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Diminished Expression of the Type II Receptor for TGFβ (TGFβRII) in T Lymphocytes from Patients with Sezary Syndrome Is Not Due to Mutations in the Receptor's Poly-A Tract: Limitations of the Standard RT-PCR in cDNA Sequence Analysis of Homopolymeric Base Stretches

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Abstract. Peripheral blood lymphocytes from patients with Sezary syndrome (SzS) frequently demonstrate decreased surface expression of transforming growth factor β receptor II (TGFβRII). The mechanism of this low TGFβRII expression remains unknown. Because mutations within the poly-A tract of the TGFβRII sequence (nucleotides 709–718) were shown to result in diminished TGFβRII expression in other types of malignant tumors, we examined the sequence of the TGFβRII poly-A tract in two SzS-derived cell lines and in peripheral blood SzS cells from 17 SzS patients and 4 control, healthy individuals using DNA sequencing and single-stranded conformation polymorphism (SSCP) analysis. A standard bidirectional, automated sequence analysis of the RT--PCR-generated cDNA TGFβRII fragment showed a heterogenous population of the normal length, 10-, with admixed, shortened, 9-base poly-A stretches. Surprisingly, this mixture was present not only in the cells from 5 SzS patients and 2 SzS cell lines, but also in cells from 2 healthy control individuals. Importantly, the proportion of the shortened, 9-base fragments was markedly reduced or practically eliminated when the procedure was modified by usage of high-fidelity DNA polymerase, labeled primers and/or cloned RT-PCR products, which indicates that the presence of the shortened, 9-base fragments represented a procedural phenomenon rather than a true deletional mutation within an allele of the TGFβRII gene. Accordingly, SSCP analysis of genomic DNA did not reveal any mutations within the poly-A tract-containing region. These results indicate that a mechanism different from mutations in the polyadenine tract underlies the diminished TGFβRII expression in SzS cells and that the results of an unmodified, direct sequence analysis of homopolymeric base streaches in RT-PCR-derived cDNA should be interpreted with caution.

Key words: type II receptor for TGFβ; cutaneous T cell lymphoma; Sezary syndrome; poly-A tract sequence analysis.

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Introduction

Cutaneous T cell lymphoma (CTCL) is a progressive, clonal lymphoproliferative disorder of atypical T lymphocytes[22]. In the clinically more advanced cases of CTCL, atypical Sezary T cells are frequently present in the peripheral blood. When the circulating Sezary cells number at least 1000/mm^3 or show loss of a pan-T cell marker, CD4 to CD8 T cell ratio reaches 10:1, and/or circulating clonal or cytogenetically abnormal T cells are detected, a diagnosis of Sezary syndrome (SzS) is rendered[20].

The loss of ability to respond to transforming growth factor β (TGFβ), which is a potent inhibitor of normal T cell growth, may be potentially oncogenic[1]. Our previous study[2] demonstrated that SzS cells often display a low response to the growth-inhibitory effects of TGFβ, apparently resulting from the low surface expression of the type II receptor for this cytokine (TGFβRII). The molecular mechanism responsible for the diminished TGFβRII surface expression in such SzS cells remains, however, unknown. One candidate mechanism was described by MARKOWITZ et al.[12], who found mutations in cDNA coding for TGFβRII in cell lines and tumor tissues from patients with hereditary nonpolyposis colorectal cancer[12]. These mutations were clustered within a stretch of 10 adenine bases (nucleotides 709–718) and represented 1- and 2-base deletions. All colorectal carcinoma cell populations carrying these mutations had greatly diminished expression of surface TGFβRII.

In the present investigation we analyzed peripheral blood mononuclear cells (PBMC) and cell lines derived from SzS patients for possible mutations within the poly-A tract. Detailed structural analysis of cDNA by bidirectional, automated sequencing and of the genomic DNA by single-stranded conformation polymorphism (SSCP) demonstrated an absence of any mutations within this region, although the automated cDNA sequencing demonstrated in both patients and control healthy individuals the presence of not only the normal 10-base poly-A stretches, but also shortened 9-A repeats. The frequency of such shortened poly-A repeats was methodology dependent. The biological and experimental implications of these findings are discussed.

