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A Potential Screening Tool for IPEX Syndrome

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ABSTRACT

IPEX syndrome is a rare, inherited condition characterized by immune dysfunction, polyendocrinopathy, enteropathy, and X-linked recessive inheritance. Patients typically present in infancy with severe diarrhea and failure to thrive. Most children die by 1 year of age without therapy. The diagnosis is established by genetic analysis, which often takes several weeks to complete and can sometimes delay crucial immunosuppressive treatment. We attempted to develop a screening tool that allows rapid identification of patients with IPEX syndrome using immunocytochemical staining of FOXP3+ cells in bowel biopsies. We found that 2 patients with classic IPEX syndrome due to protein-truncating mutations in FOXP3 had markedly decreased staining of FOXP3+ T cells in the lamina propria and lymphoid aggregates. One patient with a mild, late-onset presentation and a missense mutation in FOXP3 had intact staining of FOXP3+ cells. This screening test provides a valuable tool for diagnosing IPEX syndrome in extremely ill patients who may not tolerate a delay in therapeutic intervention.

Key words: IPEX syndrome, FOXP3, regulatory T cells, immune dysfunction, polyendocrinopathy, enteropathy

INTRODUCTION

IPEX is a clinical syndrome characterized by immune dysfunction, polyendocrinopathy, enteropathy, and X-linked recessive inheritance. This syndrome was first described in 1982 in a large family with 19 affected males across 5 generations as an X-linked syndrome with diarrhea that was lethal in most male infants by the age of 3 years [1]. Many of the affected males presented with the classic triad of enteropathy, endocrine abnormalities, and dermatitis plus other autoimmune features, such as hemolytic anemia [1]. Since then, other families have been identified with males presenting in the first few months of life with severe diarrhea, diabetes mellitus, eczema, thyroiditis, and severe infections. This severe syndrome of systemic autoimmunity is caused by defects in the forkhead box protein FOXP3. The gene for FOXP3 is located in the centromeric region of the X chromosome [2,3] and is critical for the development of CD4+CD25+ regulatory T cells, which play a significant role in maintaining peripheral T-cell tolerance.

The animal model of IPEX is the scurfy mouse, which is a naturally occurring mutant strain, discovered in 1959. It has many phenotypic

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similarities to IPEX, such as enteropathy, dermatitis, X-linked inheritance, and death, typically within a month of birth [4]. The gene causing the scurfy phenotype was identified as the mouse *FOXP3* gene [5], which led to the discovery of human *FOXP3*.

The highly conserved *FOXP3* gene is composed of 11 translated exons that encode a protein of 431 amino acids in humans and 429 amino acids in mice. The human and mouse *FOXP3* genes have 86% homology. The gene is expressed principally in the CD4+CD25+ regulatory T-cell subset [5].

In humans, several different mutations in *FOXP3* have been identified. Most of the mutations reported to date are missense mutations in the C terminal forkhead DNA-binding domain of the protein [6–9]. Other mutations affect the leucine zipper and N-terminal proline-rich domain [10]. Two mutations have been found causing a milder, late-onset form of the disease. These mutations affect either the intron-exon splice junction or the polyadenylation signal of the gene [5,6]. In addition, there are multiple patients with the IPEX phenotype who have no identifiable mutations, although 10% of patients without mutations were found to have decreased mRNA expression levels [10], suggesting that defects in the regulation of *FOXP3* could lead to a similar phenotype.

The laboratory findings in patients with IPEX are typically nonspecific and inconsistent. If present, they are generally related to the autoimmune abnormalities. Immunologically, IPEX patients appear remarkably normal. They typically have normal white blood cell counts, T-cell subsets, B-cell subsets, complement, and neutrophil function but may have eosinophilia [2,10,11]. Immunoglobulins IgG, IgA, and IgM are typically normal, but IgE is often markedly elevated. Antibody responses to vaccines are typically normal. As the disease progresses, these patients develop multiple autoantibodies to various organs, including the small bowel mucosa, thyroid, and islet cells of the pancreas.

