Thyroid papillary carcinoma: preliminary evidence for a germ-line single nucleotide polymorphism in the Fas gene

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Abstract

The expression of Fas in thyroid tumours and Graves’ disease was analysed by mRNA transcript expression. As compared with unaffected thyroid tissue, Fas expression was enhanced in Graves’ disease, adenomas, and papillary carcinomas. This pattern was also reflected in immunohistochemical studies. The PCR single-strand conformational polymorphism (SSCP) method and DNA sequencing were used to analyse Fas exons 1–9. The study was carried out on five different histotypes of thyroid tumours (n=93) and tissue from Graves’ disease patients. As compared with a group of healthy blood donors (n=64), a significant association (P=0.006) emerged between papillary thyroid carcinoma and a silent single nucleotide polymorphism (SNP, 988C→T) in exon 7 of the Fas gene. Other forms of thyroid pathology were not associated with the above polymorphism. Patients with neoplasia showed the same SNP in tumour tissue, in the unaffected contralateral thyroid lobe, and in peripheral blood cells. Thus, the 988C→T polymorphism appeared to be of germ-line origin.


Introduction

Fas (Apo-1/CD95) is a 45 kDa type I transmembrane glycoprotein in the tumour necrosis factor receptor superfamily. With its ligand (FasL, CD95 L), Fas can induce apoptosis in cells of the immune system and in a variety of fetal and adult tissues (Green et al. 2003, Tibbetts et al. 2003). The 68 amino acid cytoplasmic portion of CD95 is called the ‘death domain’ (Lenardo et al. 1999). Binding of ligands to CD95 triggers the recruitment of the cytosolic proteins FADD and the inactive caspase-8 proenzyme to form a complex with the death domain. A proteolytic cascade then leads to apoptosis (Fischer et al. 2003). The Fas gene is located on chromosome 10 (Inazawa et al. 1992) and comprises nine exons and eight introns. The transmembrane domain is coded by exons 6 and 7, while the cytoplasmic region is encoded by exons 7–9 (Cheng et al. 1995). Fas is expressed both as a cell surface and a soluble protein. Whereas surface Fas induces the apoptotic response, soluble Fas protects cells from apoptosis (Ladeda et al. 2001, Liu et al. 2002).

The expression of Fas does not necessarily predict the cell susceptibility to killing. Fas-mediated apoptosis may be blocked by several mechanisms, including the production of soluble Fas, the reduced expression of Fas at the cell surface, alterations of factors upregulating Fas expression, such as p53 (Lin et al. 2002, Tibbetts et al. 2003). Germ-line Fas mutations appear to contribute to the pathogenesis of rare autoimmune processes (Lin 2001, Dong et al. 2002; Rieux-Laucat et al. 2003). The most important germ-line Fas gene alterations have been detected in the rare autoimmune lymphoproliferative syndrome (ALPS; Rieux-Laucat et al. 2003). A few cases are consequences of homozygous null mutations that lead to complete Fas deficiency (ALPS 0). Some cases are instead due to mutations within the intracellular domain or, more rarely, in the extracellular region of Fas.

Somatic Fas mutations have been described in malignancies of the lymphoid lineage, including multiple myeloma, adult T-cell leukaemias, childhood T-cell lymphoblastic leukaemias and non-Hodgkin’s lymphomas (Beltinger et al. 1998, Gronbaek et al. 1998, Tawara et al. 2003), as well as in primary lymphomas of the thyroid, nose and skin (Takakuwa et al. 2001, 2002, van Doorn et al. 2002). In solid tumours, somatic Fas mutations have been described in bladder cancer (Lee et al. 1999a), skin squamous cell carcinoma (Lee et al. 2000), malignant melanoma (Shin et al. 1999), non-small cell lung cancer (Boldrini et al. 2002) and cervical carcinoma (Lai et al. 2003).

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Cell death pathways have attracted interest also in thyroid cancer in consideration of the possible therapeutic use of apoptosis-inducing ligands (Mitsiades et al. 2003). By immunohistochemistry, Fas is essentially not expressed in normal thyrocytes, but it is upregulated in certain histotypes of thyroid tumours (Arscott et al. 1999, Basolo et al. 2000). To investigate Fas alterations in thyroid neoplasia, we studied benign and malignant thyroid tumours in comparison with the unaffected tissue obtained from the contralateral lobe. Fas expression has been evaluated by mRNA transcript analysis and immuno- 

histochemistry. Gene mutations have been investigated by the single-strand conformational polymorphism (SSCP) analysis of Fas exons followed by DNA sequencing.