Materials and Methods

Patients and healthy controls. Seventeen patients with SzS were studied. All had erythroderma, lymphadenopathy, and peripheral blood involvement by morphologic, molecular and/or cytogenetic criteria. The percent of circulating SzS T cells ranged 21–90% (median 37%) based on morphology, and 27–99% (median 85%) based on CD4$^+$ T cell count. In all cases a clonal population was detected by T cell receptor gene DNA rearrangement (γ chain) and/or cytogenetic analysis. Four healthy members of our Department served as normal controls.

Cell lines. The cutaneous T cell lymphoma lines used in this study: SZ-4, kindly provided by Dr. T. Abrams from Hahnemann University (Philadelphia, PA), and HUT102B, have been described previously in detail[25]. Whereas the SZ-4 cell line clearly represents SzS cells, the nature of the HUT102B line remains controversial due to the HTLV-1 positivity. Genomic DNA preparations from 2 colorectal carcinoma cell lines (VACO-5 and HCT116), previously described as carrying TGFβRII mutations[12], were obtained from Dr. S. Markowitz (Case Western Reserve University, Cleveland, OH).

Cell isolation and culture. PBMC for flow-cytometric and nucleotide-sequence analysis were obtained from blood as described[2, 25, 26] by centrifugation on Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient. PBMC for SSCP study were obtained by leukapheresis. These cells were also subjected to Ficoll-Paque gradient centrifugation and stored in liquid nitrogen in freezing medium (80% RPMI1640/20% FBS/10% DMSO). To determine the cell-surface expression of TGFβRII, the PBMC (2×10^6/ml) were cultured alone or with a combination of phytohemagglutinin P (Sigma; 1 μg/ml), phorbol 12-myristate 13-acetate (0.5 ng/ml), and 10% (vol/vol) MLA-144 cell supernatant as a source of growth factors (including IL-2 but not TGFβ). This combination has been previously found to stimulate optimal proliferative responses in SzS T cells[2]. Cultures were maintained in 24-well flat-bottom plates (Becton Dickinson, Mountain View, CA) for 24 h at 37°C and 5% CO_2 in the supplemented 10%FBS/RPMI 1640/10% FBS medium.

Flow cytometry analysis. Surface staining for TGFβRII expression was performed as described[2]. Briefly, cultured cells were washed and resuspended at 1×10^6 cells/tube. To reduce non-specific antibody binding, the cells were preincubated with an unlabeled rat IgG (Sigma, St. Louis, MO) and normal goat serum (Sigma). Control IgG derived from a non-immunized rabbit, CD4-PE, or polyclonal rabbit anti-human TGFβRII (UBI, Lake Placid, NY) were then added. The cells were incubated for 30 min on ice, washed, and incubated with a secondary antibody, FITC-labeled goat anti-rabbit IgG (Sigma). Dual staining with the CD4-PE antibody (Immunotech, Westbrook, ME) was performed according to the manufacturer's directions. Cells were analyzed on a FACSort (Becton Dickinson, Mountain View, CA) using LysisII or CellQuest software.

RT-PCR amplification and cloning of the TGFβRII cDNA fragment. The complete nucleotide sequence of the human TGFβRII[11] was obtained from the GenBank data base (NCBI, Bethesda, MD; accession number: M85079). RT-PCR was performed as previously described[25, 26] with minor modifications. In brief, single-stranded cDNA was synthesized from total RNA isolated by guanidium/cesium ultracentrifugation, using 200 U SuperScriptII RNase H-reverse transcriptase (GIBCO/BRL, Gaithersburg, MD), 5 ng oligo (dT) primer (Promega, Madison, WI), 1 × first strand buffer, 0.1 M DTT, 10 mM dNTPs, and 200 U of SuperScriptII transcriptase. The mixture was incubated for 1 h at 37˚C, then the reaction was terminated by heating at 70˚C for 15 min and residual RNA was digested by incubation with RNase H (Promega) for 30 min at 37˚C. PCR was performed in 100 µl which contained 2 µl of the cDNA preparation, 1× PCR buffer, 200 µmol dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of 5' and 3' primers, 2.5 units Taq DNA polymerase (Perkin-Elmer), PCR buffer containing 200 mM Tris-HCl (PH 8.3), and 500 mM KCl. In some experiments Taq polymerase was substituted with Pfu polymerase (Stratagen, La Jolla, CA) in the appropriate PCR buffer. The PCR primers specific for the fragment of the TGFβRII cDNA sequence which contains the poly-A tract were synthesized and purified by the Nucleic Acid Facility of the University of Pennsylvania Cancer Center and had the following sequences: sense primer (5'-GAC AGT TTG CCA TGA CCC CAA) and the antisense primer (5'-TAA CGC GGT AGC AGT AGA AGA). The amplification was performed in the GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer). The conditions were: 3 min at 94˚C to completely denature the template, followed by 30 cycles: 45 s at 94˚C, 30 s at 50˚C, and 1.5 s at 72˚C followed by final 10 min incubation at 72˚C. The samples without the added SuperScriptII RNase H-reverse transcriptase served as negative controls. The amplified products were visualized in UV light by staining with ethidium bromide after electrophoresis in 1.5% agarose gel. The amplified TGFβRII DNA fragment (260 bp) was extracted from agarose gels and purified with the QIAEX gel extraction kit (QIAGEN Inc., Chatsworth, CA) as described by the manufacturer. In some experiments, the PCR products were inserted into 2.1 vector using the TA cloning kit (Invitrogen, San Diego, CA). The presence of inserts was confirmed by ECOR1 digestion and gel electrophoresis.

