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Gene expression of transforming growth factor β receptors I and II in non-small-cell lung tumors

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Abstract

Transforming growth factor (TGF)β inhibits normal epithelial cell proliferation. A decreased expression of TGFβ receptors (TβR) has been associated with loss of TGFβ sensitivity and enhanced tumor progression in many types of cancer. Although lung cancer is one of the leading causes of cancer death, a comparative analysis of TβR mRNA and protein expression in non-small-cell (NSC) lung tumors has not been performed. Lung tumor tissues and control non-lesional lung tissues were obtained from 17 patients undergoing thoracotomy for primary NSC lung tumors in clinical stage II. Each tissue sample was studied for TβRI and TβRII mRNA and immunoreactive protein expression, using a semi-quantitative reverse transcription-PCR method, and a quantitative immunohistochemistry method, respectively. TβRI protein expression was higher in tumors than in controls ($p = 0.0005$) and a similar trend was present at the mRNA level. TβRII protein expression was not significantly different between tumors and controls, however an intense peri-nuclear staining for TβRII was observed in several tumor cells. TβRII mRNA levels were lower in tumors than in controls ($p = 0.005$) and an inverse relation between TβRII mRNA and protein expression was detected in tumors ($p = 0.0013$). Our findings suggest an altered function of the TβR system in NSC lung cancer.

Keywords: Non-small-cell lung tumors; TGFβ; TGFβ receptors

1. Introduction

The transforming growth factor (TGF)β family of growth factors includes several isoforms (TGFβ 1, 2 and 3 in mammals) which are widely expressed in most tissues. TGFβ interacts with specific cellular serine/threonine kinase receptors, the TGFβ receptor type I (TβRI) and type II (TβRII) [1]. Following binding with TGFβ the TβRII forms a heteromeric complex with TβRI leading to signal transduction and activation of a variety of cellular processes. TGFβ elicits several biological activities, including inhibition of epithelial cell proliferation [2].

Lung cancer is the leading cause of cancer death in the western world. We have previously described an increased expression of TGFβ in non-small-cell (NSC) lung tumors [3]. In these tumors, TGFβ protein expression, measured by immunohistochemistry or by ELISA, has been reported to be associated with a poor prognosis [4–6]. Moreover, TGFβ plasma levels have been proposed as a useful means of monitoring lung cancer persistence and recurrence [7]. TGFβ potently inhibits the growth of non-transformed lung epithelial cells, whereas proliferation of lung tumor cell lines is frequently not inhibited by this cytokine [8–10]. Therefore, a loss of responsiveness to TGFβ could explain the fact that high levels of this cytokine seem to

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be ineffective in inhibiting the growth of lung epithelial tumors in vivo. Accordingly, the loss of growth regulation by TGFβ is considered an important step in tumor progression in several types of cancer [11–14].

A decreased expression of TβRs is considered to be one of the possible mechanisms responsible for the loss of TGFβ sensitivity and the enhanced tumor progression in many types of cancer [15–17]. However, only little data on TβR expression in NSC lung tumors has been published [5,10,18]. The presence of immunoreactivity for TβRI and/or RII has been correlated to a poor prognosis in lung adenocarcinoma [5]. On the other hand, a reduced TβRII immunoreactivity in some cases of lung adenocarcinoma [10] and in poorly differentiated adenocarcinomas and squamous cell carcinomas [18] has been described. In these studies the TβR immunoreactivity has been evaluated either qualitatively [5,10] or using a subjective scoring method [18]. The availability of instruments for computerised image analysis has rendered it possible to perform quantitative measurements of immunoreactivity. This experimental approach can reduce the discrepancies between different reports, due to the inter-observer variability. Moreover, protein expression levels determined with this method can be successfully correlated with mRNA expression levels [19,20]. We have therefore used a reverse transcription-PCR method and a quantitative immunohistochemistry method to analyse mRNA and immunoreactive protein expression of TβRI and TβRII. The aim of the study was to compare mRNA and protein levels of TβRI and TβRII in paired-NSC lung tumor and non-lesional lung tissues.

