

BRIEF REPORT

Inherited Perforin and Fas Mutations in a Patient with Autoimmune Lymphoproliferative Syndrome and Lymphoma

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SUMMARY

A 27-year-old man with the autoimmune lymphoproliferative syndrome and a large-B-cell lymphoma had heterozygous mutations in the *Fas* and perforin (*Prf1*) genes. The *Fas* mutation was inherited from his healthy father and was also carried by his healthy brother, whereas the *Prf1* mutation was inherited from his healthy mother. The combined effect of the two mutant genes may have contributed to the development of the autoimmune lymphoproliferative syndrome and lymphoma in this patient.

THE AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME (ALPS) IS A RARE disorder in children that is characterized by splenomegaly and massive lymphadenopathy with autoimmune manifestations and an accumulation of double-negative (CD3+CD4–CD8–) T cells with α/β T-cell receptors in the blood.^{1–6} In some patients, solid or hematologic cancers,^{5,6} including Hodgkin's and non-Hodgkin's lymphomas, also develop.⁷ The genetic defect responsible for this syndrome is in most cases a mutation in the *Fas* gene.^{1,2}

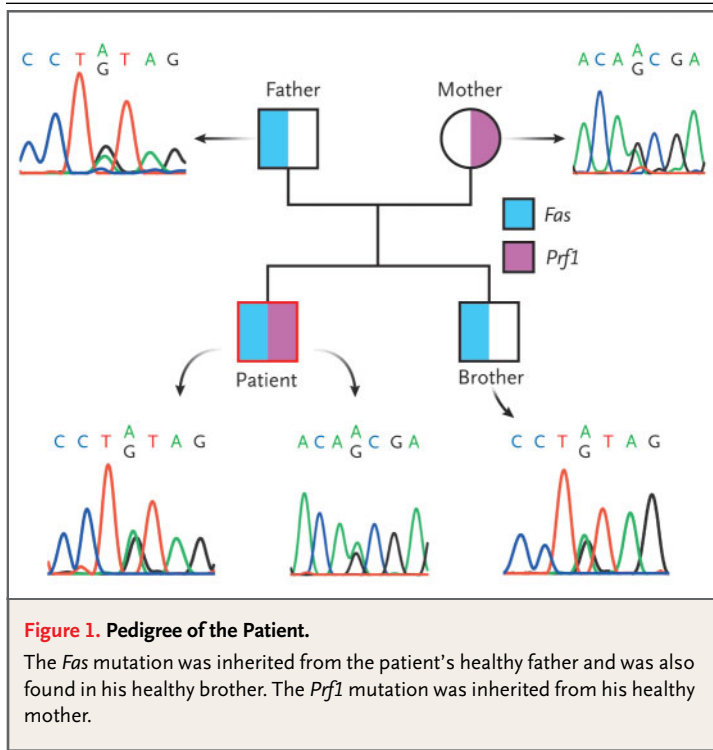
Fas (also called Apo-1 and CD95), a member of the superfamily of tumor necrosis factor and nerve growth factor receptors, induces apoptosis of lymphocytes when triggered by its ligand (FasL).⁸ The interaction between Fas and FasL causes trimerization of Fas and the formation of the death-inducing signaling complex, which initiates a cascade of caspases that culminates in the apoptotic death of the cell.⁹ The Fas–FasL system maintains lymphocyte homeostasis^{8,9}; loss-of-function mutations in the *Fas* (*TNFRSF6*) or *FasL* gene results in the accumulation of lymphocytes, as seen in mice with *lpr* and *gld* mutations¹⁰ and in patients with ALPS.^{1,6} Most patients with ALPS have mutations in *Fas* and are classified as having ALPS type Ia⁶; others have mutations in *FasL* (ALPS type Ib) or caspase 10 (ALPS type II).^{6,11,12} Autosomal recessive inheritance has been described in a minority of patients with ALPS in whom both *Fas* alleles are mutated. The form of inheritance of ALPS associated with a heterozygous *Fas* mutation is less clear, since some heterozygous carriers are asymptomatic.⁶ This variability in the

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patterns of inheritance may be due to mutations in other genes.