DNA sequence analysis. The nucleotide sequence of the TGFβRII cDNA fragment was determined by the sequencing of the PCR products either directly, after purification, or indirectly, after purification and cloning, using the ABI PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer). Twenty µl of the reaction mixture contained 9.5 µl terminator premix with AmpliTaq DNA Polymerase, 50 ng template DNA, and 3.2 pmol TGFβRII specific, sense or anti-sense primer. The reaction was carried for 25 cycles: 10 s at 96˚C, 5 s at 50˚C, and 4 min at 60˚C. Column-purified amplified products were dried by vacum centrifugation. The nucleotide sequence was determined on an automated ABI DNA sequencer (model 373A, Applied Biosystems, Foster City, CA). In some experiments a hybrid primer composed of M13 reverse primer sequences at the 5' end and the TGFβRII-specific 5' primer at the 3' end, was used for PCR amplification. The product was sequenced using M13 dye labeled primers with AmpiTaq FS DNA polymerase (Perkin-Elmer).

DNA isolation and SSCP analysis. Cryopreserved PBMC were thawed and the genomic DNA was isolated as described[25]. PCR was carried out in a standard 50 µl 1X buffer (Perkin-Elmer-Cetus) containing 1.5 µM $MgCl_2$, 0.5 mM dNTPs, 5 µCi [a-32P]dCTP, 20 pmol 5' TGFB type II primer (5'-ATAACACTAGA-GACAGTTTGCCATG), 20 pmol 3' TGFB type II primer (5'-CTGAGAAGATGATGTTGTCATTGCA), 200 ng of genomic DNA and 0.25 units of Amplitaq polymerase (Perkin-Elmer, Norwalk, CT). Reactions were overlaid with mineral oil and amplified for 35 cycles under the following conditions: 95˚C for 30 s, 60˚C for 45 s, and 72˚C for 90 s. The final cycle included a 72˚C, 7 min extension step. After PCR, 5 µl of PCR product was mixed with 5 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and denatured for 5 min at 95˚C. Samples were immediately placed on ice for 5 min and were then loaded onto a 6% polyacrylamide (19:1 acrylamide to bis-acrylamide)/0.5X TBE gel with 10% glycerol. Electrophoresis was carried out in 0.5X TBE buffer at 25 W for 4 h in front of a fan. Gels were subsequently dried under vacuum and subjected to autoradiography.

Results

Detection of TFGβRII cell-surface expression

Our previous studies have shown that mitogen-stimulated peripheral blood lymphocytes from SzS patients frequently display low cell-surface expression of TGFβRII[2]. To extend this finding, as well as to deter-

Table 1. Cell surface expression of TGFßRII in mitogen-stimulated $CD4^+$ PBMC from Sezary patients, cell lines, and control, healthy individuals

	PBMC source		
	Sezary cells (%)	CD4 cells (%)	TGFßRII (δ MFI)
Patient No.: 1	70	79	1.12
2	90	90	1.19
3	32	96	1.34
4	66	85	3.35
5	22	27	3.88
6	21	53	5.59
7	27	76	10.41
8	34	96	12.46
Cell lines: SZ-4	NT	92	3.55
HUT102B	NT	94	6.49
Healthy person No.: 1	<1	39	12.02
2	<1	44	12.08
3	<1	51	14.26
4	<1	48	17.12

MFI – mean fluorescence index (ratio of mean fluorescence intensity of the anti-TGFßRII polyclonal rabbit antibody to the control, nonimmune polyclonal antibody).

