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Dysgammaglobulinemia Associated With Glu349del, a Hypomorphic *XIAP* Mutation

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Abstract

Background: X-linked lymphoproliferative syndrome type 2 is a rare hereditary immunodeficiency caused by mutations in the *XIAP* gene. This immunodeficiency frequently results in hemophagocytic lymphohistiocytosis, although hypogammaglobulinemia and dysgammaglobulinemia are also common.
Objective: We identified 17 patients from 12 Japanese families with mutations in *XIAP*. The Glu349del mutation was observed in 3 patients, each from a different family. Interestingly, these patients exhibited dysgammaglobulinemia but not hemophagocytic lymphohistiocytosis. We conducted an immunological study of patients carrying Glu349del and other mutations to elucidate the pathogenic mechanisms of dysgammaglobulinemia in patients with mutations in the *XIAP* gene.
Patients and Methods: We performed an immunological study of 2 patients carrying the Glu349del mutation and 8 patients with other mutations.
Results: Flow cytometry showed that the percentage of memory B cells in patients with a mutation in *XIAP* was lower than that observed in the healthy controls. The patients with the Glu349del mutation had a lower percentage of memory B cells than those with other mutations. Ig production was reduced in patients with the Glu349del mutation. Increased susceptibility to apoptosis was observed in the patients with other mutations. Susceptibility to apoptosis was normal in patients with Glu349del. Microarray analysis indicated that expression of Ig-related genes was reduced in patients with the Glu349del mutation and that the pattern was different from that observed in the healthy controls or patients with other mutations in *XIAP*.
Conclusions: Patients carrying the Glu349del mutation in the *XIAP* gene may have a clinically and immunologically distinct phenotype from patients with other *XIAP* mutations. The Glu349del mutation may be associated with dysgammaglobulinemia.

Key words: X-linked lymphoproliferative syndrome. *XIAP*. Dysgammaglobulinemia. Hypomorphic mutation.

Resumen

Antecedentes: El síndrome linfoproliferativo ligado al cromosoma X (XLP) tipo 2, está causado por la mutación del gen *XIAP*. Se trata de una inmunodeficiencia hereditaria rara. Frecuentemente, los pacientes con XLP2 padecen linfohistiocitosis hemofagocítica (HLH) y disgammaglobulinemia.
Objetivo: Se han evaluado diecisiete pacientes japoneses, provenientes de doce familias con mutaciones *XIAP* y tres pacientes con la mutación Glu349del. Curiosamente, estos últimos pacientes desarrollaron una disgammaglobulinemia pero no HLH. Para dilucidar el fondo patogénico de la disgammaglobulinemia en pacientes con mutación del gen *XIAP*, se llevó a cabo un estudio inmunológico de estos pacientes.
Pacientes y métodos: Pudieron concluir el estudio inmunológico dos pacientes con la mutación Glu349del y ocho pacientes con otras mutaciones.
Resultados: Mediante análisis de citometría de flujo se observó que la proporción de linfocitos B de memoria en los pacientes con la mutación XIAP fue menor que la observada en los controles. Los pacientes con la mutación Glu349del tuvieron una menor proporción de linfocitos B de memoria que aquellos con otras mutaciones. Los pacientes con la mutación Glu349del presentaron menor producción de inmunoglobulinas. Los pacientes con la mutación Glu349del mostraron una susceptibilidad normal a la apoptosis, mientras que en los portadores de otras mutaciones se observó una mayor susceptibilidad a la muerte celular. El análisis de microarray indicó que los pacientes con la mutación Glu349del tenían disminuida la expresión de genes relacionados con las inmunoglobulinas y un patrón diferente de la observada en los controles normales o en pacientes con otras mutaciones de genes de *XIAP*.
Conclusiones: Los pacientes portadores de la mutación en el gen Glu349del *XIAP* pueden tener un fenotipo clínicamente e inmunológicamente diferente que los pacientes con otras mutaciones *XIAP*. La mutación Glu349del puede estar asociada con disgammaglobulinemia.

Palabras clave: Síndrome linfoproliferativo ligado al cromosoma X. Gen *XIAP*. Disgammaglobulinemia. Mutación hipomórfica.