Materials and Methods

Patients and healthy blood donors

Thyroid tumours were derived from a series of patients receiving surgical treatment at the Department of Surgery of the University of Pisa. Initial carcinoma treatment was total or near-total thyroidectomy in all patients, regardless of the histotype. Histological diagnosis was made at the Department of Oncology of the same University according to Fonseca & Sobrinho-Simoes (1995). Malignant tumours from 70 patients included 27 papillary thyroid carcinoma (PTC), 11 follicular carcinoma (FC), 16 poorly differentiated thyroid carcinoma (PDC) and 17 undifferentiated carcinomas (UC). Adenomas were collected from 22 patients (micro- and macrofollicular histotypes). Normal thyroid tissue from the unaffected thyroid lobe of each patient has been used for comparison. Thyroid tissue from 20 patients with Graves’ disease was also evaluated. Blood samples were obtained in Na-EDTA sterile tubes from selected thyroid cancer patients as well as from 64 healthy adult blood donors at the Blood Bank of the University of Pisa.

Fas mRNA transcripts

Total RNA was extracted from fresh tissue samples using a kit from QIAGEN (Valencia, CA, USA). cDNA was synthesized from 2·5 µg of template RNA using a cDNA synthesis kit for RT-PCR based on avian moloney virus (AMV) RT (Boehringer-Mannheim, Monza, Italy). Published Fas transcript primers (Mitra et al. 1996) were as follows: forward 5’-CAAGTGACTGACATCAACT CC-3’ and reverse 5’-CGGTTGTTTTTCCTTCTGT GC-3’. The Fas mRNA amplicon size was 549 bp. PCR molecular biology reagents were from Perkin–Elmer Applied Biosystem (Monza, Italy). Reactions were carried out in a 50 µl final volume, including 10X reaction buffer, MgCl₂, dNTP, 20 pmol primers, and 2·5 units of Taq polymerase. Samples were denatured at 97 °C for 2 min before 28 amplification cycles (30 s at 94 °C, 60 s at 55 °C and 90 s at 72 °C), which were followed by final extension at 72 °C for 5 min. DNA products were analysed on 2% agarose gel using ethidium bromide and a u.v. transilluminator. GAPDH mRNA was amplified as a control; the relative amplicon size was 412 bp.

Fas immunohistochemistry

Tissue samples were formalin-fixed, paraffin-embedded, and stained with haematoxylin and eosin. For immunohistochemistry, paraffin sections (3–5 µm) were de-waxed in xylene, dehydrated through graded alcohols, and blocked with 5% non-immune mouse or rabbit serum in PBS with 0·05% sodium azide for 5 min. Mouse monoclonal antibody against APO1/Fas (clone 11 G10; Novocastra, Newcastle, UK) was added at 1:1000 dilution for 10 min. After incubation with biotinylated anti-mouse secondary antibody for 15 min followed by streptavidin–biotin complex for 15 min (Catalyzed Signal Amplification System; DAKO, Copenhagen, Denmark), sections were developed for 5 min with 0·01% 3–3’-diaminobenzidine tetrabydrochloride, 0·01% hydrogen peroxide in 0·05 M Tris–HCl buffer pH 7·6, counterstained with haematoxylin, dehydrated and mounted. Sections were examined independently by two pathologists (F.B. and P.F.) for Fas immunoreactivity.