The end organ damage in IPEX is due to unregulated autoreactive T-cell responses that are thought to arise due to the absence of regulatory T cells. IPEX patients are typically treated with immunosuppressive drugs, such as steroids, cyclosporine, FK506, and rapamycin, drugs that work

primarily to suppress activated autoreactive T cells [12–14]. They are currently the initial therapy of choice to control symptoms and allow patients to undergo bone marrow transplantation with as little autoimmune disease as possible [11,15]. The most successful transplants are performed prior to the development of damage to the affected organs [12,15,16], although even in optimal circumstances bone marrow transplantation has been disappointing in IPEX.

Early diagnosis is critical to prevent permanent end organ damage; however, diagnosis typically requires mutation analysis in the absence of a characteristic family history. This study defined a simple screening test, easily performed on paraffin-embedded biopsy specimens.

MATERIALS AND METHODS

Immunocytochemical staining of *FOXP3* in bowel mucosa

This study was approved by the Internal Review Board. The pathology database at The Children's Hospital of Philadelphia was searched for any pathologic material from patients with known IPEX syndrome. Hematoxylin and eosin-stained slides of gastrointestinal biopsies or autopsy material from the selected patients were examined and histological data collected. Gastrointestinal biopsies were also examined to identify histologically normal tissue for comparison. Additional pathology database searches were performed to identify patients with chronic diarrheal, allergic, and other autoimmune disorders to serve as diseased controls.

Immunohistochemical analyses were performed on formalin-fixed paraffin-embedded tissue cut at 4 to 5 microns. Antigen retrieval was performed by heating slides in a pressure cooker in EDTA (Zymed, San Francisco, CA, USA) buffer-pH 8. Primary antibody, goat polyclonal *FOXP3* (Abcam, Cambridge, MA, USA), was applied at 1:400 dilution for 30 minutes at room temperature. Secondary antibody was biotinylated rabbit anti-goat antibody (Vector Labs, Burlingame, CA, USA). Standardized avidin-biotin complex technique was used for detection. The presence of nuclear staining was considered positive. Cells with nuclear staining were quantified by direct inspection.

Table 1 Clinical features of 3 IPEX syndrome patients

	Patient 1	Patient 2	Patient 3
Age of presentation	Birth	Infancy	Infancy
Current age	Deceased at 79 days	Alive at 4 years	Alive at 8 years
Endocrine symptoms	Hypo/hyperglycemia	None	Hypoglycemic seizures
Other autoimmune phenomena	None	None	Immune, thrombocytopenia, anemia of chronic disease, osteopenia
Dermatitis	Lacy rash at 1 week of age	Mild eczema	Eczema during 1st year of life
IgE (0–200 IU/ml)	5320 IU/ml	134 IU/ml	6 IU/ml
FoxP3 IHC stain	Rare to no FOXP3+ cells	No FOXP3+ cells	FOXP3+ cells present
Mutation	Splice mutation at 5' end of exon 7 of splice acceptor site: IVS7-1G>A	Deletion and frameshift mutation with premature stop codon del1061C	Missense mutation Q70H
FOXP3 phenotype	Abnormal mRNA splicing	Frameshift with loss of the C-terminal portion of the forkhead domain	

Genomic DNA isolation, polymerase chain reaction (PCR) amplification of FOXP3, and sequence analysis

Genomic DNA was isolated from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Each exon, including all intron-exon junctions, was amplified from genomic DNA by PCR with specific flanking intron primer pairs as previously reported [17]. PCR was performed as follows: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. PCR products were separated by 1% agarose gel electrophoresis, purified with the QIAquick PCR Purification Kit (Qiagen), and directly sequenced using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Boston, MA, USA) on an automated ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). A 348-bp DNA fragment encompassing the 1st polyadenylation signal 880-bp downstream of the termination codon was also amplified and sequenced as described above, using specific primer pairs previously described [7].