Introduction

X-linked lymphoproliferative syndrome (XLP) is a rare immunodeficiency characterized by extreme vulnerability to Epstein-Barr virus infection that frequently results in hemophagocytic lymphohistiocytosis (HLH). Other clinical features of XLP include lymphoproliferative disorder, dysgammaglobulinemia, recurrent fevers, hemorrhagic colitis, and lymphoma [1]. XLP is associated with 2 genes: XLP1 is caused by a mutation in the *SH2D1A* gene, which encodes the signaling lymphocyte activation molecule-associated protein [2]; XLP2 is caused by a mutation in the *XIAP/BIRC4* gene, which encodes the X-linked inhibitor of apoptosis (XIAP) [3].

XIAP is a member of the IAP family and has 3 baculovirus IAP repeat (BIR) domains and a RING domain. It appears to inhibit both stress and death receptor–induced apoptosis via direct inhibition of distinct caspases. It also inhibits the activation of procaspase 9 and the activity of processed caspase 9. XIAP is a potent inhibitor of active caspases 3 and 7, which suppress death receptor–induced apoptosis [4]. In addition to its inhibitory effects on caspases, XIAP also plays a role in several signaling pathways, including Smad, NF-κB, and JNK [5-7]. The manifestations of XIAP-deficient patients may be related to a loss of the function and/or expression of XIAP [5,8].

Hypogammaglobulinemia is one of the most common clinical manifestations of XLP2, and approximately 20% to 30% of XIAP-deficient patients develop this condition [1,9,10]. Hypogammaglobulinemia in patients with XIAP deficiency is sometimes transient and occurs secondary to immune-mediated destruction of the humoral immune system [5]. Hypogammaglobulinemia in patients with XLP1 and XLP2 can result in a condition that is clinically indistinguishable from common variable immunodeficiency (CVID) [11,12]. One study demonstrated that male patients with CVID rarely carry a mutation in *SH2D1A* and are thus diagnosed with XLP1 [13]. However, Salzer et al [12] analyzed 28 male CVID patients and found no XIAP mutations. It is worth mentioning that a hypomorphic XIAP mutation ($XIAP^{G466X}$) was recently found to be related to $CD40L^{G219R}$, which is in turn associated with reduced ability to trigger class switch recombination and can cause X-linked variable immunodeficiency [14,15].

We previously reported cases of Japanese patients with XIAP deficiency [9]. In a subsequent cohort, we identified 3 patients from unrelated families with the same mutation (Glu349del) in the *XIAP* gene. All of these patients presented with dysgammaglobulinemia but not with HLH or colitis, which were common in patients with XIAP deficiency. There may be a correlation between the phenotype and a mutation in the *XIAP* gene. In this study, we report the immunological and functional findings of patients with the Glu349del mutation in order to elucidate the pathogenic mechanisms underlying hypogammaglobulinemia in patients with XIAP deficiency.

Patients and Methods

Patients

Blood samples and clinical information were collected from patients and healthy controls. We previously reported 9 patients from 6 unrelated families with XIAP deficiency in Japan [9]. Thereafter, patients with suspected XIAP deficiency or hypogammaglobulinemia were assessed for mutations in the *XIAP* gene, and 8 additional patients from 6 families were identified. In the present study, we divided the patients into 2 groups (patients with the Glu349del mutation and patients with other *XIAP* mutations) in order to investigate potential correlations between the Glu349del mutation and the phenotype of hypo/dysgammaglobulinemia. The Glu349del

mutation was identified in 3 of these patients. However, only 2 patients underwent the immunological study: 1 patient had to receive a hematopoietic stem cell transplant because of complicated severe aplastic anemia. Eight patients with other mutations were evaluated in this study.

Flow Cytometry and Monoclonal Antibodies

The proportions of memory B cells and follicular helper T (T_HF) cells were analyzed using flow cytometry. After informed consent was obtained, 5 to 10 mL of heparinized venous blood was collected from each patient and the healthy adult volunteers. The blood was processed and studied within 24 hours. Peripheral blood mononuclear cells (PBMCs) were prepared using density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich, Inc). For the phenotypic analysis, the PBMCs were stained with a combination of the following monoclonal antibodies (mAbs): anti-CD3-PE, anti-CD4-FITC, anti-CD8-FITC, anti-CD20-FITC, and anti-CD45RO-PE (Dako Cytomation); anti-CD27-FITC and anti-CXCR5-FITC (BD Biosciences); anti-IgD-PE (Southern Biotech); and anti-CD16-FITC, anti-CD19-PC5, anti-CD19-PE, anti-CD45-PC7, anti-CD56-PE, anti-CD45RA-PE, and anti-CD4-PC5 (Beckman Coulter, Inc). The stained cells were analyzed using a flow cytometer (FC500; Beckman Coulter KK).