Detection of Fas alterations by PCR-SSCP and DNA sequencing

Tissue samples were mechanically disrupted in liquid nitrogen, lysed and digested with proteinase-K. DNA extraction was then performed using the spin column procedure (Qiamp Tissue Kit; Qiagen). DNA was extracted from blood samples with the Qiamp DNA blood mini kit (Qiagen). The screening PCR–SSCP analysis of Fas mutations was performed with regard to exons 1–9 according to a standard procedure. DNA was used as a template in a 20 µl PCR mixture consisting of 10 mM Tris–HCl, 50 mM KCl, 1·5 mM MgCl₂ (pH 8·3), 0·2 mM dNTPs, 8 pmol of sense and antisense primers, and 1 U of AmpliTaq DNA Polymerase (Perkin–Elmer, Applied Biosystems, Foster City, CA, USA). PCR analysis of the Fas exons 1–9 was performed as previously described (Lee et al. 1999b). The amplicon size for exons 1, 2, 3, 4, 5, 6, 7, 8 and 9 (A, B, C, which indicates alternative primer pairs for the exon 9, comprising different extent of the flanking introns) was 171, 249, 192, 228, 180, 145, 164, 117, and 163, 175, 191 bp, respectively. Cycling conditions were as follows: initial denaturation (94 °C, 5 min), then 40 cycles (denaturation, 94 °C for 40 s; annealing, 49–60 °C for 40 s; synthesis, 72 °C for 40 s), followed by a final extension of 5 min. Amplification products were separated on 2% agarose gel and visualized by ethidium bromide staining. PCR products were diluted
1:1 with denaturing solution (1% xylene cyanol, 1% bromophenol blue, 0.1 mM EDTA and 99% formamide), boiled for 5 min and placed immediately in ice to prevent the annealing of single strand products. SSCP screening was carried out on the GenePhor Electrophoresis Unit using GeneGel Excel 12.5/24 (12.5% T, 2% C), according to manufacturer’s instructions (Pharmacia Biotech). Electrophoresis was performed at 18°C, 600 V, 25 mA, 15 W for 80 min. Gels were stained with PlusOne Silver Staining Kit (Pharmacia Biotech), according to manufacturer’s instructions. Altered migration patterns in two or three independent PCR-SSCP runs were considered as indicative of DNA mutations. PCR products showing mobility shifts were then purified with the QIAquick PCR Purification Kit (QIAGEN). Both strands of the amplified products were sequenced on an ABI Prism 310 automated sequencer using cycle sequencing kits (Applied Biosystems, Foster City, CA, USA). DNA sequences were compared with those of the normal CD95 gene in the GenBank database using the Basic Alignment Search Tool (BLAST) software available at the National Center for Biotechnology Information.

Statistical analysis (Statistica; StatSoft, Tulsa, OK, USA) was performed using Fisher’s exact test; differences were considered significant when the P value was less than 0.01.

Table 1 Fas transcript expression and Fas immunoreactivity in thyroid tissue from patients with Graves’ disease and patients with thyroid tumours of different histotypes. Tumour samples are compared with the unaffected tissue of the contralateral thyroid lobe.

<table>
<thead>
<tr>
<th>Histotype</th>
<th>mRNA transcripts*</th>
<th>Immunoreactivity†</th>
<th>P values‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positives/total (%)</td>
<td>No. positives/total (%)</td>
<td></td>
</tr>
<tr>
<td>Normal thyroid tissue§</td>
<td>3/45 (7%)</td>
<td>2/70 (3%)</td>
<td>—</td>
</tr>
<tr>
<td>Graves’ patients</td>
<td>5/11 (45%)</td>
<td>4/11 (36%)</td>
<td>0.005</td>
</tr>
<tr>
<td>Adenoma</td>
<td>12/22 (54%)</td>
<td>11/22 (50%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PTC</td>
<td>23/27 (83%)</td>
<td>24/27 (89%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FC</td>
<td>5/9 (55%)</td>
<td>5/9 (55%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PDC</td>
<td>2/16 (12%)</td>
<td>2/16 (12%)</td>
<td>NS</td>
</tr>
<tr>
<td>UC</td>
<td>1/17 (6%)</td>
<td>2/17 (12%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*FAS mRNA transcripts were detected by RT-PCR (RNA samples were not available for all cases).
†Immunoreactivity represents the proportion of positive cells (negative: ≤5% positive cells; positive: >5% positive cells).
‡In the case of transcript expression, statistical significance was evaluated (Fisher’s exact test) for pathological thyroid samples vs the normal thyroid tissue obtained from the contralateral lobe of thyroid tumours. NS, not significant (P>0.01).
§Normal thyroid tissue was obtained from the unaffected contralateral lobe of tumour cases.

Results

Expression of Fas transcripts in pathological thyroid tissue

As shown in Table 1, RT-PCR analysis showed that Fas was expressed in 5/11 Graves’ disease cases, 12/22 adenomas, 23/27 PTC cases, 5/9 FC cases, 2/16 PDC cases and 1/17 UC cases. When compared with the unaffected tissue obtained from contralateral thyroid lobes of tumour cases, Fas expression was significantly upregulated in the Graves’ disease group, adenomas, and papillary and follicular carcinomas. Expression of GAPDH mRNA was also evaluated. Samples expressed comparable levels of GAPDH transcripts (Fig. 1). Results of transcript analysis have been confirmed by immunostaining. The two methods gave discordant results in only five cases. The Fas antigen was localized at the cell surface and in the cytoplasm with a homogeneous distribution in the majority of neoplastic cells. Up-regulation of immunoreactive Fas in Graves’ disease has been already reported (Sera et al. 2001).