NT – not tested.

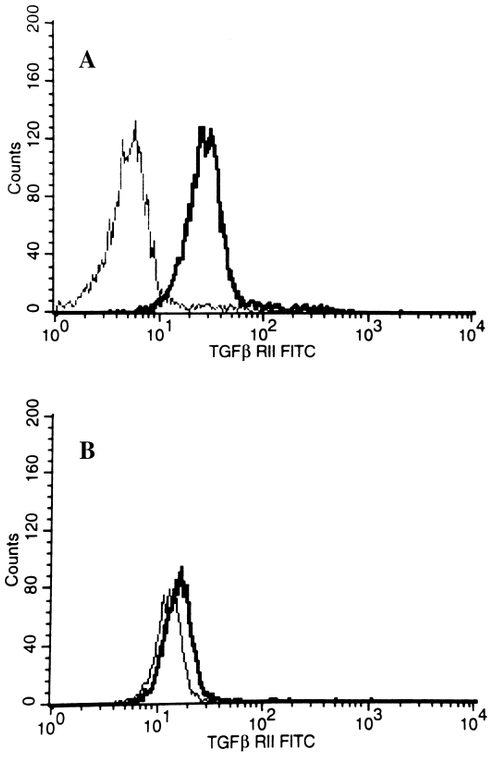

Fig. 1. Flow-cytometry analysis of the TGFβRII cell-surface expression by SzS-derived and control, normal cells. Mitogen-stimulated, PBMC from a healthy donor (**A**) and SzS patient (**B**) were double stained with anti-CD4 and anti-TGFβRII antibodies and the $CD4^+$ fraction was analyzed for TGFβRII expression

mine the TGFβRII expression in the group of patients available for the present study, a similar flow cytometry analysis was performed in 8 patients, 2 SzS cell lines, and 4 control, healthy individuals (Table 1, Fig. 1). Six of these 8 patients demonstrated low surface levels of TGFβRII in response to mitogen stimulation as measured by the net change in mean fluorescent intensity (MFI), which most accurately accounted for the absolute differences in surface staining among samples from many individuals. The other two patients did not demonstrate the diminished surface expression of TGFβRII. This 3:1 ratio of patients with low TGFβRII surface expression on $CD4^+$ PBMC is consistent with our previous findings and, as before, did not strictly correspond with number of Sezary cells or $CD4^+$ cells. Finally, two SzS-related, malignant T cell lines, SZ-4 and HUT102B expressed low surface concentrations of TGFβRII which were comparable to the concentrations seen in the majority of SzS patients.

Bidirectional, automated nucleotide sequence analysis of the TGFβRII poly-A tract

To determine if poly-A tract mutations similar to the ones found in colorectal carcinoma[12] are responsible for the low cell-surface expression of TGFβRII in SzS cells, we performed a direct, automated sequence analysis of the relevant cDNA fragment from 5 SzS patients, 2 cell lines, and 2 healthy control individuals. In the first set of experiments, the SzS cells displayed a heterogenous population of the normal length, 10-A, and shortened, 9-A cDNA stretches (see Fig. 2A and B for representative data and Table 2A for the data summary). The presence of this mixture was interpreted by the DNA sequencer software as either the replacement of the last adenine (position 718) by guanine (a base normally present in position 719) or as an unidentified base (N), which represented a mixture of A and G or G and C signals present in position 718 and 719, respectively. However, several observations indicated that this finding did not represent a true deletional mutation, but was due to a procedural artefact. First, the cDNA sequence from the position 720 onward remained unchanged, this suggesting the presence of only a minor 9-A cDNA subset which interfered with the proper identification of a low pick of 718A present in most of the transcripts, arguing against the relevance of such a minor population. The presence of this mixture was interpreted by the DNA sequencer software as either the replacement of the last adenine (position 718) by guanine (an apparent "duplication" of the base which followed the poly-A tract: 719G; Fig. 2A) or as