The mechanism of lymphomagenesis in patients with ALPS is also unclear. Defective Fas-mediated apoptosis may impair the body's ability to protect against B-cell cancers,⁷ but faulty immune surveillance may also be responsible.¹³ In this regard, the integrity of the perforin-granzyme pathway of cell-mediated killing appears to be critical.¹⁴ Perforin, a pore-forming molecule in granules of cytotoxic lymphocytes, is indispensable in the granule-exocytosis pathway of killing, and is thought to have a role in protection against viral infections and tumors that is mediated by T cells and natural killer cells.¹⁴ Indeed, an increased incidence of lymphomas has been associated with perforin deficiency in mice.¹⁵ We describe a patient with ALPS and mutations in *Fas* and the perforin gene (*Prf1*) in whom a non-Hodgkin's lymphoma developed.

CASE REPORT

A 27-year-old man presented with bulky cervical lymphadenopathy. Splenomegaly had been identified when he was three years old. Four years later, intestinal obstruction due to mesenteric lymphadenopathy and severe thrombocytopenia requir-

ing splenectomy developed. At the age of 18 years, he received a diagnosis of chronic hepatitis C infection, but viremia persisted despite treatment with interferon alfa. At the age of 25 years, he underwent a biopsy of a cervical lymph node because of adenopathy, which disclosed reactive follicular hyperplasia; a bone marrow biopsy did not reveal lymphoma. ALPS was diagnosed, and the patient underwent excision of cervical lymph nodes. Two years later, cervical lymphadenopathy recurred, with fever, weight loss, and itching; a lymph-node biopsy was diagnostic of a T-cell-rich, histiocyte-rich, diffuse large-B-cell lymphoma, according to the World Health Organization classification. Staging demonstrated multiple sites of involvement, including the liver and bone marrow. After two cycles of doxorubicin, prednisone, and vincristine and two cycles of dexamethasone, cytarabine, and cisplatin, worsening liver function precluded further chemotherapy. Shortly thereafter, the patient died of progressive lymphoma.