2. Results

2.1. TβRI

TβRI mRNA levels were assessed in samples from NSC lung tumors and from non-lesional lung tissues by semi-quantitative RT-PCR (Fig. 1). They were normalised against a cell cycle and induction-independent gene, the nucleoprotein 7B6 [21,22]. TβRI mRNA was detected in all tissues. Its levels were higher in tumors (mean ± SD: 24.12 ± 49.30 AU; range: 0.001–195.11 AU) than in non-lesional tissues (mean ± SD: 4.542 ± 5.193 AU; range: 0.20–18.1 AU) although, because of the large inter-individual variation, the difference did not reach significance (Wilcoxon test, $p = 0.163$) (Figs. 1 and 2A).

Immunohistochemical analysis of TβRI expression was then performed in samples from tumors and from non-lesional lung tissues, using the polyclonal antibody R-20 (Fig. 3A, C). In the alveolar wall of normal tissue samples, only a few TβRI positive cells could be found, including alveolar, endothelial and mononuclear cells (Fig. 3A), while the bronchial epithelial cells showed a uniform and moderate TβRI positivity (data not shown). Neoplastic cells were characterised by an intense staining for TβRI, while the stroma, both in normal and in neoplastic tissues, showed a very low level of immunoreactivity (Fig. 3A, C). No staining was observed both in normal and in neoplastic tissue using the anti-TβRI antibody pre-incubated with the corresponding immunogen peptide (Fig. 3E, F). We then performed a quantitative analysis of the immunoreactivity using a computerised image analysis method. The intensity of the staining was normalised against the cell number, which is a critical parameter due to the great variability of cellularity encountered in lung specimens. In this way, we were able to compare the immunoreactive protein levels in tumors and in non-lesional tissues, as well as to correlate the mRNA and the protein levels. The TβRI protein scattergram (immunoreactivity of 10 areas chosen at random, normalised against cell number) is shown in Fig. 2B. Immunoreactive TβRI protein expression was significantly higher in tumors (mean ± SD: 10.514 ± 4.237 AU; range: 4.76–20.89 AU) than in non-lesional tissues (mean ± SD: 5.004 ± 3.337 AU; range: 0.86–11.82 AU) (Wilcoxon test: $p = 0.0005$). Tumor histotypes did not differ significantly in TβRI mRNA levels or in TβRI protein expression (factorial ANOVA, respectively, $p = 0.69$, $p = 0.43$). No correlation between mRNA and protein levels was

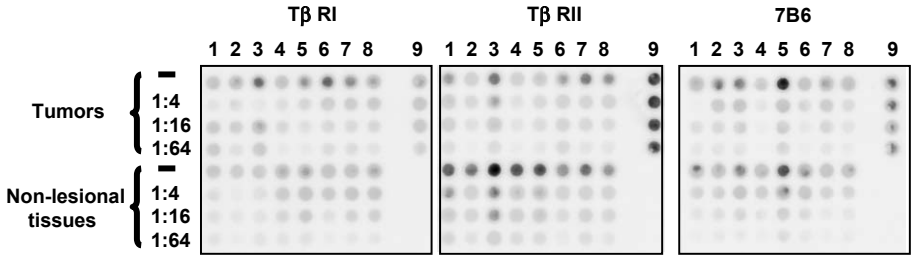

Fig. 1. TβRI and TβRII mRNA expression in paired-tumor and non-lesional lung tissues. Total RNA extracted from tumors and from non-lesional lung tissues, was reverse transcribed. The resulting cDNA was PCR-amplified at four different dilutions using primers specific for TβRI, TβRII or for 7B6. PCR products were dot blotted to a nylon membrane, hybridised with ^{32}P end-labelled oligonucleotide probes specific for TβRI, TβRII and 7B6 cDNA. Results from a representative dot blot experiment, performed using cDNA from eight patients (lanes 1–8).