Table 2. Direct, automated sequence analysis of the poly-A tract (sense strand positions: 709–718) and the corresponding antisense strand poly-T tract in the TGFßRII cDNA derived from SzS and control cells

A. Sense strand/position		706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	
Reference sequence	5’	A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	G-	C-	C-	3’
Patient No.: 1		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**G-**	G-	C-	C-	
2 – exp. 1		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**G-**	G-	C-	C-	
– exp. 2		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**N-**	C-	C-	
3		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**G-**	G-	C-	C-	
4 – exp. 1		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**G-**	G-	C-	C-	
– exp. 2		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**N-**	C-	C-	
Cell lines: SZ-4		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**N-**	C-	C-	
HUT102B		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**N-**	G-	C-	C-	
Healthy person No.: 1		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**N-**	C-	C-	
2		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**N-**	C-	C-	
B. Anti-sense strand Reference sequence	5’	G-	G-	C-	T-	T-	T-	T-	T-	T-	T-	T-	T-	T-	C-	C-	T-	3’
Patient No.: 1		G-	G-	C-	T-	T-	T-	T-	T-	T-	T-	T-	T-	**C-**	C-	C-	T-	
2		G-	G-	C-	T-	T-	T-	T-	T-	T-	T-	T-	T-	**C-**	C-	C-	T-	
3		G-	G-	C-	T-	T-	T-	T-	T-	T-	T-	T-	T-	**C-**	C-	C-	T-	
4		G-	G-	C-	T-	T-	T-	T-	T-	T-	T-	T-	T-	**N-**	C-	C-	T-	
5		G-	G-	C-	T-	T-	T-	T-	T-	T-	T-	T-	T-	**C-**	C-	C-	T-	
Cell lines: SZ-4		G-	G-	C-	T-	T-	T-	T-	T-	T-	T-	T-	T-	**N-**	C-	C-	T-	
HUT102B		G-	G-	C-	T-	T-	T-	T-	T-	T-	T-	T-	T-	**C-**	C-	C-	T-	
Healthy person No.: 1		G-	G-	C-	T-	T-	T-	T-	T-	T-	T-	T-	T-	**C-**	C-	C-	T-	
2		G-	G-	C-	T-	T-	T-	T-	T-	T-	T-	T-	T-	**N-**	C-	C-	T-	