RESULTS

A search of the pathology database at The Children's Hospital of Philadelphia identified 3 patients with a diagnosis of IPEX syndrome who had undergone endoscopy and bowel biopsy and

for whom there was paraffin-embedded tissue available for staining. A brief summary of their clinical features is provided in Table 1.

Case descriptions

Patient 1

This male infant was born at 30 weeks' gestation due to chorioamnionitis and premature rupture of membranes. Immediately at birth, he developed a copious green fluid discharge from his rectum and nasogastric tube, which subsequently evolved into a secretory diarrhea. At 2 weeks of age, he developed ascites. An exploratory laparotomy revealed an inflamed appendix, and the patient underwent an appendectomy. After the laparotomy, he developed a stricture and never tolerated oral feeds. He remained intubated throughout his hospital course and died at 79 days of age from sepsis and multiorgan system failure.

Immunologic evaluation revealed an elevated IgE of 5320 IU/ml (normal 0–15 IU/ml) and normal IgG of 979 mg/dl (normal 218–636 mg/dl). Serum antienterocyte antibody analysis was positive in the patient and negative in his mother. Based on this data, IPEX was suspected, but treatment was delayed due to the uncertainty of the diagnosis. He was ultimately started on FK506 shortly before he died.

Examination of his appendix revealed an increased heterogeneous population of lymphocytes in the lamina propria and submucosa with

obliteration of mucosal crypt architecture and complete loss of FOXP3 staining (Fig. 1). The autopsy revealed dramatic gross findings in the gastrointestinal tract, including diffuse mucosal flattening in the stomach and small bowel. The large intestine was cord-like and firm, with near occlusion of the lumen proximally and marked luminal narrowing of the distal half. Histologically, there was diffuse mucosal destruction throughout the stomach, small intestine, and colon, with complete loss of columnar epithelium. The mucosa was diffusely replaced by organizing granulation tissue with capillary and fibroblastic proliferation. An increase in chronic inflammatory cells, composed predominantly of CD3+ lymphocytes and plasma cells, was noted throughout the destroyed mucosa, with the inflammatory infiltrate focally extending into the superficial submucosa. The muscularis propria was not involved and contained a normal complement of ganglion cells. The mucosal and submucosal T cells were almost entirely negative for FOXP3 expression. In addition, the pancreas was remarkable for nearly complete destruction of exocrine tissue and replacement by loose fibrosis with scattered CD3+ lymphocytes. Lymphoid organs, such as the thymus, lymph nodes, and spleen, revealed depletion of lymphoid cells.

Postmortem, this patient was identified as having a splice site mutation at the 5' end of exon 7 of the *FOXP3* gene, which is predicted to result in a nonfunctional splice acceptor site, leading to abnormal mRNA splicing and loss of functional protein.

Patient 2

This 4-year-old male with chronic diarrhea that began in infancy was first brought for medical attention at the age of 2 years when he presented with bloody diarrhea. He has been managed with nasogastric feeds and infliximab and most recently with an ileostomy, mercaptopurine, and corticosteroids. He had no endocrine symptoms; autoimmune studies, which have included such antithyroid antibodies as antithyroglobulin and antithyroid peroxidase and such diabetes autoantibodies as insulin, GAD65, and CA512, continue to be negative. His immunoglobulin levels remain normal, except for his IgE, which has increased with age to 134 IU/ml. He had an excellent response to his tetanus and diphtheria vaccines, as

well as a booster to the pneumococcal vaccine. His T-cell subsets are normal.

Pathologic examination of the small bowel revealed partial villous atrophy and significant infiltration of lymphocytes in the lamina propria of the small intestine and colon with near absent staining for FOXP3 (Fig. 2). Genetic analysis revealed a frameshift mutation in FOXP3 that leads to loss of more than half of the forkhead domain. The deletion of a single nucleotide (1061C) causes a frameshift after amino acid 354, resulting in the addition of 34 amino acids prior to a premature stop codon, resulting in loss of the forkhead domain.

