the differential diagnosis of ES showed no immunoreactivity with Ak15, Ak16. Except weak signal detected on MEG01 and Hela. As expected, p30/32 ^{mic2} antigen highly expressed on all ET cell lines. p30/32 ^{mic2} was also detectable in a broad variety of tumour and normal cells, however , at a lower level. Thus p30/32 ^{mic2} was over expressed in ET cells as described in other studies [28 29].

4 Discussion

Ewing's tumour (ET), i.e., ES, PNET, and Askins tumour, are the second most common primary osseous malignancies in childhood or adolescence [60]. The use of multiagent chemotherapy significantly improved the survival of ET-patients[61 62 63 64]. However, one third of the patients relapse and the outcome of patients with primary disseminated disease remains poor [65 66]. The detection of specific *EWS/ETS* gene rearrangement and antigen p30/32 [mic2] has become important diagnostic technique in the clinical practice. But, with increasing valuable of this technique in the diagnosis of ET, its limitation is becoming evident[23 24]. Beside this, there is some, but not yet conclusive evidence shows that a specific *EWS/ETS* rearrangement (*EWS/Fli1* type1) is associated with a higher survival rate. The identification of new tumour-specific markers will offer novel insights and approaches towards the classification of tumours with important prognostic and/or therapeutic implications.

In a recent study, 16 monoclonal antibodies have been generated against ET[31]. The antigen of the mAb16 were cloned by immunoscreens of an ET-specific expression library so that this antigen were called *ETAA16* for Ewing's Tumour associated antigen 16 [32].This mAb shows no important cross-reactivity and detects its corresponding antigen in paraffin-embedded biopsies [31]. Thus *ETAA16* was proposed to be a favourable ET specific antigen. However, the ET-specificity has been demonstrated by immunohistochemistry only. The sequence of the cloned cDNA of *ETAA16* gives the possibility to check expression pattern at a transcriptional level by more sensitive RT-PCR analysis. Aim of the present study was to investigate the expression of the *ETAA16* transcript in different cell types by RT-PCR analysis, furthermore, to evaluate if *ETAA16* might be a potential molecular marker in diagnosis of ET and might offer a new potential therapy.

RT-PCR assays were used to analyze transcript of *ETAA-16* in 95 samples including 14 Ewing sarcoma samples, 16 tumour biopsies, 6 normal human tissues, 17 normal cell lines and 48 other pediatric small round cell tumours. In the present study, the expression of *ETAA16* is detected in a wide variety of tumour and normal cells. In all 14 ET cell lines and 5 tumourbiopsies, an

ETAA16 specific ampification product is detectable with four different primer pairs (Figure 2,3), hybridise in different regions of the cDNA (Figure 1). Previous experiments using immunhistochemical techniques showed the *ETAA16* antigen was detected on the surface of ET only [31]. The present study shows that the *ETAA16* transcript is expressed in other small round cell tumours as neuroblastomas and osteosarcomas as well as in cell lines from various tumour and normal tissues (Figure 3, Table 6). The only negative tested samples were DNA from trachea, lung and heart (Figure 4). These results suggest that *ETAA16* is ubiquitously transcribed in cells and the detection of the expression of this gene at RNA level will not be useful for differentiating *ET* from other tissues as well as small round-cell tumours of childhood.

Since *ETAA-16* gene is broadly expressed in cell lines on the RNA level (Table 6), the surface expression of *ETAA-16* antigen was re-evaluated in same tumour samples and cell lines by FACScan flow cytometry. The results showed the translocation to the cell surface of *ETAA16* antigen is only detected in *EWS/Fli-1* positive ET cell lines as well as two other non-ET cell lines MEG01 and Hela. However, the *ETAA16* antigen is widely distributed on an intracellular protein level from the work of Dr. A. Borowski by intracellular protein staining analysis.

