

Analysis of genetic defects in patients with the common variable immunodeficiency phenotype in a single Taiwanese tertiary care hospital

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Background: Seven known genetic defects, including Bruton tyrosine kinase (*Btk*), *CD40L*, and signaling lymphocyte activation molecule–associated protein (*SAP*) (all X-linked) and inducible costimulator molecule (*ICOS*), transmembrane activator and calcium-modulator and cytophilin ligand interactor (*TACI*), B-cell–activating factor of the tumor necrosis family receptor (*BAFFR*), and *CD19* (all autosomal recessive), were found in patients with the phenotype of common variable immunodeficiency (CVID).

Objective: To investigate these 7 candidate protein expressions and candidate gene sequences for comprehensive analysis of known genetic defects in patients with CVID.

Methods: These 7 candidate protein expressions were evaluated by flow cytometry or Western blot, and candidate genes were evaluated by direct sequencing.

Results: Of 9 CVID patients from a single Taiwanese tertiary care hospital, we identified 2 cousins with decreased *Btk* expression who had a mutated (Asp521Val) kinase domain of *Btk* (1694A>T in exon 15) and 1 patient with decreased *CD40L* expression who had a mutated (Thr254Met) extracellular domain of *CD40L* (782T>C in exon 5).

Conclusion: This comprehensive approach revealed that, in Taiwan, in some patients mild forms of X-linked agammaglobulinemia and hyper-IgM syndrome caused the CVID phenotype. No mutations of *SAP*, *ICOS*, *TACI*, *BAFFR*, and *CD19* were identified in this study, although selection bias among the small study population and genetic variation may exist.

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INTRODUCTION

Common variable immunodeficiency (CVID), which is characterized by varying degrees of defective innate and adaptive immunity,¹ is the second most common primary immunodeficiency disease after IgA deficiency. To define this heterogeneous disorder of CVID, the Pan American Group for Immunodeficiencies and the European Societies for Immunodeficiencies guidelines for diagnosis require (1) a significant decrease in either 2 of the 3 major immunoglobulin

isotypes, (2) abnormal specific humoral antibody responses to vaccines, and (3) the exclusion of molecularly defined causes of hypogammaglobulinemia.² This third criterion may pose practical difficulties, because some syndromes with hypogammaglobulinemia resemble the CVID phenotype. These syndromes encompass X-linked agammaglobulinemia,^{3–8} X-linked hyper-IgM syndromes (HIGM1),^{9–11} and X-linked lymphoproliferative syndrome (XLP).^{12,13}

In addition to these 3 X-linked resembling syndromes, the monogenetic basis of CVID was discovered and located within 3 relative signaling pathways. The first pathway is inducible costimulator molecule (*ICOS*) signaling. *ICOS* signaling, which is in need of *CD40* signaling, augments class switch recombination and germinal center formation.^{14–16} Mutation of *ICOS* decreases generation and/or expansion of memory B cells.^{17,18} The second pathway is a proliferation-inducing ligand (*APRIL*), B-cell–activating factor of the tumor necrosis factor family (*BAFF*) and transmembrane activator and calcium-modulator and cytophilin ligand interactor (*TACI*), B-cell maturation protein A, and *BAFF* receptor (*BAFFR*) signaling. *TACI* and *BAFFR*, which are mainly expressed on B cells and bind *BAFF* and *APRIL*, contribute to residual class switch recombination, independent of *CD40* signaling.¹⁹ Mutations of *TACI* and *BAFFR* interrupt *BAFF* and *APRIL* signaling and thus impair plasma cell development and survival.^{20–23} The third pathway is *CD19* signaling. Mutations of *CD19* disturb the downstream function of the

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Table 1. Designed Primers for Complementary and Genomic DNA of the *TACI*, *BAFFR*, and *CD19* Genes