In Vitro Ig Production Assay

The PBMCs were stimulated and cultured as previously described [16] in order to investigate their ability to produce Ig. They were then suspended in culture medium (RPMI 1640, Sigma-Aldrich) supplemented with 10% fetal calf serum and antibiotics at a concentration of 5×10^5/mL. Two hundred microliters of PBMCs was placed in 96-well round-bottom plates (BD Pharmingen) and stimulated with CpG ODN 2006 (InvivoGen, 1 μg/mL) and CD40 ligand (CD40L) (R&D Systems, Inc, 2 μg/mL) and cultured for 10 days. The Ig levels in the culture supernatants were measured using ELISA.

Induction of Cell Death and Apoptosis Assay

The PBMCs were stimulated with phytohemagglutinin (PHA, Sigma-Aldrich, 5 μg/mL) and rh-IL-2 (R&D System, 100 IU/mL) in RPMI 1640 supplemented with 10% fetal calf serum. After 3 days of culture, the PHA was eliminated, and the cells were cultured in culture medium with 100 IU/mL of rh-IL-2. PHA-induced T-cell blasts were obtained. From day 9 to day 13 of the culture, activation-induced cell death (AICD) assays were performed. For T-cell receptor–mediated induction of apoptosis, 48-well plates were coated with anti-CD3 mAbs (clone: Hit3a [BD Pharmingen], 10 μg/mL) in 150 μL of PBS or PBS only for 2 hours at 37ºC. One washing step with PBS was performed before incubation. Three × 10^5 cells were incubated in duplicate in 300 μL of culture medium with 100 IU/mL/well of rh-IL-2 for 48 hours at 37ºC. Two × 10^5 cells were incubated for 18 hours at 37ºC in duplicate in 96-well plates in 200 μL of culture medium with 100 IU/mL of rh-IL-2. Apoptosis was quantified using an annexin V staining assay (Sigma-Aldrich) to detect apoptotic cells with an intact membrane and externalized phosphatidylserine residues. The cultured cells were harvested, washed twice with cold PBS, and resuspended in 100 μL of annexin V binding buffer (BD Biosciences). The cells were then stained with annexin V and analyzed using flow cytometry. The specific percentage of apoptosis was calculated according to the following formula: 100 × [experimental cell death (%) – spontaneous cell death (%)]/[100 – spontaneous cell death (%)] [3]. The data shown are the mean of the duplicate measurements.

Analysis of Mutations in the XIAP Gene

Exon 4 of *XIAP* was amplified using PCR (forward primer, 5'-TGGCTCCTTAGAAGTACTGA-3'; and reverse primer, 5'-CTGCCCAGCTAGCTCTCATC-3') according to standard methods. We also amplified exon 4 of *XIAP* obtained from 170 healthy Japanese controls to exclude single-nucleotide polymorphisms (SNPs). The PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), and the sequencing analysis was performed on an Applied Biosystems Prism 310 Capillary Sequencer (Applied Biosystems).

Extraction of mRNA From Whole Blood

Whole blood samples (400 μL) were obtained from 2 patients carrying the Glu349del mutation (Patients 4 and 9), 6 patients with other *XIAP* mutations (Patients 1, 5, 6.1, 7.1, 7.2, and 8), and 7 controls. The samples were conserved in RNAlater (Ambion, Inc) following the manufacturer's instructions and stored at −20°C. Following complete thawing, the RNAlater was eliminated using centrifugation. Total RNA was extracted from the blood cells using a RiboPure-Blood Kit (Ambion, Inc) and treated with DNase I (Ambion, Inc) for 30 minutes at 37ºC to remove residual genomic DNA. The whole blood mRNA consisted of a relatively large population of globin mRNA transcripts. Because globin mRNA interferes with expression profiling of whole blood samples, α- and β-globin mRNA were depleted from the total RNA preparations using a GLOBINclear Kit (Ambion, Inc).

