**ABSTRACT**

**Objective**

Murine models (MRL/gld/gld mice) and recent evidence in humans suggest a possible role of Fas and Fas ligand (Fas-L) germline mutations in the pathogenesis of autoimmune-related lymphoproliferation, including adult cases. In this study, the presence of Fas and Fas-L germline mutations was investigated in a consecutive series of adult patients with lymphoproliferative disorders occurring in the context of Sjögren’s syndrome (SS) and type II mixed cryoglobulinemia (MC).

**Methods**

11 patients (8 primary SS and 3 type II MC; F/M: 10/1; mean age 64 yrs.) were investigated. All patients were suffering from atypical lymphoproliferative disorders or MALT lymphoproliferative lesions (mean duration 3.5 yrs.). Four patients later developed a malignant lymphoma. DNA from peripheral blood mononuclear cells from 11 patients and 10 controls was tested for germline mutations in the Fas gene (exons 4, 8 and 9) and Fas-L gene (exon 4) by the polymerase chain reaction-single strand conformation polymorphism (SSCP) method.

**Results**

All DNA samples from both patients and controls showed amplification of Fas and Fas-L specific fragments. Identical SSCP migration patterns were observed in all the cases, indicating the lack of mutations in the whole series.

**Conclusion**

Although it cannot be excluded that Fas and Fas-L mutations might be present in exons different from those analyzed, our data do not support the hypothesis that germline mutations in these genes are responsible for a major subset of lymphoproliferative syndromes in adult patients with SS and type II MC. Additional studies would be worthwhile in SLE-related lymphoproliferation, which is, however, a subset of limited clinical relevance when considering all cases with autoimmune-related lymphoproliferation.

**Key words:**

Fas, Fas ligand, Sjögren’s syndrome, cryoglobulinemia, lymphoma.

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**Introduction**

The Fas receptor (APO1/CD95) and its ligand (Fas-L) are transmembrane proteins that belong to the tumor necrosis factor (TNF) family of receptors and ligands, respectively. Upon engagement with Fas-L, cells expressing Fas rapidly undergo apoptosis by the activation of an intracellular signaling pathway dependent on a distinct cytoplasmic motif, the so-called “death domain”. The Fas/Fas-L system has been shown to play a crucial role in the regulation of apoptotic cell death, particularly in the clonal deletion of autoreactive T cells in the periphery and in the activation-induced cell death of mature T lymphocytes. Moreover, Fas/Fas-L interactions also mediate the apoptosis of B cells, monocytes, granulocytes and other non-hemopoietic cells and are involved in tissue homeostasis and in the host response to viruses and tumor cells. The in vivo physiopathological relevance of this apoptosis-inducing system has been highlighted by the observation that mice with homozygous mutations within their Fas (MRL/lpr) and Fas-L (MRL/gld) genes develop massive lymphadenopathy and splenomegaly, and show an increased occurrence of autoimmune manifestations (1). Of note, disorders associated with Fas or Fas-L mutations have also been recently detected in humans (2–4). Children with the Canale-Smith syndrome, or autoimmune lymphoproliferative syndrome (ALPS), have clinicopathological features similar to those of MLR/lpr mice, including early-onset splenomegaly and lymphadenopathy, the accumulation of CD3+ TCRα/β+ CD4- CD8- T lymphocytes, and a variable degree of autoimmune manifestations (2, 3). These patients carry heterozygous germline mutations in the Fas gene that are thought to produce dominant negative Fas mutants able to interfere with the function of normal Fas receptors. In addition, preliminary studies suggest that some families with Fas mutations have increased frequency of lymphomas (4). Furthermore, Wu et al. (5) have recently described a heterozygous mutation in the extracellular domain of Fas-L in 1 of 75 adult patients with systemic lupus erythematosus (SLE). Of the 20
SLE patients in this series who were followed prospectively for the development of lymphoproliferation, 12 exhibited lymphadenopathy during the disease. The patient with the mutant Fas-L allele developed a lymphadenopathy whose size and extent were the largest observed in the series. This patient was considered to be a case of adult ALPS (5). These findings led the authors to hypothesize that a proportion of adult patients with autoimmune diseases and lymphoproliferation may exhibit heterozygous mutations in Fas or Fas-L genes. To address this issue, we have investigated Fas and Fas-L germline mutations in a series of consecutive adult patients with autoimmune disease and lymphoproliferative disorders of long duration, which eventually did or did not evolve into malignant lymphoma.