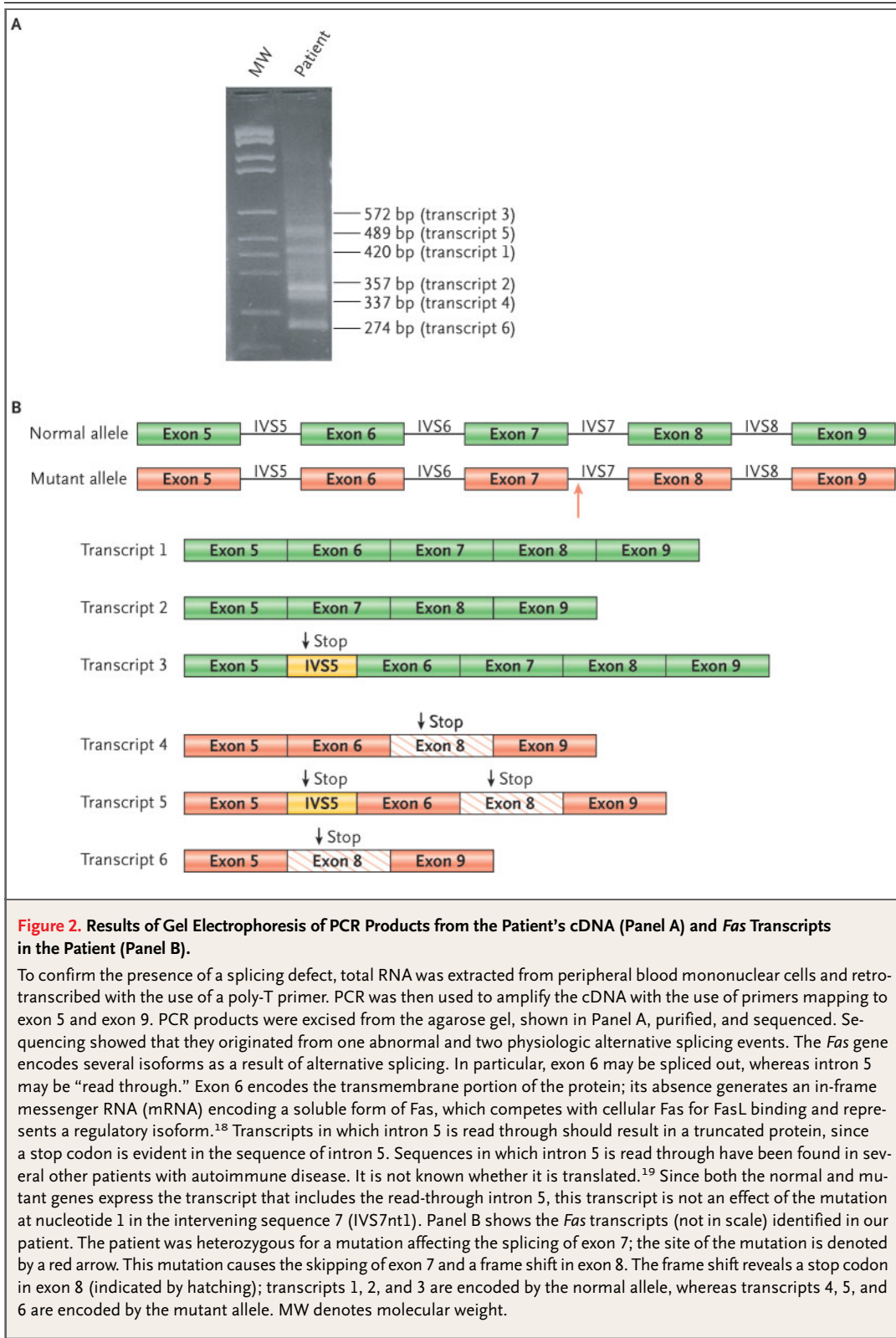
METHODS

FLOW CYTOMETRY

Lymphocyte subpopulations in peripheral-blood mononuclear cells were analyzed by means of flow cytometry. Fas-mediated apoptosis of phytohemagglutinin A-activated lymphoblasts, which were cultured for 18 days, was evaluated by means of cytofluorimetric determination of the binding of fluorescein isothiocyanate-labeled annexin V (Bender MedSystem) after treatment of the cells with the IgM anti-Fas monoclonal antibody CH-11 (MBL). The perforin content of peripheral-blood mononuclear cells was determined in fixed and permeabilized cells (Cytofix-Cytoperm, BD PharMingen) with the use of a phycoerythrin-conjugated antibody against perforin (BD PharMingen).

SEQUENCING OF THE FAS GENE

Peripheral-blood mononuclear cells were obtained from the patient and his first-degree relatives after they had provided written informed consent. Genomic DNA and total RNA were extracted according to standard methods. The *Fas* gene (exons and intron boundaries) was sequenced with the use of genomic DNA as a substrate for amplification by means of the polymerase chain reaction (PCR). As described previously,¹⁶ reverse-transcriptase (RT) PCR was performed from total RNA with the use of primers mapping within exon 5 (5'AAGGAA-



TGCACACTCACCAG3') and exon 9 (5'CAATGTGTCATACGCTTCT3') as forward and reverse primers, respectively. PCR products were subjected to agarose-gel electrophoresis, excised from the gel, and sequenced.