Microarray Analysis

Gene expression profiling of the PBMCs obtained from both patients and controls was performed on an HG-U133A plus 2.0 array spotted with 54,674 probe sets (Affymetrix). A sample preparation of the array hybridization was carried out according to the manufacturer's instructions. In short, biotin-labeled complementary RNA was prepared from 50 ng of globin-depleted total RNA using a 3'-IVT Express Kit (Affymetrix) and hybridized to the array. The arrays were scanned with a probe array scanner. The data files (CEL files) resulting from the analysis performed with the Affymetrix GCOS software package were imported into the GeneSpring GX 11.0 software program (Agilent Technologies Inc) to extract genes that were differentially expressed in the samples. The statistical analysis was performed using ANOVA with multiple testing correlations (Benjamini Hochberg FDR). A fold change value greater than 1.5 (upregulated) or less than 1.5 (downregulated) measured against the control samples was considered to be biologically relevant. Hierarchical clustering analysis was also performed using GeneSpring GX.

Table 1. Clinical and Genetic Data of Patients With *XIAP* Gene Mutations

Patient No.	Age at Presentation	Current Age	*XIAP* Mutation	XIAP Protein	Clinical Features	Ig Levels IgG	IgA	IgM	Treatment
1	20 mo	8 y	Arg238X	R	Recurrent HLH, splenomegaly, encephalitis, colitis	1860[a]	189	437[a]	PSL, CsA, Dex, tocilizumab, HSCT
2.1	7 mo	Deceased	Arg381X	–	Recurrent HLH, splenomegaly, hypogammaglobulinemia	ND	ND	ND	PSL, CsA, Dex, HSCT
2.2	3 mo	Died of colitis	ND	ND	Colitis	ND	ND	ND	ND
3.1	2 mo	13 y	Trp217CfsX27	–	Recurrent HLH	ND	ND	ND	PSL, CsA, Dex
3.2	-	18 y	Trp217CfsX27	–	Asymptomatic	ND	ND	ND	No
5	6 mo	5 y	Del of exon 1-2	+	Recurrent HLH, colitis	894	610a	189	PSL, CsA, Dex, IVIG Infliximab
6.1	17 mo	3 y	Asn341YfsX7	R	HLH, splenomegaly, colitis	1311	154	41[b]	IVIG, Dex
6.2	15 mo	15 y	Asn341YfsX7	R	Recurrent HLH, splenomegaly	823	47[b]	78[b]	PSL
7.1	7 y	14 y	Ile494Asn	+	Recurrent HLH, splenomegaly Colitis	822	ND	ND	Infliximab, TAC, colostomy
7.2	3 y	17 y	Ile494Asn	+	Recurrent HLH	1064	ND	ND	PSL
7.3	12 y	47 y	Ile494Asn	+	Colitis, malignant skin tumor	ND	ND	ND	ND
8	1 mo	5 y	Gln283X	–	Recurrent HLH, JIA	ND	ND	ND	PSL as needed
11	4 mo	2 y	Arg222X	R	Recurrent HLH, splenomegaly	769	23	23	PSL, CsA, Dex, IVIG VP-16, HSCT
12	3y	10 y	c.1056+1G>A	–	Recurrent HLH	ND	ND	ND	PSL
4	2 mo	16 y	Glu349del	+	Hypogammaglobulinemia	563[b] On IVIG	<4[b]	67	IVIG
9	7 y	15 y	Glu349del	+	IgM deficiency	562[b] On IVIG	258	<10[b]	IVIG
10	14 y	16 y	Glu349del	+	Hypogammaglobulinemia, aplastic anemia	479[b]	29[b]	239	IVIG, HSCT

Abbreviations: CsA, ciclosporin A; Dex, dexamethasone; HLH, hemophagocytic lymphohistiocytosis; HSCT, hematopoietic stem cell transplantation; IVIG, intravenous immunoglobulin; JIA, juvenile idiopathic arthritis; ND, no data; PSL, prednisolone; R, residual expression; TAC, tacrolimus; VP-16, etoposide.
[a]Higher than age-matched Japanese controls [27,28].
[b]Lower than age-matched Japanese controls.

Statistical Analysis

The statistical significance of the differences was determined using the *t* test. The differences in frequencies of polymorphisms between the patients and controls were assessed using the χ^2 test. *P* values of <.05 were considered to be significant.