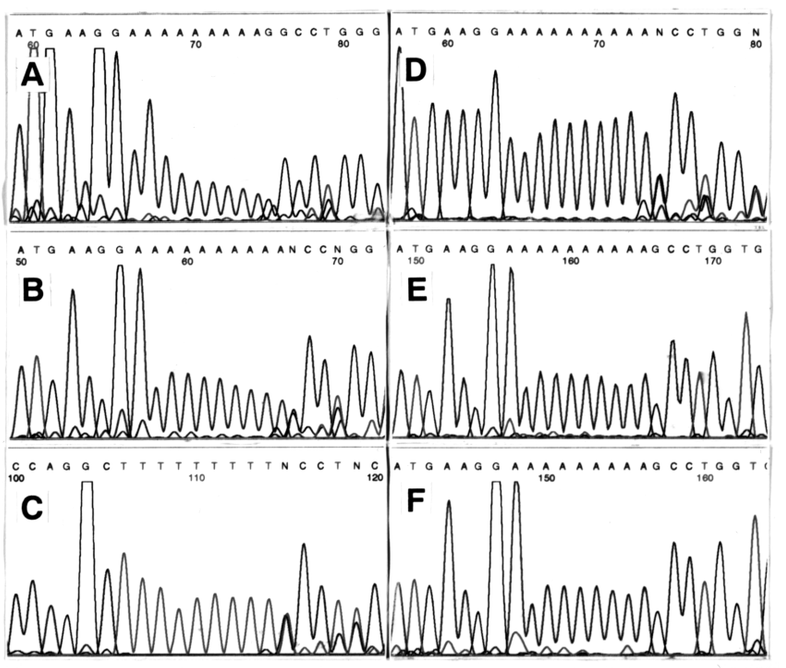

Fig. 2. Automated sequence analysis of the TGFβRII cDNA containing poly-A tract (positions 709–718). Direct sequencing of RT--PCR-generated cDNA from a SzS patient no. 1 (**A**). Direct sequencing of RT-PCR--generated cDNA from healthy person no. 1 (**B**). Direct sequencing of the poly-T tract in the corresponding, anti-sense cDNA strand from healthy person no. 1. (**C**). Sequence analysis modified by employment of the dye-labeled primers (cDNA from patient no. 3; **D**). Sequence analysis of the cloned RT-PCR product (cDNA from patient no. 2, clone 1; **E**). Sequence analysis of the cloned RT-PCR product (cDNA from healthy person no. 1, clone 5; **F**)

Table 3. Modified automated sequence analysis of poly-A tract (positions 709–718) in TGFßRII cDNA derived from SzS and control cells

Position		706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	
Reference sequence	5'	A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	G-	C-	C-	3'
A. Pfu DNA polymerase																		
Patient No.: 2		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	N-	C-	C-	
5		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	N-	C-	C-	
Healthy person No.: 1		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	N-	C-	C-	
2		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	N-	C-	C-	
B. Dye-labeled primers																		
Patient No.: 1		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**C-**	C-	C-	
2		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**N-**	C-	C-	
3		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**N-**	C-	C-	
Healthy person No.: 1		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**N-**	C-	C-	
C. Cloned cDNA																		
Patient No.: 2 -clone 1		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	G-	C-	C-	
-clone 2		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	G-	C-	C-	
-clone 3		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	G-	C-	C-	
-clone 4		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	G-	C-	C-	
-clone 5		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**0-**	C-	C-	
Healthy person No.: 1 -clone 1		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	G-	C-	C-	
-clone 2		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	G-	C-	C-	
-clone 3		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	G-	C-	C-	
-clone 4		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	G-	C-	C-	
-clone 5		A-	G-	G-	A-	A-	A-	A-	A-	A-	A-	A-	A-	A-	**0-**	C-	C-	

A – substitution of the Taq DNA polymerase with Pfu polymerase in the cDNA amplification. **B** – usage of the dye-labeled primers in the PCR-aided sequencing. **C** – cloning of the amplified cDNA

an unidefined base (N), which represented a mixture of A and G signals, present in the position 718 or 719 (Fig. 2B). Again, several observations indicated that this finding did not represent a true deletional mutation, but was due to a procedural artefact. Second, 718G could not be identified in PBMC from two normal individuals (Fig. 2B), suggesting the presence of a minor subset of 9-A cDNA also in these samples. It is rather unlikely that healthy individuals with normal TGFβRII surface expression would harbor such a mutation. Third, the signals within the poly-A tract showed a gradually decreasing strength which resulted in a very weak signal for the most distant adenins (Fig. 2A and B), which suggested that the last adenine simply might have not been identified sometimes in the sequencing reaction. Finally, there was a variability in the 10-A to 9-A ratio between the experiments, since the same RNA samples from 2 SzS patients yielded the 10-A, 718A, 719N rather than the 9-A, 718G pattern (Table 2A, patients no. 2 and 4, exp. 2) in the repeat experiments. This procedural dependence of the 10- to 9-base ratio was further stressed by the analysis of the poly-T tract in the reciprocal, anti-sense cDNA strand. All the samples tested (5 patients, 2 cell lines, and 2 healthy persons) showed a high proportion of the shortened, 9-T fragments (Fig. 2C and Table 2B) as a result of an apparent T→C substitution (with "duplication" of the C following the poly-T tract) or the presence of an unreadable base (N: a mixture of T and C).

In an attempt to correct this phenomenon, several modifications of the sequence analysis were performed. Substitution of the Taq DNA polymerase with a high-fidelity Pfu polymerase in the amplification step appeared to correct the problem to a large degree, since all 10-As could be now identified in all samples tested (2 patients and 2 healthy persons: Table 3A). However, the inability to identify the following base (N instead of G in position 719) persisted. This indicated that the proportion of the 9-A fragments was significantly reduced, but still present. Next, we substituted the 5'