SEQUENCING OF THE PERFORIN GENE

Genomic DNA was isolated from peripheral-blood mononuclear cells, and exons 2 and 3 of the coding region of the *Prf1* gene were amplified with the use of standard PCR conditions. The primers used for amplification have been described previously.¹⁷ PCR products were purified with the use of Microcon PCR filter units (Millipore) and then sequenced in an automatic sequencer (ABI 3730XL, Applied Biosystems).

CYTOTOXICITY ASSAYS

The natural killer activity of peripheral-blood mononuclear cells was assessed by means of standard chromium-51–release assays with the use of the K562 cell line, which is sensitive to natural killer cells, as a target. Results are expressed as the percentage of cell-specific lysis. Anti-CD3–dependent cytotoxic activity was measured against *Fas*-deficient L1210-3 target cells (kindly provided by P. Golstein, Marseille, France), according to a previously described method.¹⁷

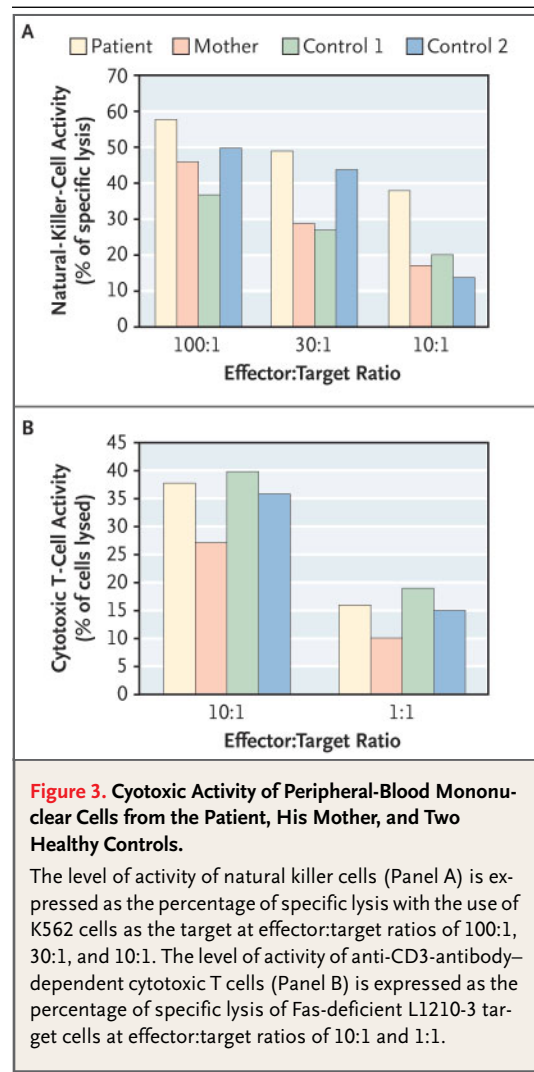
RESULTS AND DISCUSSION

Our patient with ALPS and a non-Hodgkin's lymphoma carried heterozygous mutations of *Fas* and *Prf1*, both of which are located on chromosome 10.^{1,17} The *Fas* mutation, inherited from his healthy father, was also found in a healthy brother, whereas the *Prf1* mutation was detected in his healthy mother (Fig. 1).

The diagnosis of ALPS in this patient was made on the basis of three criteria proposed by the National Institutes of Health ALPS group⁵: chronic accumulation of nonmalignant lymphoid cells, an increase in double-negative α/β + T cells in the blood (32 percent of all CD3+ T cells, as compared with less than 2 percent in normal donors), and defective in vitro *Fas*-mediated apoptosis (3 percent of cells were positive for annexin V, as compared with 33 percent in one healthy subject). The patient's *Fas* gene had a novel point mutation in intron 7 (the substitution of A for G), affecting a canonical splicing site (IVS7 at nucleotide 1) (Fig. 1 and 2). This mutation causes the skipping of exon