PCR-SSCP analysis of the Fas gene and DNA sequencing

In a search for genomic Fas alterations in thyroid tumours, the electrophoretic mobility of amplified DNA fragments of exons 1–9 was examined by SSCP. Among 93 thyroid tumours analysed by the PCR-SSCP method, no altered migration patterns were observed in exons 1–6 and 8 and 9. An exception was a single case (no. 7, undifferentiated thyroid carcinoma) showing an altered migration pattern of exon 3. In this case, DNA sequencing revealed a silent mutation at codon 222 (third nucleotide 568 A to G) coding for threonine (Protein Accession NP_690611).

In contrast, aberrant migration bands of exon 7 were detected in 42/93 tumour samples (Fig. 2). Exon-7 variants were confirmed by direct DNA sequencing. The only detected mutation was the previously described rs2234978 single nucleotide polymorphism (SNP), nucleotide 988 (GenBank accession number NM_000043.3). The detected change was in the third nucleotide of codon 214 that codes for threonine (Protein Accession NP_690611).
Accession NP_690611) and leads to a silent mutation ACC to ACT (988C→T). As shown in Table 2, 19/27 (70%) cases of PTC harboured the 988C→T SNP. The 988C→T SNP found in the tumour tissue was found in the unaffected tissue of the contralateral lobe and in peripheral blood cells. Thus, the detected SNP was of germ-line origin. Table 2 shows also that the 988C→T SNP was present at a frequency of 37% in a control group of healthy blood donors. As compared with the latter control group, the prevalence of the 988C→T SNP was significantly increased only in the patients diagnosed with PTC (P=0·006). The prevalence of the above polymorphism was not significantly increased among patients with Graves’ disease.

The genotypes and zygotic status of Graves’ patients, tumour patients and healthy controls relative to the 988C→T SNP are summarized in Table 3. Again, as compared with the healthy control group, the frequency of the normal C988 allele was selectively reduced in PTC patients (P=0·006).

### Discussion

SNP analysis is widely used for association studies to map genetic variation and to determine the frequency of a given SNP and verify its association with disease (Eriksson et al. 2003).

Fas gene alterations of the promoter region (A670G) have been reported in systemic lupus erythematosus (LES; Bolstad et al. 2000, Kanemitsu et al. 2002) and multiple sclerosis (van Veen et al. 2002). Novel silent polymorphisms of exon 7 (T297C and A416G) are weakly associated with LES (Horiuchi et al. 1999). In thyroid pathology, somatic Fas mutations have been detected in infiltrating lymphoid cells from Hashimoto’s thyroiditis patients, but not in thyrocytes (Dong et al. 2002). Evidence that the Fas-mediated apoptotic pathway is disrupted in a high percentage of cases of chronic lymphocytic thyroiditis and thyroid lymphoma has been recently published (Takakuwa et al. 2001). The reported somatic mutations are localized

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**Table 2** Fas exon 7 988C→T SNP in healthy blood donors, Graves’ disease patients and thyroid tumours of different histotypes. The 988C→T polymorphism was defined by direct DNA sequencing.

<table>
<thead>
<tr>
<th>Histotype</th>
<th>Affected thyroid tissue</th>
<th>Normal contralateral lobe</th>
<th>Peripheral blood</th>
<th>P values†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (healthy blood donors)</td>
<td>NA</td>
<td>NA</td>
<td>24/64 (37%)</td>
<td>—</td>
</tr>
<tr>
<td>Graves’ patients</td>
<td>10/20 (50%)</td>
<td>8/22 (36-3%)</td>
<td>5/10 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>Adenoma</td>
<td>8/22 (36-3%)</td>
<td>8/22 (36-3%)</td>
<td>5/10 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>PTC</td>
<td>19/27 (70%)</td>
<td>19/27 (70%)</td>
<td>7/11 (64%)</td>
<td>0·006</td>
</tr>
<tr>
<td>FC</td>
<td>4/11 (36-3%)</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>PDC</td>
<td>2/16 (12%)</td>
<td>2/16 (12%)</td>
<td>2/10 (20%)</td>
<td>NS</td>
</tr>
<tr>
<td>UC</td>
<td>9/17 (53%)</td>
<td>9/17 (53%)</td>
<td>3/6 (50%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Peripheral blood samples were available only for a subset of tumour patients. The same mutation found in the tumour was consistently detected both in the unaffected contralateral thyroid lobe and in peripheral blood cells.

