

Fas GENE MUTATIONS IN THE CANALE-SMITH SYNDROME, AN INHERITED LYMPHOPROLIFERATIVE DISORDER ASSOCIATED WITH AUTOIMMUNITY

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ABSTRACT

Background The Canale-Smith syndrome is a childhood disorder characterized by lymphadenopathy and autoimmunity. The similarity between this syndrome and that in mice with the lymphoproliferation (*lpr*) phenotype or the generalized-lymphoproliferative-disease (*gld*) phenotype led us to investigate whether it too is caused by mutations of the *Fas* gene (*lpr* mice) or the Fas ligand (*gld* mice), which regulate apoptosis in lymphocytes.

Methods We studied four patients with the syndrome and their families. T-lymphocyte phenotypes were analyzed, and the susceptibility of activated T cells to Fas-mediated apoptosis in vitro was determined. Mutations of *Fas* were sought by nucleotide-sequence analysis.

Results Patients with the Canale-Smith syndrome had increased numbers of circulating double-negative T cells (>20 percent) and profoundly impaired apoptosis of activated T cells incubated with an anti-Fas antibody. Three novel *Fas* mutations were identified, all of which were heterozygous and predicted to impair signal transduction by Fas. Autoimmune manifestations of the disease, such as hemolytic anemia and thrombocytopenia, persisted into adolescence. Two patients followed into adulthood had intermittent lymphadenopathy, which diminished over time. Neoplasms developed in both, and one died of hepatocellular carcinoma at the age of 43.

Conclusions Patients with the Canale-Smith syndrome have mutations in *Fas* — a fact that implicates this gene in the accumulation of lymphocytes and the autoimmunity characteristic of the syndrome. (N Engl J Med 1996;335:1643-9.)

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THE Canale-Smith syndrome, first described in 1967,¹ is an uncommon cause of lymphadenopathy in children.²⁻⁴ Patients with the syndrome present within the first two years of life with lymphadenopathy, hepatosplenomegaly, hemolytic anemia, and thrombocytopenia. Lymph-node biopsy reveals nonspecific hyperplasia with increased numbers of lymphocytes, plasma cells, and histiocytes.¹ The response to corticosteroids and immunosuppressive drugs varies, and the long-term prognosis is not known; only one patient has been followed into adolescence.

The presence of hypergammaglobulinemia and autoantibodies against erythrocytes and platelets led Canale and Smith¹ to postulate that the syndrome

had a primary immunologic basis. We studied four patients with the disorder, and all four had mutations in the “death domain” of the Fas receptor (also called APO-1 and CD95). The death domain is the cytoplasmic region of the Fas protein that transduces the intracellular signals required to initiate programmed cell death (apoptosis). Fas is particularly important in the apoptosis of activated lymphocytes⁵ and macrophages.⁶ In mice, mutations in the Fas receptor (*lpr*; lymphoproliferation phenotype) or its ligand (*gld*; generalized-lymphoproliferative-disease phenotype) are associated with massive lymphadenopathy and lupus-like autoimmunity.⁵ These animals also have large numbers of T cells with down-regulated CD4 and CD8 surface molecules (“double-negative” T cells). Normally, the Fas pathway triggers apoptosis in the cells. We found increased numbers of these unusual T cells in patients with the Canale-Smith syndrome.

CASE REPORTS

Patient 1

Patient 1 is a 43-year-old woman whose presentation and early history have been described previously.¹ At the age of 15, a left suborbital mass developed, which consisted of chronic inflammatory tissue with numerous foreign-body-type multinucleated giant cells. Two years later, thrombocytopenia resulted in severe menometrorrhagia. At the age of 21, pelvic masses were detected and a lymphangiogram revealed extensive bilateral iliac and para-aortic lymphadenopathy. Ten years later, the patient underwent laparotomy for an enlarging abdominal mass, which was found to be a lymph-node aggregate weighing 234 g and measuring 9.5 by 7 by 5 cm. Immunophenotyping of the cells revealed 87 percent CD3+ T cells (pan-T cells; normal range, 48 to 67 percent), 20 percent CD4+ T cells (normal range, 29 to 48 percent); 15 percent CD8+ T cells (normal range, 15 to 27 percent), and 67 percent HLA-DR+ cells (normal range, 6 to 25 percent). These results are consistent with an excess of activated, double-negative T cells (CD3+, CD4-, CD8-). The patient continues to have mild cervical, axillary, and intraabdominal lymphadenopathy. She also has chronic hepatitis B and hepatitis C infections and persistent hypergammaglobulinemia: IgA, 488 mg per deciliter (upper limit of normal, 382); IgM, 239 mg per deciliter (upper limit of normal, 277); and IgG, 4240 mg per deciliter (upper limit of normal, 1685). She has had multiple neoplastic lesions: a breast adenoma (at the age of 22), three thyroid adenomas (at 15, 32, and 36 years of age), and two basal-cell carcinomas (at 22 and 41 years of age). No autoimmunity or lymphadenopathy has occurred in her parents, her six siblings, or her son.