Patient 3

This 8-year-old male began having chronic diarrhea and failure to thrive at the age of 2 months. He also suffered from recurrent infections of the sinuses, ears, and lungs, as well as frequent viral infections (e.g., influenza). His respiratory illnesses have occasionally required intravenous antibiotics. He had 1 episode of immune thrombocytopenic purpura. He also has developmental motor delay and suffers from occasional seizures associated with hypoglycemia. He has not developed diabetes and does not require insulin. His height and weight hover around the 5th percentile, and he suffers from anemia of chronic disease, eczema, and osteopenia.

He had a small bowel biopsy at 3 months of age that showed inflammation and flat villi. He was started on total parenteral nutrition at 4 months of age and switched to nasogastric tube with Neocate feeds at 2 years of age. At 5 years of age he began having weight loss, abdominal pain, and diarrhea. A colonoscopy showed lymphoid hyperplasia and colitis. This patient is now receiving sirolimus and has decreased diarrhea and abdominal pain. IgG levels are persistently low, ranging from 370 to 515 mg/dl (normal 539–1597 mg/dl). IgE levels are normal. He has intermittently received intravenous immunoglobulin therapy for hypogammaglobulinemia. He has had adequate but not robust humoral responses to diphtheria and tetanus immunizations.

Biopsy specimens revealed intact staining for FOXP3 in the lymphocytes throughout his gastrointestinal mucosa (Fig. 3). Sequence analysis of *FOXP3* revealed a missense mutation in exon 1 of the gene, resulting in a substitution of histidine for

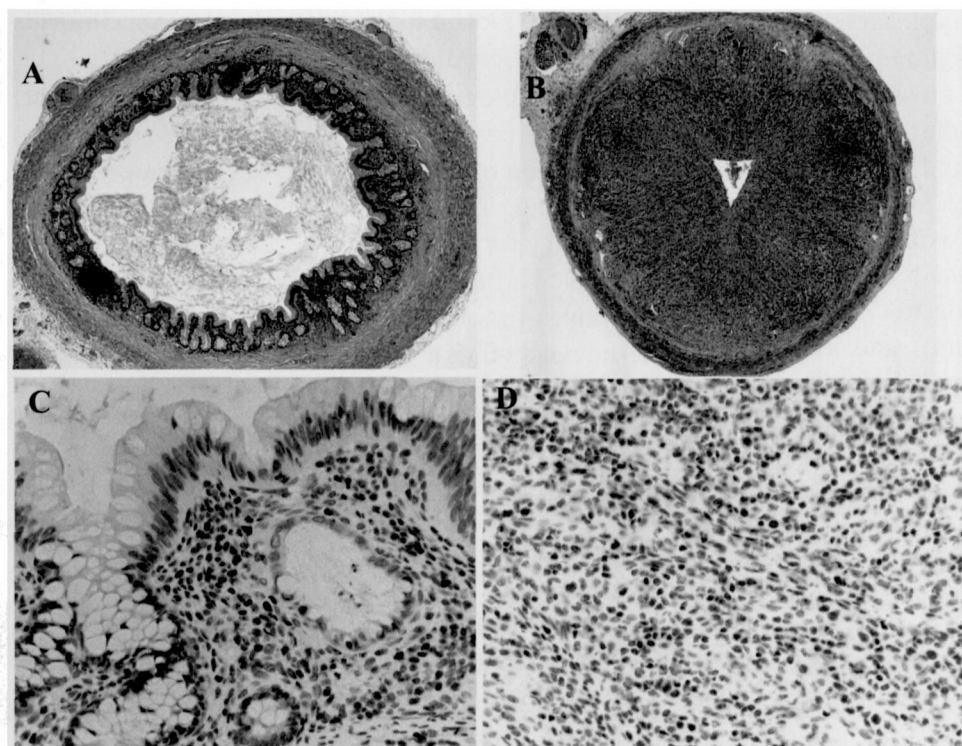


Figure 1. A. Appendix from a normal newborn with well-preserved mucosa and normal sparse lymphoid tissue (hematoxylin and eosin, original magnification $\times 50$). B. Appendix from patient 1 showing abundant lymphoid tissue with effacement of normal mucosal architecture (hematoxylin and eosin, original magnification $\times 50$). C. Normal newborn appendix showing FOXP3 nuclear staining of numerous mucosal lymphocytes. (original magnification $\times 200$) D. Appendix from patient 1 shows no staining for FOXP3 in the lymphoid cells (original magnification $\times 200$).

glutamine at amino acid 70 (Q70H) within the N-terminal proline-rich domain.