Results

Clinical Manifestations of Patients With XIAP Mutations

The patients' clinical and genetic data, including those of the previous cases [9], are presented in Table 1. Patient 2.2 died of colitis at 4 years of age. Although we did not have enough clinical information or samples from this patient because of his early death, his symptoms strongly suggested that he had a XIAP deficiency associated with colitis because his uncle was also the maternal uncle of patient 2.1. Seventeen patients from 12 families with *XIAP* mutations were identified in Japan. Eleven patients (65%) had HLH, and 5 patients (29%) had colitis. Four patients (24%) had dysgammaglobulinemia, and 3 patients (patients 4, 9, and 10) had the same Glu349del mutation in the *XIAP* gene and exhibited normal XIAP protein expression. The mean and median age at presentation in patients with other *XIAP* mutations were 7 years 1 month and 7 years (range, 2 months to 14 years) in patients with the Glu349del mutation and 2 years 5 months and 1 year 3 months (range, 1 month to 12 years [except for patient 3.2 who had no clinical manifestations]). Intriguingly, none of the patients with the Glu349del mutation had HLH. Although 1 patient (patient 10) also developed aplastic anemia, it is worth mentioning that dysgammaglobulinemia was the only clinical feature observed in the other 2 patients. Patient 10 underwent hematopoietic stem cell transplantation owing to severe aplastic anemia, and the other patients were treated with intravenous immunoglobulin replacement therapy.

Table 2. Lymphocyte Subsets of Patients With the Glu349del Mutation and Other Mutations[a]

Patient No.	Total Lymphocytes	CD3	CD4	CD8	CD19	CD16/ CD56
4	1930	1460	740	840	30[b]	130
9	1790	1590	930	590	40[b]	80
1	3290	1960	1350	560	1050	50[b]
3.1	1200[c]	1020	540	480	70b	20[b]
5	2020	1610	1110	410[a]	180b	110
6.1	5150[c]	2960	2370[c]	690	1500[c]	340
7.1	2270	1760	760	870	440	70
7.2	1430	1310	690	530	20[b]	80
8	5150	4340	2120	1500	530	20
11	2770[b]	1710[b]	1020[b]	630	860	30[b]

[a]The absolute lymphocyte and subset counts (number of cells per microliter) were calculated by multiplying percentages.
[b]Lower than age-matched controls [17].
[c]Higher than age-matched controls.

Glu349del Mutation in the XIAP Gene in Healthy Individuals

Fifty-six patients with suspected XIAP deficiency or hypogammaglobulinemia were assessed for the *XIAP* gene mutation. Nine patients (patients 1, 2, 3, 5, 6, 7, 8, 11, and 12) were diagnosed with XIAP deficiency without the Glu349del mutation, and 3 of the remaining patients (patients 4, 9, and 10) had the Glu349del mutation (Table 1). Next, we sequenced exon 4 of the *XIAP* gene in 170 healthy Japanese individuals to rule out the Glu349del mutation as a possible SNP. Two and 4 individuals had heterozygous and homozygous Glu349del mutations, respectively. The Glu349del mutation revealed a SNP in the Japanese population. This mutation was recently observed in dbSNP138 (rs199683465) (http://www.ncbi.nlm.nih.gov/SNP/). Although the Glu349del mutation is a SNP, it could be associated with hypo/dysgammaglobulinemia.

Numbers of Switched Memory B Cells and T_HF Cells Were Lower in Patients With the Glu349del Mutation

The $CD19^+$ B-cell counts decreased with age in both of 2 patients with the Glu349del mutation (patients 4 and 9) and in 3 of the 8 patients with other *XIAP* mutations (patients 3.1, 5, and 7.2) (Table 2). In some of the patients with other *XIAP* mutations, the treatment may have exercised an effect on lymphocyte counts and lymphocyte subset counts. There were no differences in B-cell counts between patients with the

Table 3. B-cell Subsets and T_HF Cells in the Patients With Glu349del Mutation and Other Mutations

	$CD19^+$ B Cells, %			$CD4^+CD45RA^-$ T Cells, %
	IgD^+CD27^-, %[a]	IgD^+CD27^+, %[a]	IgD^-CD27^+, %[a]	$CXCR5^+$, %[b]
Glu349del (P4, P9)	92.3, 87.9	2.6, 4.3	3.9, 2.1	5.3, 16.7
Other mutations (n=8)	77.2 (10.3)[c]	7.7 (3.8)[c]	10.8 (7.2)[c]	13.7 (12.8)[c]
Control subjects (n=6)	54.6 (13.3)	22.3 (8.8)	20.4 (7.0)	31.2 (3.2)

[a]Relative numbers of B-cell subsets within $CD19^+$ B cells, mean (SD).
[b]Relative numbers of $CXCR5^+$ cells within $CD4^+CD45RA^-$ memory T cells, mean (SD).
[c]*P*<.01, statistical significance of the difference compared with the controls.