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Patient 2

Patient 2, a man who has been described previously,¹ presented at the age of 43 with hepatitis associated with hepatitis C infection. A liver biopsy revealed hepatocellular carcinoma, and he died one month later. He had been treated with corticosteroids and mercaptopurine from the ages of 4 to 12, with substantial, but incomplete, regression of lymphadenopathy. The lymphadenopathy gradually diminished during adolescence and was infrequent and mild during adulthood.

Patient 3

Patient 3 is the eight-year-old son of Patient 2. He presented at seven months with a syndrome identical to that of his father. A lymph-node biopsy revealed atypical T-cell hyperplasia with angioimmunoblastic features; the bone marrow aspirate contained no atypical cells. Ultrasonography demonstrated a mass of lymph nodes in the porta hepatis.³ Worsening thrombocytopenia required splenectomy at the age of two years. Subsequently, autoimmune hemolytic anemia and thrombocytopenia were controlled with methotrexate (2.5 mg per week). At present, the child has massive lymphadenopathy (predominantly cervical) but is otherwise well. Serum immunoglobulin levels are normal except for IgA, which is slightly elevated (267 mg per deciliter; upper limit of normal, 202).

Apart from his father, no family member has a history of lymphoproliferative or autoimmune diseases.

Patient 4

Patient 4, an eight-year-old boy, was found to have splenomegaly at 4 months and hemolytic anemia and neutropenia (absolute neutrophil count, 154 per cubic millimeter [normal, 500 to 8500]) at 10 months of age. A bone-marrow biopsy was normal. Because of generalized lymphadenopathy, a lymph-node biopsy was done. It revealed reactive follicular hyperplasia with 24 percent CD4+ T cells (control value, 38 percent), 11 percent CD8+ T cells (control value, 29 percent), 42 percent CD2+ T cells (control value, 79 percent), and 55 percent CD19+ T cells (control value, 7 percent). At three years of age, hypergammaglobulinemia, a positive direct Coombs' test, anti-smooth-muscle antibodies, and an antinuclear-antibody titer of 1:320 with a nucleolar pattern were detected.

At 3½ years of age, profound thrombocytopenia developed; the patient responded to intravenous immune globulin but required splenectomy. At surgery, massive splenomegaly and mesenteric lymphadenopathy were found. Currently, the boy has generalized lymphadenopathy but is otherwise well; serum immunoglobulin levels are normal. The family history is unremarkable except for a grandmother who had multiple sclerosis and a grandfather who had Guillain-Barré syndrome in 1994. The patient has a healthy dizygotic twin.

METHODS**Flow Cytometry**

Peripheral-blood mononuclear cells were isolated by density-gradient centrifugation and analyzed by flow cytometry^{6,7} (FACScan, Becton Dickinson, San Jose, Calif.) with the following primary antibodies: unconjugated rabbit polyclonal antihuman Fas (N18, Santa Cruz Biotechnology, Santa Cruz, Calif.); biotin-conjugated anti-CD3, anti-CD4, anti-CD8, and anti-CD19 (Caltag, South San Francisco, Calif.); biotinylated anti-CD56 (Southern Biotechnologies, Birmingham, Ala.); and phycoerythrin-conjugated anti-CD4, anti-CD8, and anti-CD25 (Becton Dickinson). The secondary reagents used were streptavidin conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch, West Grove, Pa.) or Tricolor (Caltag); and donkey antirabbit IgG conjugated to fluorescein isothiocyanate or phycoerythrin (Jackson ImmunoResearch). Double-negative T cells were detected by three-color staining with anti-CD3, anti-CD8, and anti-CD4 monoclonal antibodies.