†The significance level was evaluated (Fisher’s exact test) for each group of affected thyroid tissue vs the data for the group of normal blood donors. NS, not significant (P>0·01). NA, not applicable; ND, not done.
in the cytoplasmic region of Fas (death domain). Low-frequency somatic Fas mutations have also been detected in bladder and prostate tumours (Lee et al. 1999h, Takayama et al. 2001). More recently, somatic Fas mutations (exon 9) have been reported in a high percentage of testicular germ cell malignant tumours, none in benign lesions (Takayama et al. 2002).

Two common Fas gene polymorphisms (670 G→A in the promoter region and 154C→T in exon 7) have shown no significant association with Graves’ disease and Hashimoto thyroiditis (Stuck et al. 2003). The silent exon 7 polymorphism that has been detected in this study (988C→T) has also been reported in patients with marginal zone B cell lymphoma (Bertoni et al. 2000) and in LES patients (SNP: ss4479789). It is of interest that the frequency of the 988C→T polymorphism observed in the normal population used as a control by the latter investigation (178 control individuals from the NIH Polymorphism Discovery Resource) is quite similar to that found in our study. This indicates that there is no substantial variation of the 988C→T SNP among normal individuals of different ethnic backgrounds.

In conclusion, the present data show a significant association between the germ-line 988C→T polymorphism and the papillary forms of thyroid carcinoma. Though the detected 988C→T polymorphism within exon 7 is silent at the amino acid level, it may affect the expression of Fas itself or be linked to other genetic abnormalities playing a role in PTC pathogenesis. In this regard, it should be recalled that PTCs are characterized by a significant increase in the expression of Fas that is, however, also found in Graves’ disease, thyroid adenoma and follicular carcinoma. Unknown factors are responsible for the enhanced expression of Fas in different forms of thyroid pathology (Sera et al. 2001).

The association of a coding region silent polymorphism with a human disease is not unprecedented. The silent 102C→T polymorphism of the 5-HT2A receptor gene has been implicated in the pathogenesis of schizophrenia (Polesskaya & Sokolov 2002) and that of the angiotensin-2 receptor has been shown to enhance cardiovascular risk in hypertension (Jones et al. 2003).

Acknowledgements
This work was supported by grants from AIRC (Milan, Italy) and MIUR (Rome, Italy).

Funding
The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


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Table 3 Genotype and allele frequencies of Fas 988C→T SNP in healthy blood donors, Graves’ disease patients and thyroid tumours of different histotypes

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Control group (healthy blood donors, n=64*)</th>
<th>Graves’ patients (n=20†)</th>
<th>Adenoma (n=22‡)</th>
<th>PTC (n=27‡)</th>
<th>FC (n=11‡)</th>
<th>PDC (n=16‡)</th>
<th>UC (n=17‡)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>40 (63%)</td>
<td>10 (50%)</td>
<td>14 (63-6%)</td>
<td>8 (30%)</td>
<td>7 (63-6%)</td>
<td>14 (87%)</td>
<td>8 (47%)</td>
</tr>
<tr>
<td>CT</td>
<td>5 (6%)</td>
<td>9 (45%)</td>
<td>6 (27-2%)</td>
<td>15 (56%)</td>
<td>2 (18-2%)</td>
<td>2 (13%)</td>
<td>5 (29%)</td>
</tr>
<tr>
<td>TT</td>
<td>4 (6%)</td>
<td>1 (5%)</td>
<td>2 (9-1%)</td>
<td>4 (15%)</td>
<td>2 (18-2%)</td>
<td>0</td>
<td>4 (24%)</td>
</tr>
</tbody>
</table>

**Genotype**

<table>
<thead>
<tr>
<th>Major allele frequency</th>
<th>C (%)</th>
<th>P values§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>78.1</td>
<td>—</td>
</tr>
<tr>
<td>Graves’ patients</td>
<td>72.5</td>
<td>NS</td>
</tr>
<tr>
<td>Adenoma</td>
<td>77.2</td>
<td>NS</td>
</tr>
<tr>
<td>PTC</td>
<td>57.4</td>
<td>0.006</td>
</tr>
<tr>
<td>FC</td>
<td>72.7</td>
<td>NS</td>
</tr>
<tr>
<td>PDC</td>
<td>93.7</td>
<td>NS</td>
</tr>
<tr>
<td>UC</td>
<td>61.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Peripheral blood cells.
†Thyroid tissue from Graves’ patients.
‡Tumour tissue samples.
§Significance level (using Fisher’s exact test): major allele frequency in the thyroid pathology group vs the group of normal blood donors. NS, not significant (P>0.01).


Received 1 June 2004
Accepted 8 June 2004