Glu349del mutation and patients with other mutations (data not shown). We analyzed naïve and memory B-cell subsets (Table 3). We also analyzed T_HF cells defined as $CXCR5^+CD45RA^-CD4^+$ T cells, because T_HF cells provide help to B cells [18]. Counts for memory B cells and T_HF cells in patients with *XIAP* mutations were lower than those observed in the healthy controls. In the case of memory B cells, this tendency was observed in patients with the Glu349del mutation. Otherwise, no tendency toward a decrease in T_HF counts was observed.

Patients With the Glu349del Mutation Produced Low Levels of All Ig Classes In Vitro

To determine the level of Ig production, PBMCs were stimulated with CpG and CD40L, and the supernatant was measured using ELISA. The levels of all Ig classes were lower in patients with the *XIAP* gene mutation than in the controls. Production of all Ig classes was higher in XIAP-deficient patients than in the healthy controls (Table 4). It is worth mentioning that Ig production in patients with the Glu349del mutation observed in the CpG- and CD40L-stimulated cultures was lower than in patients with other *XIAP* mutations. Reduced in vitro levels of Ig production were observed in some of the patients with other *XIAP* mutations who did not have hypogammaglobulinemia. Patients with the Glu349del mutation tended to have lower Ig production in vitro than patients with other *XIAP* mutations.

Normal Susceptibility to Apoptotic Stimuli in the Patients With the Glu349del Mutation

AICD is observed in XIAP-deficient patients [3]. We analyzed the degree of apoptosis induced by AICD in patients with the Glu349del mutation compared with that observed in the patients with other mutations and in the healthy controls. Patients with other *XIAP* mutations exhibited increased AICD (Figure 1), whereas patients with the Glu349del mutation did not. These results suggest that the apoptotic mechanism in patients with the Glu349del mutation was different from that of patients with other *XIAP* mutations.

Immunoglobulin-Related Gene Expression Was Decreased in Patients With the Glu349del Mutation

Microarray analysis revealed that the gene expression patterns of patients with the Glu349del mutation were different from those of the controls and the patients with other *XIAP*

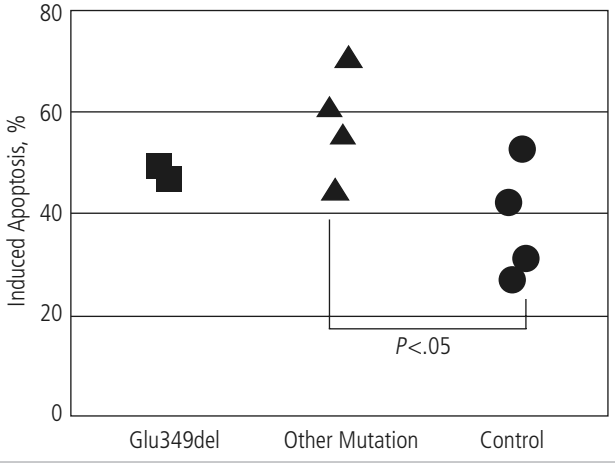

Figure 1. No enhanced activation-induced cell death of T lymphocytes in the patients with the Glu349del mutation. We evaluated induced apoptosis of T-cell blasts in 4 healthy controls, 2 patients with the Glu349del mutation, and 4 patients with other *XIAP* mutations. The cells were stimulated with anti-CD3 mAbs. Apoptosis was quantified using an annexin V staining assay to detect apoptotic cells. The data shown are the mean of duplicate measurements.