Cell Culture and Analysis of Fas and Fas-Ligand Function

T cells were activated with the anti-CD3 monoclonal antibody OKT3 (ascites fluid, 1:1000 dilution) and interleukin-2 (20 U per milliliter) for seven to eight days before the assays. To examine the function of Fas, the activated T cells were incubated with IgG3 anti-APO-1⁸ (kindly provided by Peter Krammer, German Cancer Research Institute, Heidelberg, Germany) or control monoclonal antibody for 16 hours,⁷ and cell viability was measured by the Alamar Blue assay.⁶ The function of Fas ligand was evaluated by a chromium-release assay, with activated T cells (>95 percent CD3+) and target cells consisting of a mouse L1210 B-cell lymphoma cell line, transfected with murine Fas in either the sense or antisense orientations (kindly provided by W. Clarke, UCLA, Los Angeles).⁹ Effector cells were incubated with targets labeled with chromium-51 for six hours in the presence of anti-CD3, and the extent of specific lysis was calculated.⁶ The assay was also performed in the presence of magnesium ethyleneglycol-bis-(β -aminoethyl-ether)-*N,N,N',N'*-tetraacetic acid to block calcium-dependent, perforin-mediated cytotoxicity, as described elsewhere.¹⁰

Nucleotide Sequence of Fas and Fas-Ligand DNA and Analysis of Single-Strand Conformation Polymorphisms

Complementary DNA (cDNA) was prepared from anti-CD3-activated T cells,¹¹ and Fas and Fas-ligand (*FasL*) gene sequences were amplified with the polymerase chain reaction (PCR) by the primers^{12,13} shown in Table 1. For *Fas*, a 694-bp 5' cDNA fragment, spanning sequences that encode the extracellular and transmembrane domains (primer pair 1 and 14 in Table 1), and a 506-bp 3' cDNA fragment, encoding transmembrane and cytoplasmic domains (primers 11 and 4 in Table 1), were generated. The PCR products were cloned into pGEM-T (Promega, Madison, Wis.), and bidirectional sequencing of inserts was performed with consensus T7 and Sp6 primers. The results were assembled as directed by the Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Foster City, Calif.) and analyzed on an automated sequencer (model 377, ABI, Foster City, Calif.).

To verify mutations, genomic DNA was isolated from peripheral-blood leukocytes.¹⁴ Up to 200 ng of DNA was used as a tem-

TABLE 1. OLIGONUCLEOTIDE PRIMERS USED IN STUDIES OF THE *FAS* AND *FASL* GENES.

PRIMER	SEQUENCE	LOCATION OF cDNA*	ANNEALING
			TEMPERATURE
		bp	°C
<i>Fas</i>			
1	GGAGTTGGGAAGCTCTTC- ACTT	151-174	57
14	AGGATTTAAGGTTGGAGATT	845-826	57
11	GATCCAGATCTAACTTGGGG	700-719	57
4	CACTCTAGACCAAGCTTTGG	1206-1187	57
23	GGCCGGAACCTTTCAGAATA	Intron 7	58
17	CATGGTTTTCACTAATGGGA	Intron 8	58
49	ATGTTGACTTGAGTAAATATAT- CACC	871-896	58
47	CTTCCATGAAGTTGATGCC	1055-1036	58
<i>FasL</i>			
142F	ATGCAGCAGCCCTTCAATTAC	142-162	53
507R	GTGCATCTGGCTGGTAGACT	507-488	53
448F	CAGCTCTCCACCCTACAGAA	448-467	55
1011R	GGAAAGAATCCCAAAGTGCT	1011-992	55

*The locations of cDNA are given with respect to the relevant transcriptional start sites reported for *Fas*¹² and *FasL*.¹³ The intron 7 and 8 sequences used to design primers 23 and 17, respectively, were derived from the corresponding entries for *Fas* in the GenBank data base (accession code X81335).