TGFβRII-specific primer with a hybrid M13/TGFβRII primer in the PCR amplification followed by dye-labeled primer sequencing to obtain a more uniform peak intensity and, therefore, enhance the weak 718A signal. This also resulted in a proper reading of all 10-As and a much improved, stronger signal sustained for the entire tract (Fig. 2D, Table 3B) in both the patients and a control. However, the G-719 still could not be identified and was read as either N or "duplicated" C-720 in some samples. We cloned the PCR-amplified products prior to the sequencing reaction. Analysis of the cloned cDNA yielded an unambiguous, single sequence for all samples tested. In most samples, the normal length sequence, which contained all 10-As followed by the easily identifiable G in position 719, was detected (Fig. 2E, Table 3C). However, occasional sequences with shortened 9-A tracts and a true 718A deletion were present in both patient and normal control samples (Fig. 2F, Table 3). These findings of low frequency and presence in normal individuals further supported the procedural derivation of cDNA with the shortened 9-base poly-A tracts.

SSCP analysis of the genomic DNA

To provide further evidence that there are no mutations within the TGFβRII poly-A tract in the SzS cells, we performed SSCP analysis of genomic DNA from such cells using primers that span the DNA fragment which includes the poly-A tract. As shown in Fig. 3, none of the 13 SzS patients tested displayed altered PCR conformers. In contrast, PCR products from control colorectal carcinoma cell lines (VACO-5 and HCT116) with previously described mutations in the polyadenine tract[12] displayed altered mobility as compared with PCR products from 2 normal donors and the SzS patients. The PCR product from the HCT116 cell line (homozygous-1 A deletion) showed a mild, reproducible downward shift, while the PCR product from the VACO-5 line (heterozygous-2 A deletions/+1 A addition) displayed multiple, aberrantly migrating conformers.

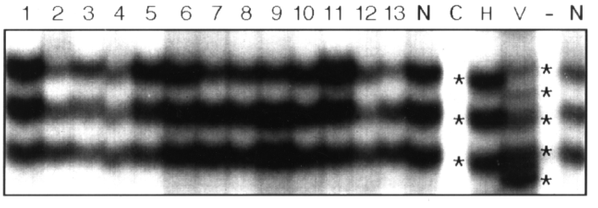

Fig. 3. SSCP analysis of the amplified genomic DNA fragment containing TGFβRII poly-A tract. DNA was derived from SzS patients (no. 1–13), normal, healthy individuals (N), and colorectal carcinoma cell lines (V: VACO-5 and H: HCT116) with previously described[19] mutations within the polyadenine tract (the asterisks denote additional and shifted bands). The control lane (C) denotes a PCR reaction with no DNA. The control lane (–) denotes a PCR reaction with no DNA polymerase added

In summary, the above findings indicate that cells from SzS patients carry no mutations within the poly-A tract of TGFβRII.

Discussion

An escape from the anti-proliferative effect of TGFβ may be important in the development of various malignant tumors[1]. Low cell-surface expression of TGFβRII by the tumor cells, which represents one of the mechanisms leading to non-responsiveness to TGFβ, was found in two types of cutaneous T cell lymphoma: $CD30^+$ anaplastic large cell lymphoma[9] and SzS[2]. In contrast to the cell surface, the intracellular expression of TGFβRII in cells from SzS patients appears comparable to the intracellular expression in cells from healthy donors[2]. The basis for the diminished cell-surface TGFβRII expression in SzS cells is unknown and so the finding that tumor cells from patients with hereditary nonpolyposis colorectal cancer display mutations within the polyadenine tract of the TFGβRII cDNA[12] prompted us to determine if mutations of this kind also occur in SzS cells. Here we report that no such mutations were found by SSCP and automated nucleotide sequence analysis, although the latter test yielded some unexpected data showing, besides the normal, 10-base poly-A tract, a subset of shortened, 9-base adenine homopolymers. Based on several observations, we conclude that this finding represented a procedural phenomenon rather than a true deletional mutation within one of the TGFβRII genomic alleles. First, the 9-A sequences comprised only a minor subset of the obtained sequences and their proportion varied from experiment to experiment even when the same cDNA was used. Second, the 10- and 9-A mixture was seen not only in the SzS patients and cell lines, but also in healthy individuals with normal expression of the TGFβRII receptor. Third, a larger proportion of the 9-base homopolymers was generated when the anti-sense (poly-T) than when the sense (poly-A) strands were sequenced, even for the same cDNA samples. Fourth, methodologic improvements, such as the use of high-fidelity DNA polymerase and dye-labeled primers, significantly reduced the proportion of the 9-A cDNA. Finally, no mutations of the poly-A tract were identified in any of the SzS samples in SSCP analysis.