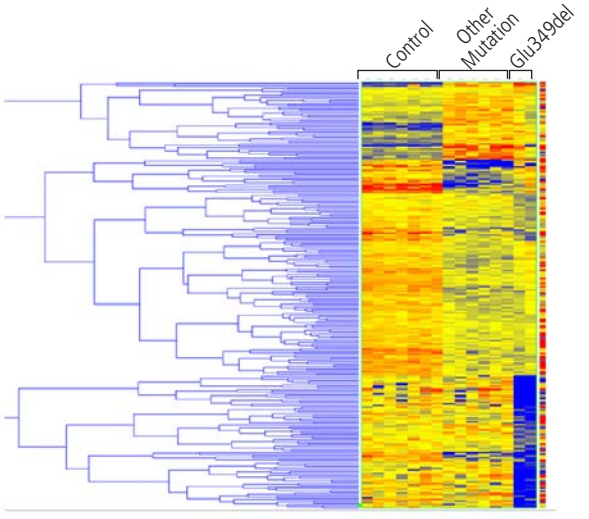

Figure 2. The gene expression patterns of patients with the Glu349del mutation were different from those of the controls and the patients with other *XIAP* mutations. Gene expression profiling of peripheral blood cells obtained from 7 healthy controls, 6 patients with XIAP deficiency, and 2 patients carrying the Glu349del mutation was performed using a microarray. The red and blue colors indicate higher and lower expression of mRNA, respectively.

Table 4. Reduced Production of All Ig Classes in Patients With the Glu349del Mutation[a]

	Glu349del (P4, P9)		Others (n=8)		Controls (n=6)	
	Non	CpG+CD40L	Non	CpG+CD40L	Non	CpG+CD40L
IgG	6.7, 9.8	12.6, 14.3	41.7 (13.2)	694.6 (374.0)[b]	49.1 (10.4)	2977.7 (711.3)
IgA	6.0, 10.0	7.9, 11.9	17.6 (4.3)	378.0 (145.0)	25.2 (6.5)	1174.2 (507.1)
IgM	8.1, 28.4	.0, 10.3	17.0 (3.4)	1521.9 (913.6)[b]	60.0 (28.0)	6843.7 (1159.1)

[a]Peripheral blood mononuclear cells (5×10^5/mL) were stimulated with CpG and CD40L and cultured for 10 days. The Ig levels in the culture supernatants were determined using ELISA. The results are shown as mean (SEM).
[b]$P<.05$, statistical significance of the difference compared with the controls.

mutations (Figure 2). In the patients with the Glu349del mutation, 37 and 31 mRNAs were expressed at 10-times lower levels than in the controls and in patients with other mutations, respectively (data not shown). Among these genes, levels of mRNA were lower in 29 genes in patients with the Glu349del mutation than in the healthy controls and patients with other gene mutations (Table 5). Intriguingly, 12 genes, including *IGHA, IGHG, IGHM, IGKV*, and *TNFRSF17*, were associated with Ig levels and the development of B cells.

Table 5. Genes Whose mRNA Levels Are 10 Times Lower Than Those of Patients With Other Mutations and Controls in Patients With Glu349del

Probe Set ID	Gene Symbol
1557242_at	
1569194_at	ZNF789
233903_s_at	ARHGEF26
207995_s_at	CLEC4M
221671_x_at	IGK@ /// IGKC
214677_x_at	IGLV1-44 /// LOC100290481
1559350_at	
238705_at	
219874_at	SLC12A8
217281_x_at	IGHA1 /// IGHA2 /// IGHG1 /// IGHG2 /// IGHG3 /// IGHM /// IGHV4-31 /// LOC100126583 /// LOC100290036
1564069_at	NCRNA00213
216401_x_at	
237625_s_at	
219518_s_at	ELL3 /// SERINC4
222320_at	
228518_at	IGHG1 /// IGHM
215121_x_at	IGLC7 /// IGLV1-44 /// LOC100290481
216874_at	DKFZp686O1327
217179_x_at	
216510_x_at	IGHA1 /// IGHG1 /// IGHM /// IGHV3-23 /// IGHV4-31
223898_at	ZNF670
232564_at	SLC9A5
237315_at	
216517_at	IGKV1D-8
216576_x_at	IGK@ /// IGKC /// LOC652493 /// LOC652694
217022_s_at	IGHA1 /// IGHA2 /// LOC100126583
215118_s_at	IGHA1
214777_at	IGKV4-1
206641_at	TNFRSF17

Discussion

In our cohort, 3 patients, each from a different family, had the Glu349del mutation in the *XIAP* gene and exhibited dysgammaglobulinemia as the major clinical manifestation. Because the Glu349del mutation was repeatedly identified, it was suspected as the possible causative SNP. Direct sequencing of exon 4 in the *XIAP* gene revealed this mutation in 6 of the 170 (3.5%) healthy Japanese individuals. Glu349del was not detected in previous studies, including polymorphism studies conducted in Korea and Australia [13,19,20]. Therefore, the Glu349del mutation may be a SNP and have undergone a founder effect in Japan. Approximately 20% of the patients with XIAP deficiency had dysgammaglobulinemia, while all of the patients with the Glu349del mutation developed dysgammaglobulinemia. Although the Glu349del mutation is a SNP that can be found in the Japanese population, it could be associated with the clinical phenotype.