Control patients

FOXP3⁺ cells were observed both in normal patients and in patients with enteropathies of other etiologies, including inflammatory bowel disease ($N = 2$), celiac disease ($N = 4$), milk protein allergy ($N = 2$), eosinophilic gastroenteritis ($N = 2$), and

lymphangiectasia ($N = 2$), and in patients with the IPEX syndrome-like phenotype, without an identifiable *FOXP3* mutation ($N = 2$) (Table 2).

DISCUSSION

We have described 3 patients with mutations in the *FOXP3* gene and have attempted to develop a screening tool to identify those with IPEX

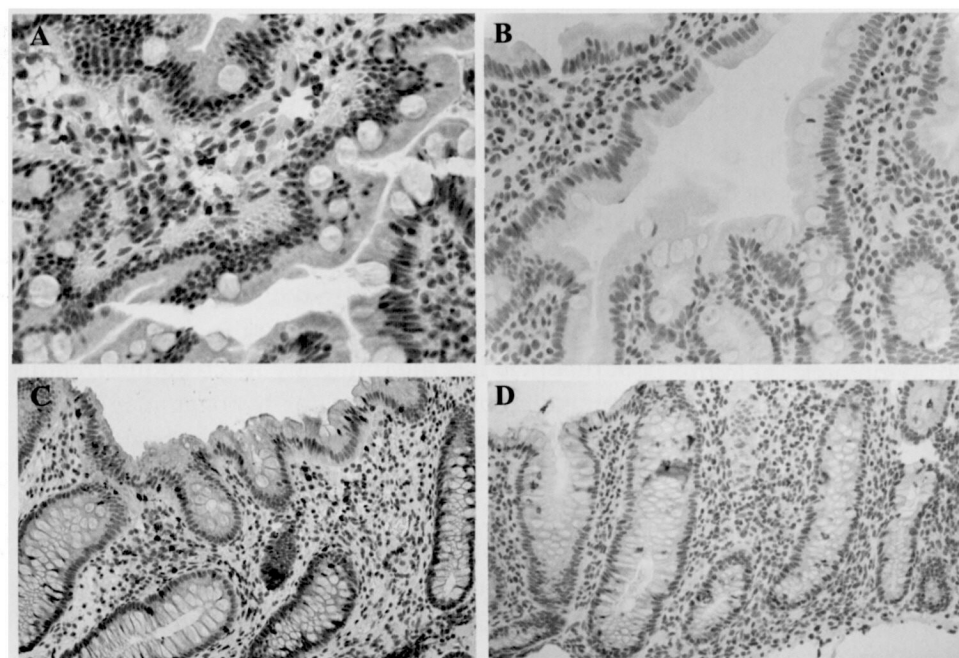


Figure 2. A. Normal small intestinal mucosa showing nuclear staining for FOXP3 in numerous lamina propria lymphocytes (original magnification $\times 400$). B. Small intestine from patient 2 showing rare nuclear staining for FOXP3 in the lamina propria lymphocytes (original magnification $\times 400$). C. Normal colonic mucosa showing nuclear staining for FOXP3 in numerous lamina propria lymphocytes (original magnification $\times 200$). D. Colonic mucosa from patient 2 showing virtually absent nuclear staining for FOXP3 in lamina propria lymphocytes (original magnification $\times 200$).