The presence of shortened, 9-base fragments most probably reflects the limited efficiency of the DNA polymerse or, less likely, reverse transcriptase in transcribing *in vitro* long same-base repeats. However, diminishing signals generated in the routine automated sequence analysis for the poly-A and poly-T tracts, as well as the enhanced signals for the bases which follow these tracts (see ABI Prism sequencing manual), also appeared to be a contributing factor leading to difficulties in correctly reading the weak signal of the terminal adenine (718A). Regardless of these technology-related difficulties encountered in both patients and normal individuals, our data, particularly the SSCP and the modified automated sequence analysis, indicate that a mechanism different from mutation in the poly-A tract is responsible for the low cell-surface expression of TGFβRII in the SzS cells. These data are in agreement with other findings indicating that TGFβRII poly-A tract mutations are highly malignancy type and/or organ specific, and related to microsatellite instability[19]. Whereas, in addition to the hereditary polyposis-related colonic carcinoma[12], they have been identified in sporadic colonic[15], gastric[23] and ampullary[7] carcinoma and gliomas[8], they have not been seen in several other types of malignancy[1, 18, 19].

Mutations in the TGFβRII gene outside of the poly-A tract have also been found to result in the aberrant expression of the receptor. Accordingly, two different mutations of the TGFβRII gene were identified in cell lines derived from squamous cell carcinomas of the upper aerodigestive tract[5]. These two mutations were missense (G→A) rather than deletional and were localized in the kinase domain of the receptor (positions 1576 and 610). They had two opposite functional consequences. The first rendered TGFβRII incapable of autophosphorylation, the second induced constitutive phosphorylation of the receptor. Both, however, resulted in a greatly diminished cell-surface expression of TGFβRII; the cytoplasmic expression of TGFβRII was not analyzed in that study. Interestingly, abundant cytoplasmic expression associated with decreased cell-surface expression has been described for other cytokine/growth factor receptors, such as the erythropoietin receptor (EpoR)[13, 21, 24]. The cytoplasmic localization of EpoR apparently resulted from two types of mutations leading to either receptor inactivation[13, 24] or ligand-independent constitutive activation[21]. Still another mechanism of the loss of cell-surface expression resulting from chromosomal translocations have been identified for several cell-membrane receptors with intrinsic tyrosine-kinase activity, such as anaplastic lymphoma kinase (ALK)[4, 26]. In these cases, the receptor gene distal regions coding for intracellular and, frequently, transmembrane domains of the receptor are fused to the proximal regions of genes coding for various intracellular proteins[4]. The resulting hybrid proteins, e.g. ALK/nucleophosmin, show abundant cytoplasmic and, sometimes, nuclear localization, but virtually no cell-surface expression[4, 26]. These proteins, however, are constitutively activated, a feature that could be detrimental to malignant cells in the case of the TGFβRII, for which inactivation rather than constitutive activation represents an oncogenic event.

Resistance to TGFβ may also be due to mutations of signaling molecules which are downstream of TGFβRII[1]. Such intracellular effectors of TGFβ-signaling include members of the Smad family[1, 3], CBP/p300[14], MDM2[16], and SnoN/Ski proteins[17]. Accordingly, at least two Smads, Smad2 and Smad4, have been identified as tumor suppressors[1, 6, 10]. Whereas any aberrations in the expression or function of such intracellular signaling molecules impact on the distribution (cell-surface vs. cytoplasm) of TGFβRII remains unclear.

In summary, we have provided evidence that SzS cells, which upon mitogenic stimulation display a diminished cell-surface expression of TGFβRII when compared with their normal cell counterparts, do not carry any deletional mutations within the poly-A tract of the TGFβRII gene. This indicates that other mechanisms which change structure, signaling, and/or cellular distribution of the receptor are responsible for the aberrant TGFβRII expression in SzS cells. We have also shown that direct sequence analysis of homopolymeric base stretches is prone to artifacts, requires technical adjustments, and should be interpreted with caution.

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