Patients and methods

Eleven consecutive patients from northeastern Italy, attending the Rheumatic Disease Unit of the University of Udine, were studied. There were 10 females and 1 male with a mean age of 64 yrs (range 46 to 79 yrs). Eight patients had primary Sjögren’s syndrome (SS) (6) and 3 had type II mixed cryoglobulinemia (MC) (Table I). Hepatitis C virus (HCV) infection (positive antibodies and serum HCV RNA) was detected in the 3 MC patients. Antinuclear antibodies were detected in 7/8 primary SS patients, with anti-SSA and/or anti-SSB positivity in all of them. All the patients were suffering from a non-malignant lymphoproliferative disorder (atypical lymphoproliferative disorders and MALT lymphoma) of lymphoproliferative lesions; mean duration 3.5 yrs.; range 1 to 5 yrs.) diagnosed according to published criteria (6, 7). Four of these patients later developed a B-cell non-Hodgkin’s lymphoma (Table I).

To detect germline mutations, analysis was carried out on DNA obtained from peripheral blood mononuclear cells (PBMCs). Genomic DNAs of PBMCs from 10 normal healthy donors were used as control samples. The investigation for Fas and Fas-L mutations was focused on those exons of the Fas and Fas-L genes where the large majority of relevant mutations occurred. These comprised: (i) exon 9 (which includes the “death domain”) (8), exon 8 (9), and exon 4 (10) of the Fas gene; and (ii) exon 4 of the Fas-L gene (5). Exons 4 and 9 of the Fas gene and exon 4 of the Fas-L gene were amplified using previously reported pairs of specific oligonucleotide primers (2, 5, 8, 10); and exon 8 of the Fas gene was amplified with the following primers: F8 5’-TATTTTTATTT GTCTTCTCTC-3’ and R8 5’-AGGTAG AATGTATGAGAAAT-3’, at an annealing temperature of 45°C (Fig. 1).

To detect mutations in these exons, both the radioactive and non-radioactive (silver-stained) polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) methods were used. This approach increased the likelihood of detecting significant genomic variations (11). Radioactive PCR was performed in a 10 µl volume containing 50 to 100 ng of genomic DNA with 0.1 µl of [α-33P]dATP (NEN Life Science Products, Boston, MA). 200 - 500 ng aliquots of genomic DNA were used as templates for PCR amplification in 50 µl of non-radioactive reaction. Both reaction mixtures contained 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 µM of dNTPs, and 0.5 µM of each primer.

For the radioactive SSCP analysis, the radiolabeled amplified DNA was mixed (1:1) with the stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol) and denatured at 95°C for 2 min followed by quenching on ice. Four µl of each diluted sample were electrophoresed on Hydrolink-MDE gels (0.5X final concentration) with or without 10% glycerol. These gels (40 x 20 x 0.04 cm) were run

<table>
<thead>
<tr>
<th>Table I. Patients’ characteristics.*</th>
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<tr>
<td>Patient (age/sex)</td>
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<td>10 (60/F)</td>
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<td>11 (72/F)</td>
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*SS: Sjögren’s syndrome; MC: mixed cryoglobulinemia; NHL: non-Hodgkin’s lymphoma; cases with (+) or without (-) evolution to malignant lymphoma.