	Forward primer (5'→3')	Reverse primer (5'→3')
<i>TACI</i> complementary DNA (Genebank No. NM012452)	GCA TCC TGA GTA ATG AGT GG	TCC TTC TCT GCC TGT CTC TT
<i>TACI</i> genomic DNA (Genebank No. NT010718)	ACA TCC AAC AGG TTG AGG	TCC TTC TCT GCC TGT CTC TT
Exon 1	TCC ACT GCT CTG TGT GTA CC	AAC CTC AGT GGC TTC TGT CA
Exon 2	AGG TTG TCT GTG CGA ATG TG	GTA AGA GGT GCC ACA CTG TA
Exon 3	CCA GAG TTC CTG CTA GTC	GGC TTC ATG CAT TGT GGA CT
Exon 4	GGA TGT GGA TTG CTT GAG	CTC TCC TCA TAT CTC TCT CC
<i>BAFFR</i> complementary DNA (Genebank No. NM052945)	AGC CTC AGT CCC CGC AGC	GGC ATG TGC ATG CTG GAG TG
<i>BAFFR</i> genomic DNA (Genebank No. NM011520)		
Exons 1–2	AGC CTC AGT CCC CGC AGC	CCA CTC TGT CTC CGT T
Exon 3	CTC CAG AGG AGT CTT CTA GG	GGC ATG TGC ATG CTG GAG TG
<i>CD19</i> complementary DNA (Genebank No. NM001770)	CCG GAG AGT CTG ACC ACC AT	TGT CAG GTT CCT CAT AG
	CCA CCA GGA AGT TCT TCA	CAC ATG CAC ACA TCC TAA GC

Abbreviations: *BAFFR*, B-cell-activating factor of the tumor necrosis family receptor; *TACI*, transmembrane activator and calcium-modulator and cytophilin ligand interactor.

signaling complex of CD19, CD21, CD81, and CD225 for B-cell development, activation, and proliferation.^{24,25} These 4 monogenetic mutations of ICOS, *TACI*, *BAFFR*, and *CD19* have been identified as causing the CVID phenotype in humans.^{1,17,18,20–26}

In this study, we comprehensively investigated the molecular basis of CVID patients in a single medical center during a 20-year period.²⁷ We analyzed gene mutations known to mimic the CVID phenotype in male patients, including Bruton tyrosine kinase (*Btk*), *CD40L*, and signaling lymphocyte activation molecule-associated protein (*SAP*). If these were wild-type genes, we also studied the known candidate genes of *ICOS*, *TACI*, *BAFFR*, and *CD19*. With this approach, we identified genetic defects in 3 of 9 patients belonging to 8 unrelated families.

MATERIALS AND METHODS

Patients

After institutional review board approval from the Human Investigation Committee and informed consents were obtained in our institute, 15 mL of venous blood was collected into heparin-containing syringes and delivered to our laboratory within 24 hours. The clinical data collected in patients included initial clinical manifestations, onset ages, family history of immunodeficiencies and/or recurrent infections, malignancies, basic immunologic laboratory tests, blood smear, and differential cell counts. Lymphocyte subpopulations (T cells, B cells, and natural killer cells) and functional

assessments of innate and adaptive immunity were evaluated as previously described.²⁷

Detection of Gene Expressions

The expression of *Btk* in platelets and *CD40L* and *ICOS* by activated T cells was evaluated by immunostaining as previously designed.^{11,28,29} Briefly, platelets were sedimented by centrifugation at 1,200g for 20 minutes and resuspended in a modified Tyrode buffer. Platelets were fixed with 4% paraformaldehyde and permeabilized with 0.1% saporin. Intracellular staining was performed with anti-*Btk* monoclonal antibody (mAb) 48 to 2H (a gift from Futatani Takeshi, MD, PhD, and Hans D. Ochs, MD), which recognized an epitope of the SH3 kinase domain. For *CD40L* and *ICOS* expression, fresh peripheral blood mononuclear cells were stained with phycoerythrin-labeled anti-CD4 mAb (Ancell, Bayport, Minnesota) at 4°C for 20 minutes, washed, and incubated overnight in the presence of phorbol 12-myristate 13-acetate (PMA; GIBCO-BRL, Gaithersburg, Maryland) at 10 ng/mL and ionomycin (Sigma, St Louis, Missouri) at 1 µg/mL. We used anti-human *CD40L* mAb (Pharmingen, San Jose, California) to identify *CD40L* expression; fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, Alabama) was used as secondary antibody. Immunostaining for *ICOS* was performed by incubating the activated cells with the *ICOS* ligand fusion protein B7RP-1 Fc (a gift from Amgen Inc, Thousand Oaks, California) at a concentration of 20 µg/mL for 1 hour

Table 2. Clinical Features of the 9 Patients With the Common Variable Immunodeficiency Phenotype

Patient No./sex	Age, y		Clinical events					Other presentations
	Onset	Current	Recurrent sinopulmonary infections ^a	Bronchiectasis	Chronic diarrhea	Septicemia	Severe skin infections ^b	
1/F	3	25	+	+				
2/M ^c	1.5	5	+		+	<i>Pseudomonas aeruginosa</i> , <i>Pseudomonas oryzae</i>	+	Lip gangrene, meningitis, and toxic megacolon
3/M ^c	16	27 (dead)	+	+				Arthritis (<i>Streptococcus pneumoniae</i> , <i>Staphylococcus aureus</i>), appendicitis, chronic hepatitis B and C, hepatocellular carcinoma, and hepatomegaly
4/F	18	27	+					
5/M	22	67	+	+				
6/F	6	9	+					
7/M	6	10	+					Alopecia, septic arthritis and abdominal aneurysm vaccine-associated poliomyelencephalitis
8/M	16	21	+	+		<i>P aeruginosa</i> , <i>S pneumoniae</i>		
9/M	7	22	+			<i>P aeruginosa</i>	+	Anal fistula and colostomy

Symbol: +, positive.