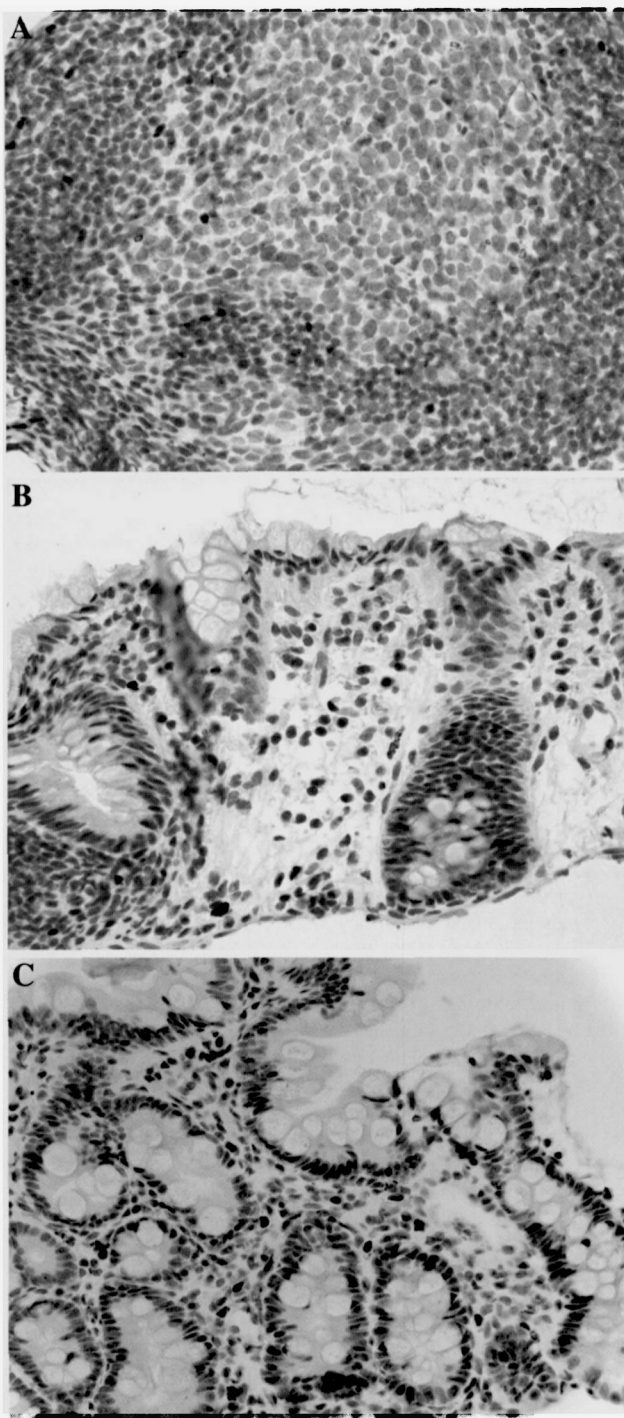


Figure 3. Patient 3. Colonic lymphoid aggregate (A), colonic mucosa (B), and small intestinal mucosa (C), all showing multiple lymphocytes with positive nuclear staining for FOXP3. This is in contrast to Figures 1 and 2, which show a near absence of FOXP3 staining in the gastrointestinal tract of the 2 patients with mutations affecting expression of all or part of the FOXP3 protein.

syndrome. Patients 1 and 2 had mutations in the *FOXP3* gene that led to abnormal mRNA splicing or truncation of the FOXP3 protein that seemed to cause more severe disease. In biopsy specimens of small and large bowel, both patients demonstrated

Table 2. Quantification of FOXP3-positive cells

	Number of cells positive for FOXP3 (per high-power field, x400)
Patient 1	0
Patient 2	0.2
Patient 3	3.6
Normal controls	Colon 10.2
IPEX-like patients (2)	6.975
Celiac disease	7.4
Inflammatory bowel disease	16.8
Milk protein allergy/eosinophilic gastroenteritis	9.35
Lymphangiectasia	3.2

an absence of lymphocytes that stained positive for FOXP3. The 3rd patient had a milder clinical course and later onset of symptoms. A missense mutation was identified in exon 1 of the *FOXP3* gene that is predicted to encode a single amino acid substitution in the FOXP3 protein. It is not yet known whether this mutation affects FOXP3 protein function. In this case, small and large intestine biopsies demonstrated FOXP3+ cells. The structure of normal FOXP3 and the predicted proteins present in these patients is shown in Figure 4.