The patients with the Glu349del mutation exhibited decreased numbers of memory B cells and reduced Ig production in vitro. Since XIAP inhibits apoptosis [5], we suspected that, in patients with the Glu349del mutation, apoptosis of B cells could be induced during B-cell development or that almost mature B cells are unable to survive following Ig class switches. However, the apoptosis assay did not reveal any significant differences between patients with the Glu349del mutation and the healthy controls. The antiapoptotic properties of the XIAP protein depend primarily on the sites of BIR2 and 3 [5]. BIR3 binds and inhibits procaspase 9, while BIR2 inhibits the activation of caspases 3 and 7 [5]. Because Glu349 is distant from these domains, the results of the apoptosis assay may contrast with those observed in XIAP-deficient patients with augmented apoptotic properties. All isotypes of Ig were produced at lower levels in patients with the Glu349del mutation. This finding may be related to decreased numbers of memory B cells.

XIAP also plays a crucial role in inflammation and innate immune responses via the MAPK and NF-κB pathways [21-23]. XIAP mediates NF-κB activation via the NOD1/2 pathway. When bacterial peptidoglycans bind to NOD1/2, a complex comprising NOD1/2, RIP2, XIAP, cIAP1, and cIAP2 is assembled, leading to ubiquitination of RIP2 [22]. Subsequently, MAPK and NF-κB are activated. The RING domain of IAPs exhibits ubiquitin-E3 ligase activity and enables ubiquitination. Patients with a mutation in the *XIAP* RING domain exhibit interference with ubiquitin ligase activity, and clinical manifestations of XIAP deficiency may be caused by impaired NOD2 signaling [24]. However, many clinical manifestations cannot be explained only by impaired NOD2 signaling. XIAP regulates several signaling pathways downstream of immune receptors [24]. XIAP also contains a ubiquitin-associated domain for binding the ubiquitin chain. A recent study reported that the ubiquitin-associated domain uses different surfaces for binding ubiquitin [25], potentially leading to the complex phenotype of XIAP deficiency. It remains unclear how the Glu349del mutation affects these pathways.

The microarray analysis provided valuable information. Compared with patients with other *XIAP* mutations and controls, expression of the B-cell differentiation pathways was downregulated in the patients with the Glu349del mutation.

Furthermore, in the mRNA of patients with the Glu349del mutation, expression of 29 genes was decreased 10 times compared with the healthy controls and patients harboring the other *XIAP* mutations. The least downregulated gene was *TNFRSF17*, which is a member of the TNF receptor superfamily and encodes B-cell maturation antigen [26]. B-cell maturation antigen is predominantly expressed on terminally differentiated B cells and has functional activity in mediating the survival of plasma cells that maintain long-term humoral immunity. Decreased expression of the *TNFRSF17* gene may be associated with impaired humoral immunity. *IGHG, IGHA*, and *IGHM* encode the γ, α, and μ heavy chains of these molecules, respectively, and decreased expression of these genes is associated with reduced Ig production. The Ig molecule is made up of 2 identical heavy chains and 2 identical light chains, either κ or λ, which are joined by disulfide bonds so that each heavy chain is linked to a light chain and the 2 heavy chains are linked together. *IGH* encodes the Ig heavy chain, and *IGK* and *IGL* encode the κ and λ light chains, respectively. Decreased expression of these genes suggests reduced frequency of somatic hypermutation and can lead to reduced Ig production and dysgammaglobulinemia in patients with the Glu349del mutation; however, the mechanisms by which the Glu349del mutation reduces the expression of these Ig-related genes have not yet been elucidated. The other genes whose expression is decreased in patients with Glu349del mutation might not be associated with the XIAP protein or the apoptosis pathway.

In conclusion, patients carrying the Glu349del mutation in the *XIAP* gene may have a clinically and immunologically distinct phenotype from patients with other *XIAP* mutations. Our findings indicate that the Glu349del mutation in the *XIAP* gene may be associated with dysgammaglobulinemia, although the mutation is a SNP in Japanese individuals. Further studies are required to clarify this issue.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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