![Fig. 1. Structure of the Fas (A) and Fas-L (B) genes and the location of the primers used for PCR amplification. The numbered black bars refer to the corresponding Fas/Fas-L exons. DD: death domain of the Fas gene.](Image)
at room temperature, 500 - 800 V for 15 to 16 hrs. For the non-radioactive SSCP analysis, an aliquot of non-radioabeled PCR products (3 µl) was mixed with 3 µl of gel loading solution (75% deionized formamide, 12 mM NaOH, 6 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), denatured at 95°C for 2 min and kept on ice until loading. Each diluted sample was electrophoresed on 20% non-denaturing polyacrylamide TBE gels (8.0 x 10 cm x 1 mm; 39:1 acrylamide:bis-acrylamide ratio). The running buffer was 1.5X TBE. The running conditions were as follows: pre-run, 300 V for 5 min; and electrophoresis, 300 V for 3 - 6 hrs. The gels were silver-stained with the QuickSilver Detection Kit (Amersham Pharmacia Biotech, Aylesbury, Buckinghamshire, UK). 

**Results**

All of the DNA samples from both the controls and autoimmune patients showed amplification of the Fas and Fas-L specific exons. SSCP analysis was performed 3 times using DNA fragments obtained from independent PCR amplifications. When compared with the healthy donors, all samples from autoimmune patients showed identical SSCP migration patterns for all specific fragments. Thus, no evidence of Fas and Fas-L germline mutations was found in the exons investigated.

**Discussion**

In murine models (1), it has been demonstrated that the development of masspive lymphadenopathy and lupus-like autoimmune disease is linked to three independent autosomal recessive mutations of the Fas receptor or its ligand. Furthermore, it has been recently proved that ALPS patients (2, 3) and adult SLE patients (5) may carry heterozygous germline mutations affecting either Fas or Fas-L, with a likely dominant negative effect. Based on these studies, we investigated the presence of Fas or Fas-L germline mutations in consecutive adult patients with autoimmune diseases and lymphoproliferative disorders (as assessed by strict pathologic criteria) which either did or did not eventually evolve into malignant lymphoma. These patients had either SS or type II MC. Although it cannot be excluded that Fas or Fas-L mutations might have been present in exons different from those analyzed, our results do not support the hypothesis that germline mutations within these two genes are responsible for a major subset of autoimmune lymphoproliferative syndromes of adult patients. This may be particularly true in the case of SS, the most highly represented autoimmune disease in our series. Recent evidence from a series of 70 primary SS patients (where each of the 9 exons of the Fas gene and the 4 exons of the Fas-L gene were analyzed) suggests that mutations in these genes are not a cause of primary SS, although the presence of SS-related lymphoproliferation was not assessed (12). It is worth noting that autoimmune-prone MRL/lpr mice show features very similar to those of SS in humans. In fact, in addition to progressive lymphadenopathy, these mice spontaneously develop an autoimmune sialadenitis closely recalling the myoepithelial sialadenitis observed in patients with SS (13). While increased apoptosis has been shown on the salivary ductal epithelium of SS patients, where these cells were Fas-positive and Bcl-2-negative, an apoptotic genes (Bcl-2) may be overexpressed in the infiltrating lymphocytes (14). These findings, together with the absence of mutations in the Fas/Fas-L genes in our SS patients, suggest that lymphoproliferation in SS is more likely to be linked to the presence of anti-apoptotic signals. Although there are significant differences between SS and type II MC, these two disorders share many clinical and immunological characteristics. Both show a similar mechanism of evolution from polyclonal B-lymphocyte activation to oligo/monoclonal B-cell expansion (6). This may culminate in the development of an overt B-cell lymphoma, but the oncogenic events implicated are still unknown (15). In line with these findings, our results suggest that germline mutations of the Fas receptor and its ligand are probably not involved in the pathogenesis of type II MC, whereas an overexpression of Bcl-2 has been detected in type II MC-related bone marrow and liver infiltrates (16). In view of these results, mutational analyses of other target genes of the Fas/Fas-L system (proteases or protein tyrosin phosphatases) or of anti-apototic genes (Bcl-2 family) should be performed to study the pathogenesis of autoimmune lymphoproliferative syndromes. It is possible that germline mutations in the Fas/Fas-L system may be specifically involved in adult SLE-related lymphoproliferation, a point which is, however, of limited clinical relevance when considering all cases with autoimmune-related lymphoproliferation. Finally, Fas or Fas-L mutations might be somatic rather than germline and therefore confined to the NHL cells. Recent findings in autoimmune-related HHLs support such a possibility (17).

**References**

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