7 and a frame shift in exon 8. The frame shift reveals a stop codon in exon 8. Abnormally spliced products lacking exon 7 were identified by analysis of complementary DNA (cDNA) (Fig. 2). The predicted protein would lack the death domain of *Fas*, which is required for the induction of apoptosis.⁹ Our patient was therefore classified as having ALPS type Ia. The same *Fas* mutation was found in his father and brother, who had no evidence of ALPS and were healthy. However, their lymphocytes had impaired *Fas*-mediated apoptosis in vitro (data not shown), as has been reported in other mutation-positive healthy relatives of patients with ALPS.³

The mutant protein was not detected in the lymphocyte lysate by Western blotting with the use of a monoclonal antibody against the extracellular portion of *Fas* (data not shown). One explanation for



this finding is that the mutant messenger RNA underwent nonsense-mediated decay, a specific RNA-degradation process that is believed to prevent the expression of peptides that could have dominant negative effects on the cell.²⁰ The fact that we detected the mutant cDNA by means of RT-PCR does not vitiate this hypothesis, because we did not use a quantitative approach.

The particularly aggressive course of the lymphoma in our patient prompted us to look for mutations in other genes besides *Fas*. Among the candidates we investigated was the *Prf1* gene. In our patient, the *Prf1* gene had a point mutation in exon 3 (755A→G), causing a change from asparagine to serine at position 252 (N252S). This mutation occurs within the membrane-attack complex, a region critically involved in the pore-forming activity of the molecule.²¹ The same mutation was found in our patient's healthy mother (Fig. 1). Biallelic *Prf1* mutations have been found in about 40 percent of cases of familial hemophagocytic lymphohistiocytosis,^{17,22-24} a rare, life-threatening immune deficiency affecting infants and young adults. A patient with this disorder and the same heterozygous *Prf1* mutation as that in our patient has been described.¹⁷ This mutation was not found in 25 Italian patients with familial hemophagocytic lymphohistiocytosis or in 50 healthy Italian control subjects (data not shown).

Peripheral-blood mononuclear cells from the above-mentioned patient with familial hemophagocytic lymphohistiocytosis and a heterozygous *Prf1* mutation¹⁷ had reduced cytotoxic activity and grossly deficient perforin levels, suggesting a second, as yet undetermined, mutation affecting perforin expression.¹⁷ By contrast, peripheral-blood mononuclear cells from our patient and his mother had normal intracellular levels of perforin (data not shown), and the levels of activity of natural killer cells and cytotoxic T cells were similar to those in healthy controls (Fig. 3). These results were not surprising, given the reported lack of a dominant

negative effect of this particular *Prf1* mutation¹⁷; moreover, the wide range of in vitro cytotoxic activity of normal natural killer cells and cytotoxic T cells makes a partial deficiency — as would be expected in persons who were heterozygous for a *Prf1* mutation — difficult to identify.

The development of non-Hodgkin's lymphoma in our patient supports the reported increase in the risk of lymphoma among patients with germ-line *Fas* mutations and ALPS.⁷ Moreover, clonal somatic *Fas* mutations have been found in B-cell lymphomas that were not associated with ALPS.²⁵ Our patient, and all other patients with ALPS in whom a lymphoma developed, carried a mutation that altered the intracytoplasmic region of the death domain of *Fas*. These changes usually cause the greatest defect in *Fas*-mediated apoptosis, have the highest penetrance, and are associated with the most severe symptoms.⁷

In conclusion, we propose that ALPS and lymphoma developed in our patient owing to a combined effect of mutations in *Fas* and *Prf1*. A perforin mutation may be one of the additional defects contributing to the development of lymphoma in patients with ALPS, possibly in conjunction with other, unknown factors. Defective *Fas*-mediated apoptosis could allow the prolonged survival of lymphocytes, which might then become targets of transforming events, whereas defects in *Fas*-mediated killing and in the granule-exocytosis pathway would impair immune surveillance for transformed cells. This proposal is consistent with evidence that immune surveillance prevents the development of lymphomas from clonal B cells in aging mice with *lpr* and *gld* mutations.¹³

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