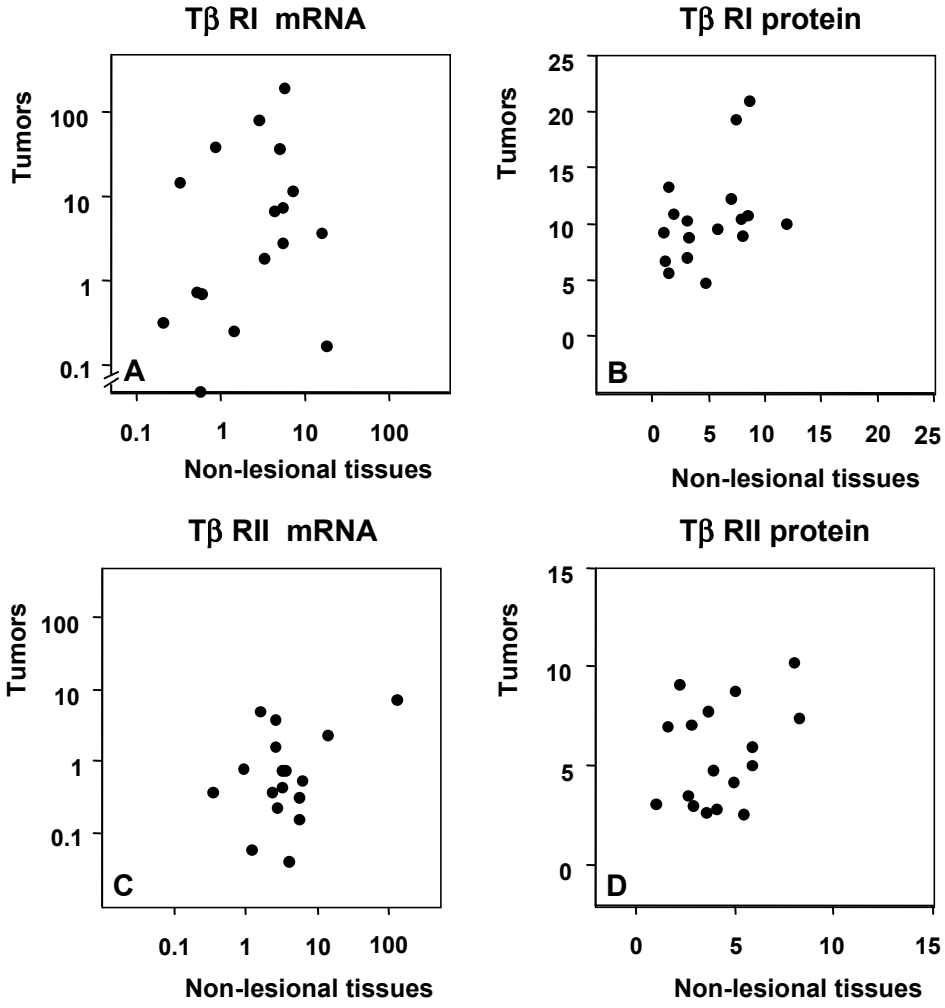

Fig. 2. Comparison of TβRI and TβRII mRNA and immunoreactive protein levels in paired-tumor and non-lesional lung tissues from patients with NSC lung cancer. In each panel, values are plotted for each paired measurement, on the vertical axis levels of the tumors and on the horizontal axis levels of the non-lesional tissues ($n = 17$). mRNA and immunoreactive protein levels are indicated in AU (different scales are used in the four panels), as defined in Section 4. TβRI mRNA expression (A): $p = 0.163$. TβRI protein expression (B): $p = 0.0005$. TβRII mRNA expression (C): $p = 0.005$. TβRII protein expression (D): $p = 0.084$.

found in non-lesional tissues (linear regression, $p = 0.59$; NS) as well as in tumors (linear regression, $p = 0.82$; NS).