^a Sinopulmonary infections include sinusitis, otitis media, and/or pneumonia.

^b Severe skin infections contain carbuncles, pustulosis, and/or cellulites.

^c Patients 2 and 3 were from the same family.

at room temperature; the cells were then washed and stained with FITC-conjugated anti-human Fc antibody (Chemicon, Temecula, California) at a final dilution of 1:200. FITC-labeled anti-human CD69 (Pharmingen) served as activation control.

Anti-TACI (Santa Cruz Bio, Santa Cruz, California) and BAFFR (eBioscience, San Diego, California) conjugated with phycoerythrin- and FITC-labeled anti-CD19 monoclonal antibodies (mouse IgG1, Pharmingen) were used to evaluate TACI, BAFFR, and CD19 expression in Epstein-Barr virus transformed lymphoblast cell lines (EBV-LCLs).^{20,21–25,30} A minimum of 10,000 events was analyzed by flow cytometry.

The expression of SAP protein was measured by Western blot. The blots were incubated with 1:1,000 anti-SAP (Santa Cruz Bio) antibodies at room temperature for 1 hour, followed by incubation for 1 hour with 1:10,000 horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Bio-source, Camarillo, California), then SAP proteins were visualized by electrochemiluminescence (Pierce, Rockford, Illinois). To verify that equal amounts of proteins were loaded on the gel, the same blots were subsequently stripped and reprobed with anti- β -actin (Sigma) as internal controls. The

results were quantified by densitometry using a GS-670 Imaging Densitometer (Bio-Rad, Hercules, California) and calculated as a ratio of SAP protein vs total anti- β -actin protein.

Gene Sequencing

Total RNA was isolated from activated peripheral blood mononuclear cells (4 hours of incubation with PMA at 10 ng/mL and ionomycin at 1 μ g/mL) with TRIzol (GIBCO-BRL). Reverse transcription of messenger RNA followed by polymerase chain reaction was performed as followed: 1 to 2 μ g of RNA in a total volume of 20 μ L was reverse transcribed into complementary DNA using oligo-dT primer and superscript RNaseH reverse transcriptase (GIBCO-BRL). Amplification was performed using 1 μ L of complementary DNA in a total volume of 20 μ L containing 0.5 U of High Fidelity Taq DNA polymerase, 1,875-mmol/L magnesium sulfate, 200- μ mol/L dNTP, 500-nmol/L each oligonucleotide primer, and $\times$ 10 buffer (Invitrogen, Gaithersburg, Maryland). Every 2 oligonucleotide primers were selected to cover the entire coding region of the *Btk*, *CD40L*, *SAP*, *ICOS*,¹¹ *TACI*, *BAFFR*, and *CD19* genes (Table 1). If a specific

Table 3. Immunoglobulin Levels, Lymphocyte Subsets, and Proliferation Tests in 9 Patients With the Common Variable Immunodeficiency Phenotype

Patient No./sex	Immunoglobulins ^a				Absolute count		Lymphocytes (%)					Lymphocyte proliferation test ^b	
	IgM, mg/dL	IgG, mg/dL	IgA, mg/dL	IgE, IU/L	Lymphocytes, /mm ³	Neutrophils, /mm ³	CD3	CD4	CD8	CD19	CD16/56	Mitogens	Antigens
1/F	<5	308	<6	<7	2,459	1,659	65	36	29	9	8	N	D
2/M	32	320	16	<7	3,965	2,246	95	59	26	2		N	N
3/M	28	228	95	<7	1,755	2,916	84	28	22	2	15	Not done	Not done
4/F	83	215	<6	<7	2,172	5,204	69	38	31	13	7	N	D
5/M	109	48	83	<7	6,435	9,625	72	42	35	10		N	N
6/F	7	48	<6	<7	2,631	3,242	82	34	29	15		N	N
7/M	7	93	<6	25	3,524	3,685	90	-	-	8	12	D	D
8/M	<5	59	<6		2,718	3,654	78	47	22	5		N	Not done
9/M	42	232	18	<7	3,459	3,258	82			12		D	Not done

Abbreviations: N, normal; D, decreased.