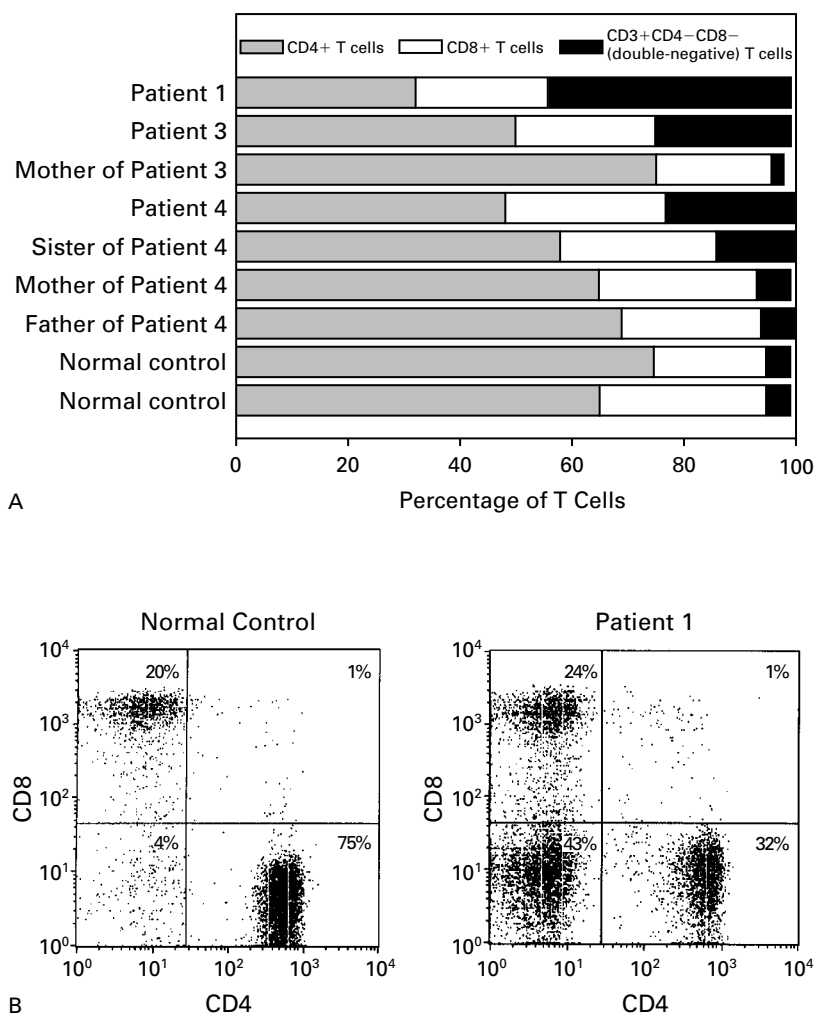


Figure 1. Analysis of Lymphocyte Subgroups in Patients with the Canale-Smith Syndrome and Their Relatives, and Control Subjects.

Panel A shows the results of a phenotypic analysis of T-cell subgroups. In Panel B, peripheral-blood mononuclear cells from Patient 1 and a normal control were analyzed by three-color flow cytometry with monoclonal antibodies specific for CD3, CD4, and CD8. CD3+ cells were gated; the percentages of double-negative cells are indicated in the lower left quadrants.

plate in a 100- μ l PCR reaction (denaturation at 95°C for one minute, annealing at 55 to 60°C for one minute, and extension at 72°C for one to two minutes) for 30 cycles. The PCR products were sequenced directly with primers end-labeled with [γ -³²P]ATP (fmol DNA cycle sequencing system, Promega). In the case of Patient 2, archival material was available from his liver-biopsy sample (kindly provided by Dr. A. Altman, Warren Hospital, Phillipsburg, N.J.), and genomic DNA was isolated as described elsewhere.¹⁵

Analysis of single-strand conformation polymorphisms was used to investigate the frequency of defined mutations in 100 unrelated subjects. Primers spanning the 5' end of the death domain (cDNA position, 871 to 1055) were used to amplify genomic DNA, as described above. The results were analyzed as reported elsewhere.¹⁶

Serologic Analysis

Serum samples were tested for antinuclear antibody by indirect immunofluorescence with Hep2 cells used as the substrate, as described previously.¹⁷ Anticardiolipin¹⁸ antibodies, antibodies

against double-stranded DNA,¹⁷ and IgM rheumatoid factors¹⁹ were detected by an enzyme-linked immunosorbent assay.