2.2. TβRII

TβRII mRNA levels were measured by semi-quantitative RT-PCR (Fig. 1). They were significantly lower in tumors (mean ± SD: 1.483 ± 2.082 AU; range: 0.04–7.42 AU) than in non-lesional tissues (mean ± SD: 10.776 ± 29.515 AU; range: 0.34–124.71 AU) (Wilcoxon test, $p = 0.005$) (Figs. 1 and 2C). The immunohistochemical analysis, performed using the polyclonal antibody C-16, showed that all cell types present in non-lesional tissues were immunoreactive for TβRII (Fig. 3B). Neoplastic cells were intensely stained for TβRII, and many of them showed a marked perinuclear localisation of the staining (Fig. 3D). No staining was observed both in normal and in neoplastic tissue using the anti-TβRII antibody pre-incubated with the corresponding immunogen peptide (Fig. 3G, H). Immunoreactive TβRII protein expression was not significantly different between tumors (mean ± SD: 5.606 ± 2.528; range: 2.6–10.2 AU) and non-lesional tissues (mean ± SD: 4.199 ± 2.047; range: 0.98–8.22 AU) (Wilcoxon test, $p = 0.084$) (Fig. 2D). Tumor histotypes did not differ significantly in TβRII mRNA levels or in TβRII protein expression (factorial ANOVA, $p = 0.39$, $p = 0.73$, respectively). In tumors (Fig. 4B; linear regression, $p = 0.0013$), but not in non-lesional tissues (Fig. 4A; linear regression, $p = 0.99$), the TβRII immunoreactive protein levels were inversely correlated to the TβRII mRNA levels.

In previous studies, performed with different anti-TβRII antibodies, a reduced TβRII immunoreactivity in some cases of adenocarcinoma [10], and in poorly differentiated adenocarcinomas and squamous carcinomas [18] has been reported. Since we did not observe