^a Numbers set in italic type indicate below the normal range. Normal ranges are from Stiehm RE. *Immunologic Disorders in Infants and Children*. 6th ed. Philadelphia, PA: Philadelphia Press; 2003. Pair-serum of polio vaccine titers was evaluated before and after 4-week vaccination in 3 patients, revealing decreased results in 2 patients (patients 7 and 8) and normal results in 1 patient (patient 9). Natural killer cell activity was measured in 4 patients (patients 6, 8, 9, and 10), and all results were normal.

^b Lymphocyte proliferation tests were stimulated in vitro by mitogens (phytohemagglutinin, concanavalin A, and/or pokeweed mitogen) for 3 days and/or antigens (*Candida albicans* or BCG vaccine) for 7 days, then incubated with 3H-thymidine.

mutation was identified in each gene, genomic DNA was amplified and confirmed again.

RESULTS

Patients Characterization

Nine patients with CVID (3 females and 6 males from 8 unrelated families) were investigated. Their ages at presentation ranged from 1.5 to 22 years. The clinical features and immunologic functions in patients with the CVID phenotype are given in Tables 2 and 3. Overall, recurrent sinopulmonary infections occurred in all patients, followed by bronchiectasia (4 patients), septicemia (3 patients with 5 episodes, including 4 with *Pseudomonas* spp and 1 with *Streptococcus pneumoniae*), severe skin infections (2 patients), and chronic diarrhea (1 patient). CVID patients had lower levels of IgG (9/9), IgM (6/9), IgA (6/9), and IgE (7/8). All were receiving regular intravenous immunoglobulin therapy. Absolute lymphocyte and neutrophil counts were normal and without reverse CD4/CD8 ratio. In vitro decreased lymphocyte proliferation to mitogens and specific antigens was observed in 2 of 8 and 3 of 6 patients, respectively. Patient 3, the uncle of patient 2, died of hepatocellular carcinoma associated with hepatitis B and C infections.

Gene Expression

To analyze *Btk* expression in platelets as an initial diagnostic tool to screen patients for mutation of *Btk* mimicking CVID, 5 of the 6 patients had normal expression, whereas patient 2 had only trace expression (2.0%; III-I in Fig 1). Platelets from his carrier mother showed a bimodal pattern (II-3 in Fig 1). Detectable expression of CD40L and ICOS by CD4⁺ cells was observed after stimulation with PMA and ionomycin for

2 hours (data not shown). After such stimulation for 24 hours, 79.8% to 98.3% of patients' activated CD4⁺ cells expressed CD40L and 72.6% to 85.4% of activated CD4⁺ cells bond B7RP-1 fusion protein, similar expression patterns to that of controls except that patient 5 had significantly decreased CD40L expression (29.6%) and mean fluorescent intensity (36.3; Fig 2).

Western blotting showed normal SAP expression in 4 selected patients (Table 4). Notable surface TACI expression (43.2% to 83.0%) from EBV-LCLs, but diminished TACI expression (4.8% to 11.2%) from peripheral blood cells was observed in patients and controls. BAFFR and CD19 were also ubiquitously expressed on both CD19⁺ lymphocytes and EBV-LCLs without additional stimulation in patients and controls.

Gene Analysis

According to complement and genomic DNA sequencing, patients 2 and 3 from the same family had missense mutations of the *Btk* gene (1694 A>T in exon 15), resulting in the substitution of Asp by Val at amino acid location 521 in the kinase domain of the Btk protein (Fig 1). As expected from bimodal Btk expression of his mother's platelets, the maternal parents of patient 2 were carriers. Asp521, functionally belonging to the RDLAARN motif (from Arg 520 to Asn 526) of Btk, constituted the catalytic loop as an indicator of tyrosine specificity of protein kinase.³¹ Mutation of Asp521 replaced by Val in this loop could change the protein stability and attenuate kinase activity as previously reported.³²

Patient 5 had a missense mutation of the Thr254Met *CD40L* gene (782 T>C in exon 5) in the tumor necrosis factor domain with lower affinity to the monoclonal CD40L antibody (Fig 2). This patient did not consent to have his