RESULTS

T-Cell Phenotypes

Phenotypic analysis of T cells from the three living patients revealed that all had higher levels of double-negative (CD3+CD4-CD8-) T cells (>20 percent) than control subjects (<5 percent) (Fig. 1A). In patient 1, almost half of all T cells in the blood had this unusual phenotype (Fig. 1B). The clinically normal twin sister of Patient 4 had intermediate levels of double-negative T cells (12 percent). Fas was detected on unstimulated T cells from all patients (data not shown).

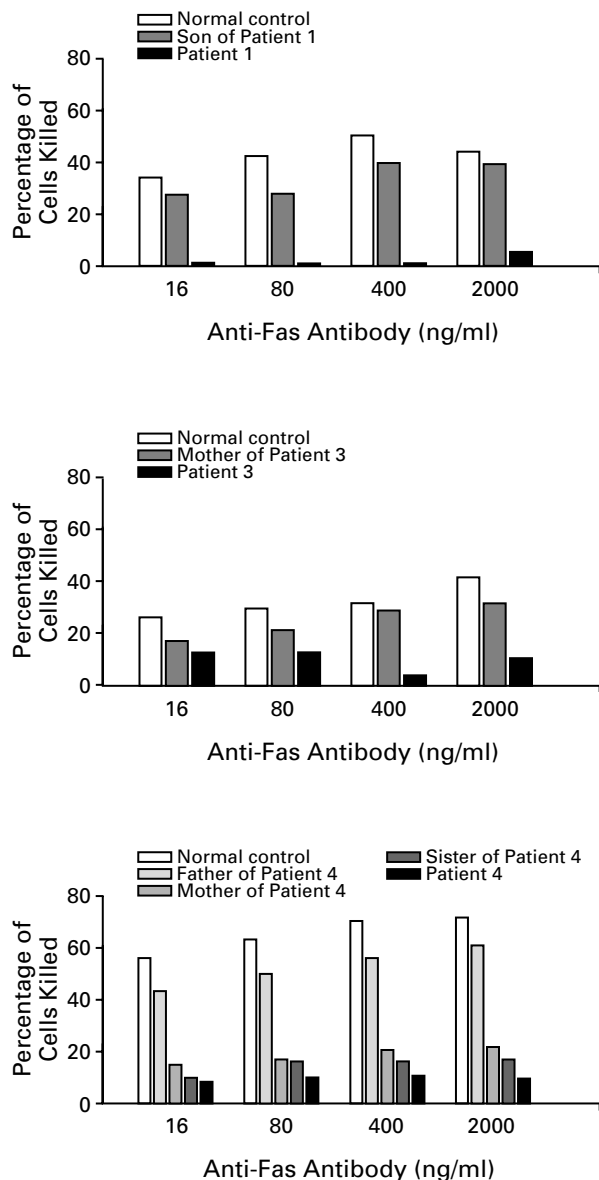


Figure 2. Fas-Mediated Apoptosis of Activated T Cells in the Patients, Their Relatives, and Normal Controls.

Peripheral-blood T cells were activated by incubation with anti-CD3 and interleukin-2 for eight days *in vitro* and then tested for Fas-mediated apoptosis.

Fas-Mediated Apoptosis

Activated T cells from the patients were almost completely resistant to apoptosis induced by ligating the Fas receptor with an anti-Fas antibody (Fig. 2). Activated T cells from the mother and twin sister of Patient 4 (both of whom were phenotypically normal) were also highly resistant to the anti-Fas antibody (Fig. 2).

To assess the function of the Fas ligand, which mediates one of the cell-mediated cytolytic pathways,

we measured the lysis of Fas-positive target cells by activated T cells. The activity of the patients' T cells in this assay was equivalent to that of T cells from normal subjects at all effector-to-target ratios (data not shown). These findings suggest that the expression and function of the Fas ligand are intact in the Canale-Smith syndrome. Consistent with this conclusion is the finding that *FasL* coding sequences, which were amplified from the patients' cDNA, cloned, and sequenced (six clones for each insert), contained no variations from the published sequence of *FasL*.¹³

Fas Mutations

Since the phenotype of the patients strongly resembled that associated with *Fas* mutations in mice,²⁰ *Fas* coding sequences were amplified from cDNA. The PCR products were cloned, and four or more clones were sequenced in both directions for each insert.