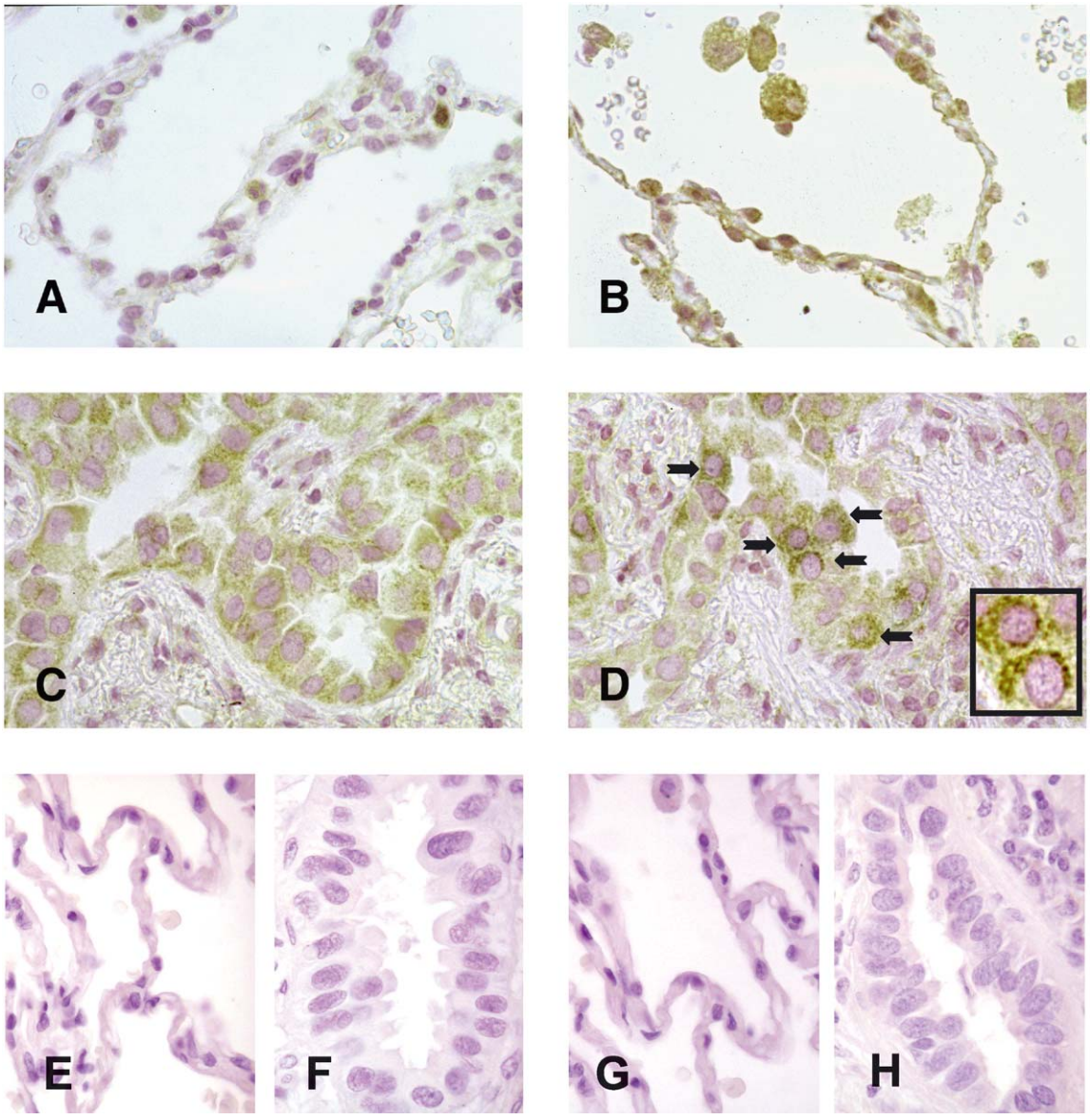

Fig. 3. Immunostaining for TβRI and TβRII in paired-tumor and non-lesional lung tissues from patients with NSC lung cancer. Non-lesional tissue (A), and adenocarcinoma (C) tested with anti-TβRI Ab. Non-lesional tissue (B), and adenocarcinoma (D) tested with anti-TβRII Ab. Non-lesional tissue (E, G), and adenocarcinoma (F, H) tested with anti-TβRI Ab (E, F) or with anti-TβRII Ab (G, H) pre-incubated with corresponding blocking peptides. Original magnification ×600. Arrows in 3D indicate tumor cells with an intense peri-nuclear staining for TGFβRII. A higher magnification (×1000) image of these cells is shown in the insert. The samples are from the same patient. The immunohistochemical analysis was performed in paired-tumor and non-lesional tissues ($n = 17$) and the protein levels are reported in Fig. 2.

a reduced TβRII immunoreactivity in tumors or a difference between adenocarcinomas and squamous carcinomas, we have studied additional six adenocarcinomas and six squamous carcinomas, by using in parallel the C-16 anti-TβRII antibody and the L-21 anti-TβRII antibody, which recognizes a different epitope of the receptor. Again, immunoreactive TβRII protein expression was not significantly different between tumors and non-lesional tissues, and between tumor histotypes. Although the staining pattern obtained with C-16 antibody was more granular than that observed with L-21, no significant differences in staining intensity between the two antibodies were observed (data not shown).

3. Discussion

TGFβ exerts a potent anti-proliferative effect on normal epithelial cells and there is evidence that loss of response to TGFβ by neoplastic epithelial cells could be an important stage in tumor progression [11–14]. Alterations of TGFβ receptors can explain this loss of

responsiveness [15–17,23]. We have studied TβRI and TβRII mRNA and immunoreactive protein expression in 17 NSC lung tumors and in their paired non-lesional tissues. Immunoreactive TβRI protein is significantly more expressed in tumors than in non-lesional tissues, and a similar trend is present at the mRNA level. Interestingly, in colon carcinoma cell lines, TβRI mRNA and protein expression is greater in cells growing exponentially than in quiescent ones [24]. In contrast, the expression of TβRII mRNA is lower in tumors than in non-lesional tissues. This is in agreement with the results of in vitro studies showing that mRNA and protein expression of TβRI and TβRII are differentially regulated [24–26].