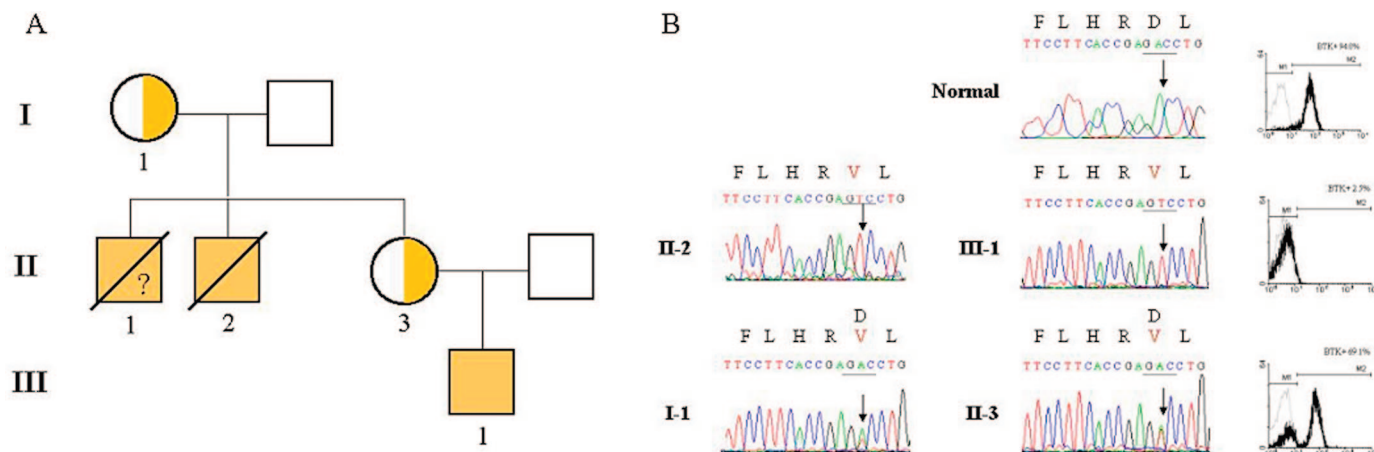


Figure 1. A, The pedigree of patients 2 (III-1) and 3 (II-2) revealed X-linked inherent diseases. Patient II-1 had meningitis and subsequent epilepsy and died at 26 years of age. He did not undergo genetic analysis. Patient 3 (II-2) had frequent sinopulmonary infections and died of hepatocarcinoma associated with hepatitis B and C at the age of 27 years. Four individuals (I-1, II-2, II-3, and III-1) underwent genetic analyses that showed mutant (Asp521Val) Bruton tyrosine kinase (Btk) protein in patients 2 and 3, whose proteins were not obviously detected by anti-Btk monoclonal antibody 48 to 2H. B, Similar bimodal expression of Btk protein in platelets was also traced to maternal carriers (I-1 and II-3).

entire family studied. A representative patient with the same *CD40L* mutation was not able to mount IgG isotype switching after receiving secondary boost of T-cell-dependent bacteriophage ψ X174.¹⁰ No mutations of the *SAP*, *ICOS*, *TACI*, *BAFFR*, and *CD19* genes were found.

DISCUSSION

Mutations of the *Btk*, *CD40L*, and *SAP* genes, which are responsible for 3 X-linked primary immunodeficiencies of agammaglobulinemia, HIGM1, and XLP could present as the CVID phenotype (Table 5). In more than 400 mutations of the *Btk* gene,³³ the genotypes did not closely correlate with the phenotypes, and considerable variation in clinical severity was observed within the affected members of the same pedigree.⁵ Patient 3 with a missense mutation (Asp521Val) in *Btk* had arthritis twice and died of hepatocellular carcinoma associated with hepatitis B and C. He probably had residual or leaky *Btk* function, leading to detectable peripheral B cells,³⁻⁷ and subsequently received the first intravenous immunoglobulin therapy at 27 years old, whereas his cousin (patient 2) with recurrent sinopulmonary infections was found to have a *Btk* mutation. Patient 5, previously reported cases with 4 unique *CD40L* mutations, had a less severe HIGM1 phenotype, similar to the CVID phenotype.¹¹ Of these 4 unique *CD40L* mutations, there were the missense (Thr254Met) mutation, the generating new initial start (Arg11stop), and few escaped normal products from 2 splicing errors (IVS2 + 2t>a and 367G>A) that were all partially recognized by monoclonal CD40L antibodies and reserved, to some extent, affinity to CD40 as previously reported.¹¹ Patients with residual CD40 signaling had fewer infections in contrast to those typical HIGM1 patients with undetectable CD40L who always presented, in infancy, as having intermittent or

persistent neutropenia, severe recurrent sinopulmonary infections, *Pneumocystis jirovecii* (carinii) pneumonia, and *Cryptosporidium*-related chronic diarrhea and sclerosing cholangitis.³⁴⁻³⁸

XLP patients had 3 distinct major characteristics: fulminant infectious mononucleosis linked to EBV infection, immunoglobulin deficiencies (mostly low IgG levels due to the decreased number of B cells), and non-Hodgkin B-cell lymphomas.^{39,40} Fluctuating serum immunoglobulins and recurrent respiratory infections in XLP patients could indicate that these patients have the CVID phenotype.^{12,13} In our few male CVID patients, none had fulminate infectious mononucleosis. No more than 1 male patient was encountered in the same family except for those patients with *Btk* mutations. Therefore, no *SAP* mutation was found.