In addition to known *Fas* gene polymorphisms,²¹ a single nucleotide change, affecting the death domain (amino acid residues 231 to 298), was demonstrated for each patient in half the sequenced 3' cDNA clones. Patient 1 had an insertion of a T at cDNA position 887, which would predict a frame shift leading to a premature termination at residue 230 (K230•). Patient 3 had a transversion of G to T at position 972, resulting in a nonconservative substitution of tyrosine for aspartic acid at residue 244 (D244Y). Patient 4 had a transversion of C to T at position 942 of the *Fas* cDNA, resulting in an in-frame premature stop codon and truncation at residue 234 (R234•).

Mutations identified in cloned DNA were confirmed at the genomic level by PCR amplification of a 1.3-kb fragment with primer pair 23 and 4 and direct cycle sequencing of the death domain by primer 17. All four patients were heterozygous with respect to their *Fas* mutation (Fig. 3).

Fas Mutations and Defective Fas-Mediated Apoptosis

The mutated *Fas* allele in Patient 3 was assumed to be derived from his father, Patient 2.¹ A *Fas* mutation in Patient 2 was detected in DNA that was extracted from an antemortem liver biopsy and amplified by PCR (primers 17 and 47). Sequencing of the cloned PCR product revealed the D244Y mutation (data not shown). No *Fas* mutations were detected in genomic DNA from the son of Patient 1 or the mother of Patient 3.

Analysis of genomic DNA from relatives of Patient 4 revealed that his mother and sister, but not his father, were heterozygous for the R234• mutation (Fig. 3C). The mutations in this family therefore segregated with the *in vitro* resistance to Fas-mediated apoptosis but not with the expression of disease.