A decreased expression of TβRII mRNA and of the corresponding protein has been reported in colon, head and neck, and gastric cancer cell lines [11,12,27]. In NSC lung tumors we find that the decrease of TβRII mRNA expression is not associated with a decrease of TβRII protein expression. Again, it is interesting to note that, due to a lower mRNA stability, proliferating colon carcinoma cells show a lower TβRII mRNA expression, compared to the quiescent ones; however, the TβRII protein expression is similar in the two growth states [24]. It is known that eight pentameric repeats AUUUA, which infer mRNA instability, are present in the 3′ UTR of the TβRII gene [28]. It is tempting to speculate that in NSC lung tumors a decreased mRNA stability could be associated with an increased protein translation and/or stability. Unfortunately, this hypothesis cannot be tested using the lung tissue samples, because this kind of measurements can only be performed on viable cells. Interestingly, in tumors but not in control tissues there is an inverse relation between TβRII mRNA and protein expression. An inverse relation between mRNA and protein expression has also been described in other systems, such as the placental D2-dopamine receptor [29] and the breast cancer cytokeratin 18 [30]. However, the mechanisms underlying these findings have not been clarified. A reduced TβRII immunoreactivity in some cases of lung adenocarcinoma [10] and in poorly differentiated adenocarcinomas and squamous cell carcinomas [18] has been reported. In these studies the tumor stage was not specified. We have not observed reduced TβRII immunoreactivity in tumors or differences in immunoreactivity between squamous cell carcinomas and adenocarcinomas and, in our study all of the patients were in clinical stage II. A significant relationship has been found between tumor stage and TβRII expression in lung adenocarcinoma [5]. Thus a difference in the tumor stage could be one of the possible reasons for these discrepancies. We have observed in many tumor cells a predominant granular peri-nuclear staining for TβRII protein. This is suggestive of a cytosolic distribution of the protein [31,32]. A cytosolic form of TβRII has been described in non-transformed [31,32] and in transformed cells [32,33]. Interestingly, MCF-7 breast carcinoma cells and mitogen-stimulated CD4+ T cells from Sezary patients, which express the cytosolic form but only little if any of the membrane form, are poorly inhibited by TGFβ1 [32,33]. In addition, in cutaneous carcinoma a predominant membranous localisation of TβRII is found in less aggressive verrucous forms, while a predominant cytoplasmic one is found in more aggressive squamous forms [34]. In MCF-7 and Sezary cells, the low TβRII surface expression is possibly caused by a defective trafficking from the cytosolic pool to the membrane [32,33]. A defective membrane localisation of TβRII could induce loss of TGFβ-mediated growth inhibition in NSC lung tumors.

This is the first study assessing TβRs mRNA and protein expression in paired-NSC lung tumor and non-lesional lung tissues in NSC. In tumors, independently from the histotype, expression of TβRI is increased, whereas expression of TβRII mRNA but not that of the protein is decreased. In addition, in tumors, we have observed a frequent peri-nuclear staining for TβRII. Our findings suggest an altered function of the TβR system in NSC lung cancer.

4. Materials and methods

4.1. Patients

The protocol of this study was approved by the local Ethics Committee. Lung tumor tissue specimens were obtained from patients undergoing thoracotomy for primary lung tumors in clinical stage II ($n = 17$: adenocarcinoma = 6; squamous cell carcinoma = 5; large cell carcinoma = 4; bronchioloalveolar carcinoma = 2). Control non-lesional lung tissue specimens of the same patients ($n=17$) were obtained from areas distal to the tumor. The volume of each tissue specimen

[1] Sequence of PCR primers and internal oligonucleotide probes:

TβRI gene. Product size 159 bp.
Forward: 5′-GGG AAA TTG CTC GAC GAT GTT C-3′
Reverse: 5′-CAC AGC TCT GCC ATC TGT TTG G-3′
Cycling: [95 °C, 2 min] ×1; [95 °C, 1 min; 60 °C, 1 min; 72 °C, 30 s] ×30; [72 °C, 5 min; 4 °C, hold] ×1.
Internal probe: 5′-CGA CGA TGT TCC ATT GGT G-3′
TβRII gene. Product size 177 bp.
Forward: 5′-TCT CGC TGT AAT GCA GTG GGA GAA G-3′
Reverse: 5′-CAA CGT CTC ACA CAC CAT CTG GAT G-3′
Cycling: [95 °C, 2 min] ×1; [95 °C, 1 min; 60 °C, 1 min; 72 °C, 30 s] ×27; [72 °C, 5 min; 4 °C, hold] ×1.
Internal probe: 5′-GAA GGA CAA CGT GTT GAG AGA TC-3′
7B6 gene. Product size 132 bp.
Forward: 5′-GTA GAC GGA ACT TCG CCT TTC TC-3′
Reverse: 5′-GGA CCT CTG CCT CAT CTT TCT TC-3′
Cycling: [95 °C, 2 min] ×1; [95 °C, 1 min; 60 °C, 1 min; 72 °C, 30 s] ×28; [72 °C, 5 min; 4 °C, hold] ×1.
Internal probe: 5′-TGA GAG CCA AGT GGA GGA AG-3′.

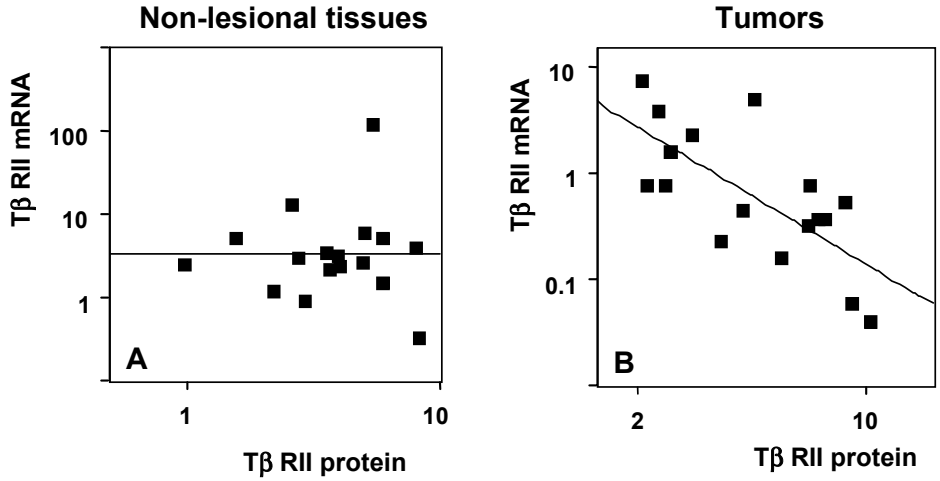

Fig. 4. Correlation between TβRII mRNA levels and immunoreactive protein levels in paired-tumor and non-lesional lung tissues from patients with NSC lung cancer. mRNA and immunoreactive protein levels are indicated in AU (logarithmic scales are used in both panels), as defined in Section 4. TβRII mRNA levels vs. TβRII immunoreactive protein levels in non-lesional lung tissues (A): linear regression, $r = 0.002$, $p = 0.99$, NS ($n = 17$). TβRII mRNA levels vs. TβRII immunoreactive protein levels in tumors (B): linear regression, $r = 0.712$, $p = 0.0013$ ($n = 17$).

was about 1 cm^3. A preliminary histochemical analysis was performed on cryosections obtained from tumor specimens. Only the specimens showing more than 70% neoplastic cells, and no necrotic areas, were selected for the study. Each tissue specimen was divided into two parts: one was snap-frozen in liquid N_2, pulverised while frozen, and kept at −80 °C until used; the other was formalin-fixed and paraffin embedded.