For the monogenetic basis of CVID, Grimbacher et al¹⁷ and Salzer et al¹⁸ identified a large deletion of the *ICOS* genes in 9 adult-onset patients, resulting in frameshift and truncation of the ICOS molecule. The affected members from 4 families were homozygous for the same 1.8-kb deletion and seemed to have a common founder from Black Forest, Germany. Both these groups also identified *TACI* mutations in 24 patients (15 females and 9 males) in whom 2 patients had IgA deficiency and the rest had CVID.^{20,21} Of 19 patients studied by Grimbacher et al, 10 had lymphoproliferation (splenomegaly or tonsillar hyperplasia) and 5 had autoimmunity (thyroiditis, pernicious anemia, and vitiligo). Of their subgroup, only 3 patients had onset ages younger than 20 years and 2 patients had a family history of CVID.²⁰ In our institute, a 12-year-old girl was diagnosed as having symptomatic IgA deficiency (IgG, 724 mg/dL; IgA, 8 mg/dL; and IgM, 71 mg/dL), presenting as recurrent sinopulmonary infections and bronchiectasis. She received *TACI* gene analysis, but wild type.

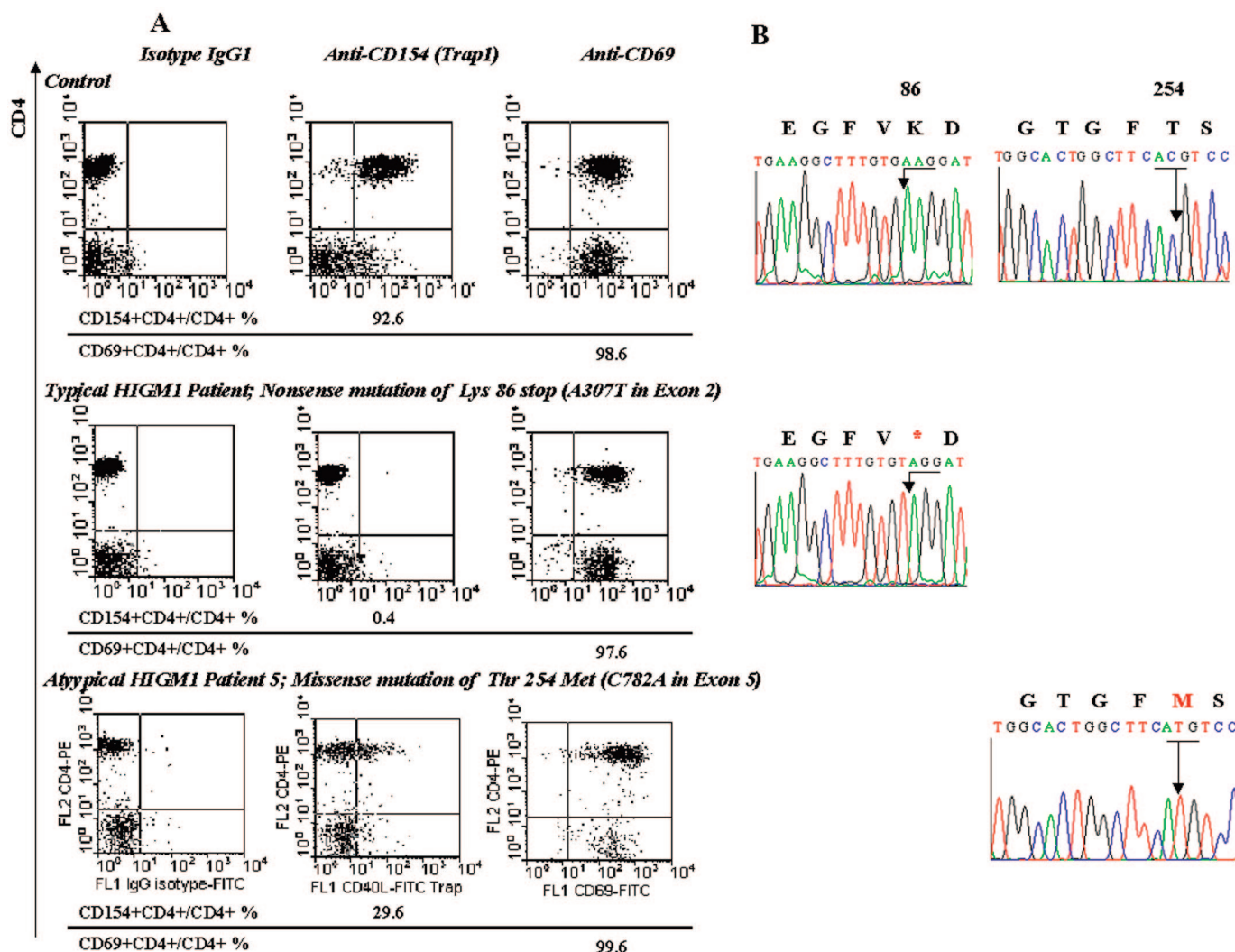


Figure 2. CD40L expression by activated CD4⁺ T cells. After stimulation with phorbol 12-myristate 13-acetate and ionomycin for 16 hours, immunostaining of cells was performed using CD40L (CD154) monoclonal antibody (mAb). CD69 served as the activation control. The immunostaining with mAb and isotype-matched control reagent were shown by flow cytometry. A typical patient with hyper-IgM syndrome (HIGM1) and pretermate (Lys86stop) CD40L (middle lane in B) lacked affinity to the mAb (middle lane in A). In contrast, an atypical HIGM1 patient (patient 5) with a missense mutation of (Thr254Met) CD40L (low lane in B) had residual 29.6% CD40L affinity to the mAb compared with that of the healthy control (low and high lanes in A). Similar results were obtained by the CD40-immunoglobulin binding capability (data not shown).