The likelihood that the *Fas* mutations we found

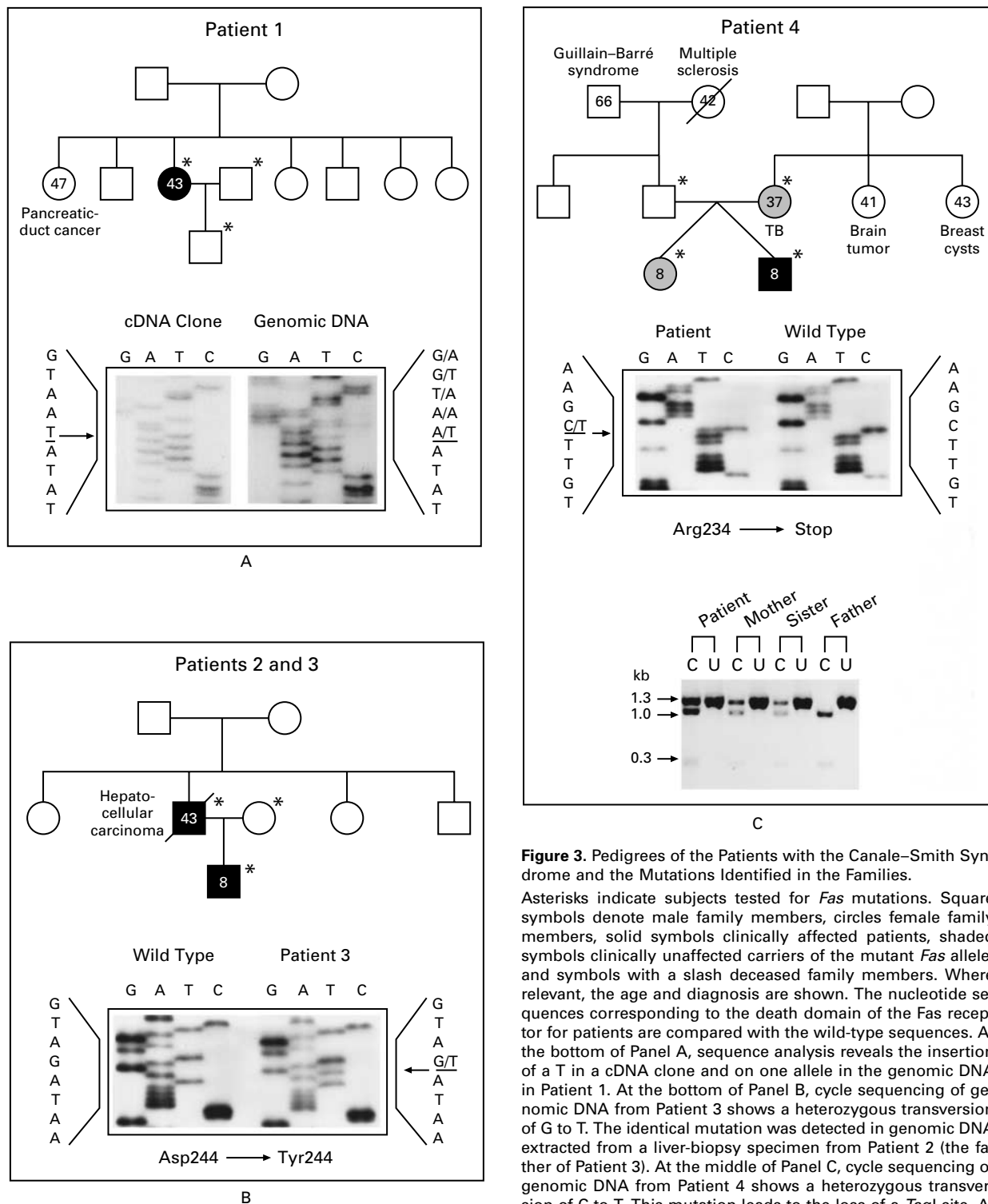


Figure 3. Pedigrees of the Patients with the Canale-Smith Syndrome and the Mutations Identified in the Families. Asterisks indicate subjects tested for *Fas* mutations. Square symbols denote male family members, circles female family members, solid symbols clinically affected patients, shaded symbols clinically unaffected carriers of the mutant *Fas* allele, and symbols with a slash deceased family members. Where relevant, the age and diagnosis are shown. The nucleotide sequences corresponding to the death domain of the Fas receptor for patients are compared with the wild-type sequences. At the bottom of Panel A, sequence analysis reveals the insertion of a T in a cDNA clone and on one allele in the genomic DNA in Patient 1. At the bottom of Panel B, cycle sequencing of genomic DNA from Patient 3 shows a heterozygous transversion of G to T. The identical mutation was detected in genomic DNA extracted from a liver-biopsy specimen from Patient 2 (the father of Patient 3). At the middle of Panel C, cycle sequencing of genomic DNA from Patient 4 shows a heterozygous transversion of C to T. This mutation leads to the loss of a *TaqI* site. At the bottom of Panel C, a 1.3-kb genomic PCR product spanning the death domain of Fas (primers 23 and 4 in Table 1) was amplified from Patient 3 and his mother, sister, and father and cut with *TaqI*. Heterozygous family members (the patient and his sister and mother) have bands at 0.1 and 0.3 kb but retain a 1.3-kb fragment, whereas the wild-type fragment (present in the father) has bands only at 1.0 and 0.3 kb. TB denotes tuberculosis, U uncut DNA, and C cut DNA.

were causally related to the Canale–Smith syndrome was supported by our failure to detect any of the mutations in 100 unrelated subjects by analysis with PCR and single-strand conformation polymorphisms (not shown).

Serologic Analysis

Serum samples from all three patients and the relatives studied in Figure 1 were negative for antinuclear, anti–double-stranded DNA, and anticardiolipin autoantibodies. Patient 1 had a high titer (positive at a dilution of 1:1000) of IgM rheumatoid factor.

DISCUSSION

We found that, like *lpr* and *gld* mice,²² patients with the Canale–Smith syndrome have increased numbers of double-negative (CD3+CD4–CD8–) T cells in the circulation and lymph nodes and profoundly impaired Fas-mediated apoptosis of activated T cells. It is thought that normally activated T cells down-regulate CD4 and CD8 molecules and are disposed of by Fas-mediated apoptosis. But in mice with a mutant *Fas* gene, they accumulate in vast numbers. Canale and Smith¹ commented that the syndrome of chronic lymphadenopathy, hepatosplenomegaly, and autoimmunity named after them did not have a genetic basis, but, prompted by the family history of Patient 3 and the striking features of the murine *lpr* phenotype present in these patients, we tested the hypothesis that a mutation of the *Fas* or *FasL* gene causes the syndrome. The four patients we studied had novel *Fas* mutations predicted to cause either truncation (K230• and R234•) or a nonconservative amino-acid substitution (D244Y) in a highly conserved region of the *Fas* death domain.^{23,24}

Fas is a member of the superfamily of tumor necrosis factor and nerve growth factor receptors. Binding to its cognate ligand causes clustering of the Fas receptor,²⁵ which recruits signal-transduction molecules to its intracytoplasmic death domain, thereby initiating programmed cell death.^{24–26} Fas is expressed on thymocytes and activated T and B cells and is thought to be primarily responsible for the apoptosis of antigen-primed, activated lymphocytes.⁵ Defective Fas function could therefore cause an accumulation of lymphocytes, including potentially autoreactive cells. These molecular abnormalities can account, at least in part, for the lymphadenopathy and autoimmunity characteristic of the Canale–Smith syndrome.