4.2. RT-PCR analysis

To measure levels of mRNA, a semi-quantitative reverse transcription-PCR method was used [35]. Total RNA (10 μg/sample), extracted from tumors, from non-lesional lung tissues, and from control LPS-stimulated peripheral blood mononuclear cells (PBMC) with the guanidine-isothiocyanate/CsCl gradient method, was reverse transcribed using random hexa-deoxy-nucleotides primers and AMV reverse transcriptase (Promega Corporation, Madison, WI, USA). The resulting cDNAs were PCR-amplified in duplicate at four different dilutions using *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD, USA) and primers specific for TβRs[1] or for the ribonucleoprotein 7B6.[1] Amplification reactions were kept within the sub-optimal range. The resulting products were dot blotted to a nylon membrane (Zeta-Probe, Biorad, Hercules CA, USA), hybridised with ^{32}P end-labelled oligonucleotide probes specific for each TβRs and 7B6 cDNA,[1] and analysed on a G250 phosphoimager (Biorad). Results were acquired as pixel density (PD)/mm^2. Half-maximum units (HMU) were defined in each membrane as 50% of the difference between maximum PD/mm^2 of LPS-stimulated PBMC and PD/mm^2 of plate background. For each sample, PD/mm^2 values for the four dilutions were plotted as a simple regression line. Dilution values corresponding to HMU (HMU-DV) were estimated by interpolation. The transcript 7B6, which encodes for the ribosomal protein L41 [21], is a cell cycle independent mRNA species [22]. HMU-DV for 7B6 transcript, obtained from the same sample and within the same reverse transcription, was used to correct for errors introduced by different tissue cellularity and by variable mRNA/total RNA ratios in activated cells. mRNA levels were expressed as arbitrary units (AU) calculated by dividing HMU-DV of the transcripts of interest by HMU-DV of the corresponding 7B6 transcripts.

4.3. Immunohistology

Formalin-fixed, paraffin-embedded sections were stained by the standard streptavidin biotin HRP method (Dakopatts, Glostrup, Denmark). The primary antibodies were anti-human TβRI (R-20) [36] and RII (C-16) [32] rabbit polyclonal antibodies (Santa Cruz Biotechnology, CA, USA). The primary anti-TβRs polyclonal antibodies pre-incubated with corresponding blocking peptides (Santa Cruz), were used as negative controls. In some experiments we used in parallel the C-16 and the L-21 (Santa Cruz) anti-human TβRII rabbit polyclonal antibodies. The sections, counter-stained with hematoxylin, were analysed with a Leica Q500IW imaging work-station. The image analysis system (IAS) was connected to a red–green–blue (RGB) colour video camera (JVC TK-C 1380 digital 1/2 inch CCD) and to a Leitz DMRB light microscope.

Pixel density data were converted to numerical values by transformation of RGB values into a binary image. According to the filtering values selected, numerical output expressed the intensity of detection for hematoxylin blue and diaminobenzidine brown. The imaging software was able to identify "isolated features", such as nuclei (hematoxylin blue staining), and to measure the total immunoreactive protein (diaminobenzidine brown staining) associated with cellular limits in a given field (total brown "field data"). Background staining values, obtained by averaging the results from areas of negative control slides, were subtracted automatically from every tissue. To correct for different cellularity, immunoreactivity was expressed as arbitrary units (AU), obtained dividing the total brown "field data" by the number of blue "isolated features" (nuclei). Ten areas of 0.96 mm^2, chosen at random, were processed for each sample, and values expressed as mean AU $\pm$ SD.

4.4. Statistical analysis

TβRs mRNA and protein levels in paired-tumor and non-lesional lung tissues were compared by non-parametric Wilcoxon signed rank test. Correlation between TβRs mRNA and protein levels in paired-tumor and non-lesional lung tissues was calculated by linear regression test performed on logarithmic values. Differences between tumor histotypes were analysed by factorial ANOVA. As the phenomena tested are likely to be biologically related and therefore not independent and because of the multiple testing involved, we applied a Bonferroni correction to the results, and only tests with a $p \leq 0.005$ were considered significant.

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