Homozygous deletion in the *BAFFR* gene was also found in a white patient with adult onset.^{22,23}

Among 4 currently identified patients with homozygous deletion in the *CD19* gene, onset ages ranged from 1 to 7 years. Three Colombian patients from the same family without consanguinity received regular intravenous immunoglobulin therapy during 3 decades when they had hypogammaglobulinemia and recurrent sinopulmonary infections.²⁵ Another Turkish patient had consanguineous parents.^{1,24,25}

Based on the update reports, adult-onset CVID seems to predominate in patients with *ICOS*, *TACI*, *BAFFR*, and *CD19* deficiency.²³ However in Taiwan, only a few CVID patients

had their conditions diagnosed in adulthood. It is possible that patients with adult-onset CVID might have been treated as having autoimmune or connective tissue diseases by rheumatologists who were not familiar with the clinical manifestations of the CVID patients, although racial variation might affect the disease patterns and the monogenetic basis of CVID.

None of the mutations of the coding regions of the *SAP*, *ICOS*, *TACI*, *BAFFR*, and *CD19* genes were found in our small population from a single institute. We cannot exclude the hypothesis that alteration or deficiency of candidate genes due to mutations in the promoter or putative regulatory intron regions could occur. However, protein

Table 4. *Btk*, *CD40L*, *ICOS*, *CD19*, *TACI*, and *BAFFR* Expression in 9 Patients With the Common Variable Immunodeficiency Phenotype^a