Understanding the biological mechanism leading to *ETAA-16* surface expression on ET cells may contribute to further understanding of the nature of ET biology. One possibility is that the mAb16 detects another molecule rather than the *ETAA16* antigen described by A. Borowski. A way of verifying the *ETAA16* as the definitive antibody-defined antigen would be a transfection analysis: the cDNA should be eukaryotic expressed in xenogenic cells–which do not express an ETAA16 transcript as CHO or COS-7. If the antigen is detectable following transfection by staining with the mAb16, the ETAA16 antigen depicts the mAb16 defined antigen. As it is proposed that the surface expression of the ETAA16 antigen is dependent on a ET specific pathway, the ETAA16 antigen should be detecatable intracellulary and not on the surface. Additional, the antigen of the mAb16 could be purified by immunoprecipitation experiment. Afterwards the antigen could be analysed by a proteomic

investigation to determine the aminoacid sequence and comparing this with the deduced aminoacid sequence of the isolated *ETAA16* cDNA[67].

The surface expression of the antigen might be regulated by the ET-specific *EWS/ETS* transcripts. As shown for the most common transcript EWS/Fli1, the chromosomal translocation results in protein containing the amino-terminal domain of *EWS* and DNA-binding domain of the transcription factor *FLI-1*. This structure suggests that the chimeric protein might be directed to the promoter region of specific genes and alters the expression of these genes[68]. In support of this, *EWS/FLI*-1 protein has been shown to be an efficient transcriptional activator when cotransfected with model reporter gene constructs containing ETS-binding sites in their promoters[69]. Furthermore, some experimental data has shown that the fused protein functions as a transcription activator on specific genes. These target genes include: 1) *MFNG*, a member of the Fringe gene family encoding secreted signalling molecules instrumental in somatic development [70,71]. 2) *EAT2* (EWS/FLI-1 –activated transcript 2) has features of a signal transduction molecule [72] . 3) *E2-C* is a cyclin-specific ubiquitin-conjugating enzyme and has been related to cell cycle control and cyclin B degradation[73]. It is thought that these target genes deregulated by *EWS/FLI*-1 protein are part of the specific characteristics for ET.

The results of this study indicates, that *ETAA16* transcription may not be affected by *EWS/FLI*-1 protein but the cell surface expression might be induced by the fusionproduct which modulates *ETAA16* or leads to a *de novo* expression of an associated antigen which causes the translocation through the cell membrane.

It is a complex way for a membrane protein to attain the surface of the cell after being translated in cytoplasm. The general pathway for the transport of nascent polypeptide to the cell surface is dependent on a series of membranous structures that include the endoplasmic reticulum(ER), the Golgi complex and the culminates in fusion of secretary/transport vesicles with the plasma membrane. The nascent polypeptide is posttranslational modified in the transport way by preteolysis, glycosylation or association with protein chaperones[74]. It is supposed that the *EWS/Fli-1* fusion protein or other ET-

specific regulatory proteins, might directly modulate the cytoplasm *ETAA16* antigen to attain the membrane, or the fusion protein leads to a *de novo* expression of an associated antigen which is needed by cytoplasm *ETAA16* antigen to attain the membrane. There is growing evidence that some protein expressed at the cell surface is associated with other cytoplasm protein. Such as the nucleolin, a multifunctional protein expressed on the cell surface where it exists in close association with the intracellular actin[75].

Although this kind of function of the EWS/ETS fusion protein was not reported before, this hypothesis underlined by our finding that the staining with mAb16 was negative in ES cells (GG-62, RM-82) lacking the *EWS/FLI-1* gene expression (Figure5, Table7). Furthermore other tumours with SRCT-like features lacking *EWS/ETS* gene rearrangements showed no immunoreactivity with Ak15, Ak16. However, further investigation has to be done to study the mechanism of the ET-restricted expression of the *ETAA16* corresponding surface antigen. First of all, coexpression analysis of *EWS/FLI-1* has to be evaluated whether the fusionproduct may influence the *ETAA16* surface expression. Immunoprecipitation analysis has to be done to look for the modulation of *ETAA16* in ET and non-ET cells. Possibly, associated proteins could be identified with this approach. In addition, two cell lines MEG01 (chronic myeloid leukemias) and Hela (cervix carcinoma) showed weak immunochemical stain with mab 16, further experiment should be included to investigate the relationships between the two cell lines with ET cells. However, the differential cell surface expression of *ETAA16* could be a interesting gene for the Ewing's Tumor diagnostic and its corresponding antibody gives the possibility for an immunotherapy of ET patients.

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Ich versichere, daß ich die vorliegende Dissertation selbst verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

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