As the clinical course of our 3 IPEX patients suggests, IPEX syndrome is a disease with a wide range of possible presentations. Patients can have a severe phenotype with chronic diarrhea, infections, and death if not treated promptly. They can also have a milder clinical course without life-threatening inflammation and nutritional compromise. Formal genotype-phenotype analysis has not yet been reported for IPEX. In this limited study, patients who had mutations causing complete absence of all or part of the FOXP3 protein had the most severe clinical course and are the most likely to be identified with this screening tool. These are the patients in whom more rapid diagnosis may allow earlier intervention and improved outcome. Patients with slower progression of disease and a milder phenotype may have FOXP3+ cells in bowel mucosa.

Although lack of staining of FOXP3+ cells in patients with severe IPEX (patients 1 and 2) appeared to be specific because FOXP3+ cells were observed both in normal patients and in patients with enteropathies of other etiologies, this immunohistochemical screening tool is not intend-

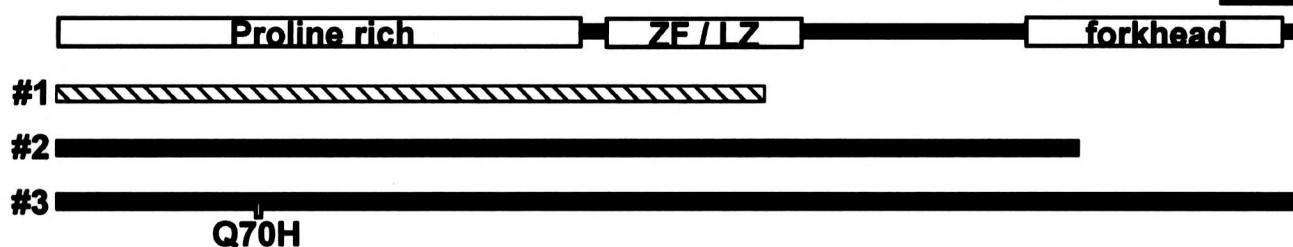


Figure 4. Normal FOXP3 protein (top line) in contrast to the predicted abnormal FOXP3 protein of patients 1, 2, and 3, respectively. The top line demonstrates normal FOXP3 with the known putative motifs: proline rich, zinc finger (ZF), leucine finger (LZ), and forkhead domain. The antibody used recognizes the indicated C-terminal epitope. Because patients 1 and 2 had either absent or truncated proteins, FOXP3 would not be detected in these individuals, in contrast to patient 3.

ed to substitute for definitive mutation analysis. Its advantages are that it could be available in any routine pathology laboratory and that it is extremely rapid. In addition, because patients in whom this diagnosis is suspected usually have had a gastrointestinal biopsy during their evaluation, this test can easily be performed on existing specimens. In contrast, mutation analysis is time consuming and currently only available at 1 center, University of Washington, Seattle, WA. Rapid results are significant in such syndromes as IPEX, which is associated with significant morbidity and mortality.

The FOXP3 or regulatory T cells (CD4+CD25+) in the normal gut mucosa are thought to be involved in maintaining immunologic balance throughout the body and the gastrointestinal tract. They are thought to balance the need to respond to foreign pathogens and not initiate the inflammatory response to harmless antigens. Dysregulation is thought to lead to inflammatory bowel disease [18,19]. In addition, syngeneic immune-deficient mice that have been injected with CD4+CD45RB cells, causing them to develop inflammatory bowel disease, can have improvement in their intestinal architecture after injection with regulatory T cells [20,21]. The absence of FOXP3 in our patients with the most severe disease could lead to aberrant regulation and uncontrolled inflammation.

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