Resistance to Fas-mediated apoptosis in vitro was found in all patients who were heterozygous for a *Fas* mutation, suggesting that the mutant alleles act in a dominant negative manner. In *lpr^g* mice a heterozygous amino-acid substitution at position 225 in the *Fas* death domain results in lymphadenopathy and autoimmunity,²⁷ and in vitro cotransfection of wild-type and mutant *Fas* genes impairs Fas-mediated apoptosis.²⁸ However, the genotype–phenotype rela-

tion is complex. Three members of the family of Patient 4 had the R234• mutation, as well as defective Fas-mediated apoptosis, but only the proband had autoimmunity and lymphadenopathy. We conclude that a single mutant *Fas* allele can affect Fas-mediated apoptosis in vitro, but by itself is insufficient to cause disease; additional factors modulate Fas deficiency, as observed in mice bearing the *lpr* mutation that have different genetic backgrounds.^{29,30}

Our findings are similar to those in the original reports of Fas dysfunction in humans, which also documented inherited, heterozygous *Fas* mutations with variable penetrance.^{28,31,32} Comparison of these five cases of a “human autoimmune lymphoproliferative syndrome”²⁸ and three cases of “human lymphoproliferative syndrome and autoimmunity”³² with the Canale–Smith syndrome suggests that all three syndromes are the same.

The major medical complications in infancy and childhood are autoimmune hemolytic anemia, thrombocytopenia, and infection due to splenectomy or neutropenia. Treatment with corticosteroids, immunosuppressive drugs, or both can reduce the degree of lymphadenopathy and improve the cytopenias. However, most of our patients underwent splenectomy during childhood to alleviate the cytopenias.¹ The waxing and waning of the lymph nodes suggests that alternative pathways of lymphocyte apoptosis can compensate for impaired Fas function for extended periods. Exacerbations of lymphadenopathy could have been precipitated by viral infections, since certain DNA viruses can inhibit apoptosis.³³ Canale and Smith¹ observed that bacterial infections frequently cause a reduction in the size of the lymph nodes; bacterial infections induce the release of cytokines such as tumor necrosis factor α that promote lymphocyte apoptosis through alternative pathways.^{34,35}

Our results show that defective Fas function is compatible with long-term survival; two of our patients have been or were under medical care for 29 years. However, lymphadenopathy, autoimmune thrombocytopenia, and complications of blood transfusion (hepatitis virus infection) have continued into adolescence and adulthood. In two patients (Patient 1 and Patient 2) neoplasms developed in adulthood. These tumors could have been related to cytotoxic drugs or hepatitis virus, but a role for *Fas* mutations requires consideration. Fas is expressed at multiple sites throughout the body, including skin, liver, and gastrointestinal tract.³⁶ Its role in nonlymphoid tissue is not known, but Fas is functional in hepatocytes^{37,38} and is up-regulated in hepatitis B and C infection.^{38,39} The failure of cytotoxic T cells to eliminate hepatitis virus through the Fas pathway could therefore have contributed to the persistence of hepatitis virus in Patients 1 and 2. Furthermore, *Fas*-knockout mice, in which the *Fas* gene is disabled, have liver hy-

perplasia, suggesting a role for Fas in controlling the growth of hepatocytes.⁴⁰ Cells bearing mutant Fas receptors at other sites may also have a growth advantage resulting from the failure of CD8 or natural killer cells to perform tumor surveillance through the Fas effector pathway.⁴¹⁻⁴³ Preliminary studies suggest that some families with *Fas* mutations have an increased frequency of lymphomas.⁴⁴

Twelve cases of lymphadenopathy and autoimmunity associated with *Fas* mutations have now been reported. The characterization of factors that modulate the clinical outcome of *Fas* mutations may lead to the identification of important susceptibility genes or environmental agents that participate in other autoimmune and lymphoproliferative disorders.

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