Patient No./sex	<i>Btk</i> (platelets)	<i>CD40L</i> (activated CD4 ⁺ lymphocytes)	<i>ICOS</i> (activated CD4 ⁺ lymphocytes)	<i>SAP/β-actin</i> (lymphocytes) ^c	MFI, % (mean ± SD) ^b				
					<i>CD19</i> ⁺ <i>TACI</i> ⁺ (PBMCs)	<i>CD19</i> ⁺ <i>BAFFR</i> ⁺ (PBMCs)	<i>CD19</i> ⁺ <i>TACI</i> ⁺ (PBMCs)	<i>CD19</i> ⁺ <i>BAFFR</i> ⁺ (EBV-LCLs)	<i>CD19</i> ⁺ (EBV-LCLs)
1/F	Not done	Not done	78.5 (38.5 ± 17.3)	Not done	9.4 (29.4 ± 28.4)	88.5 (34.9 ± 17.2)	67.5 (25.9 ± 16.2)	56.0 (35.4 ± 18.1)	98.6% (36.2 ± 18.2)
2/M	2.0% (289.9 ± 10.5) ^d	89.6 (123.8 ± 29.6)	82.5 (43.8 ± 26.8)	Not done	Not done	Not done	Not done	Not done	Not done
3/M	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done
4/F	Not done	Not done	75.3 (39.8 ± 17.5)	Not done	5.6 (25.6 ± 25.7)	88.6 (32.9 ± 18.5)	82.2 (39.4 ± 16.8)	59.3 (37.8 ± 14.5)	98.7 (39.4 ± 15.2)
5/M	87.2 (695.2 ± 34.7)	29.6 (36.3 ± 24.7) ^e	84.2 (42.8 ± 15.7)	Not done	Not done	Not done	Not done	Not done	Not done
6/F	98.0 (726.7 ± 27.4)	98.3 (158.4 ± 26.9)	72.6 (35.8 ± 19.4)	Not done	5.5 (24.9 ± 25.4)	85.1 (31.5 ± 15.8)	49.5 (19.7 ± 12.1)	59.5 (35.7 ± 16.7)	89.4 (29.7 ± 18.4)
7/M	91.8 (706.8 ± 17.4)	92.8 (156.3 ± 33.5)	85.4 (46.7 ± 18.5)	0.22–0.32	8.7 (27.6 ± 29.1)	89.3 (29.8 ± 15.4)	84.3 (25.4 ± 18.4)	39.3 (29.8 ± 15.4)	94.2 (35.4 ± 12.9)
8/M	85.4 (685.7 ± 22.9)	82.4 (127.8 ± 24.8)	77.9 (47.1 ± 14.6)	0.14–0.33	11.5 (32.5 ± 31.4)	96.0 (36.9 ± 19.1)	76.0 (23.9 ± 16.2)	46.0 (36.9 ± 19.1)	93.6 (33.9 ± 17.6)
9/M	82.4 (645.6 ± 46.8)	79.8 (126.8 ± 39.4)	75.4 (45.8 ± 21.2)	0.15–0.28	Not done	Not done	70.7 (40.7 ± 14.1)	24.8 (36.3 ± 11.5)	98.9 (37.6 ± 15.2)
Normal	84.2–98.6 (706.7 ± 38.5)	78.4–99.6 (146.3 ± 19.8)	67.7–85.6 (41.8 ± 21.8)	0.13–0.36	4.8–11.2 (24.5 ± 24.3)	77.8–98.2 (38.5 ± 26.7)	43.2–83.0 (19.3 ± 22.1)	27.8–68.2 (32.6 ± 28.4)	89.2–99.6 (39.3 ± 18.5)

Abbreviations: *BAFFR*, B-cell-activating factor of the tumor necrosis family receptor; *Btk*, Bruton tyrosine kinase; EBV-LCLs, Epstein-Barr virus transformed lymphoblast cell lines; *ICOS*, inducible costimulator molecule; MFI, mean fluorescent intensity; PBMCs, peripheral blood mononuclear cells; *SAP*, signaling lymphocyte activation molecule-associated protein; *TACI*, transmembrane activator and calcium-modulator and cytophilin ligand interactor.

^a All gene expressions were determined by flow cytometry except for *SAP/β-actin*, which was determined by Western blot.

^b Data are presented as MFI (mean ± SD) except for *SAP/β-actin*, which was presented as a ratio range.

^c Each *SAP* protein was duplicate performed in selected patients.

^d Nearly absent expression of *Btk* protein in patient 2.

^e Significantly decreased *CD40L* expression in patient 5 was detected.

Table 5. Patients With Mutations of *Btk*, *CD40L*, *ICOS*, and *TACI* Present as the Common Variable Immunodeficiency Phenotype^a

Patient No. (family No.)	Age at diagnosis, y	Genomic DNA mutation (nucleotide, nt)	Predicted effect on protein	Affected domain (exon)	IgG, mg/dL	IgA, mg/dL	IgM, mg/dL	Protein expression, %	Family history	Reference(s)
<i>Btk</i> Gene (Xq21.3) (Genebank No. NM 000061)										
4 (2)	[12]/[18]	215 G>A	Arg 28 His	PH (2)	80/370/42/319	0/48/0/20	16/18/18/25	NA	+	3
1 (1)	32	227 T>C	Leu 32 Ser	PH (2)	462	<8	<7	Absent	—	4
1 (1)	10	251–73 del (23)	Fs, stop at 40	PH (2)	28	8	11	2.7	—	4
1 (1)	14	441–2 ins 21 nt	Inframe	PH (4, 5)	40	<2	44	2.1	+	4
2 (2)	51/41	567 C>A	Cys 145 stop	TH (6)	401/556	<7/<7	15/50	NA	+	5
4 (1)	6/15/58	994 C>T	Arg 288 Try	SH2 (10)	101/108/102/429	<7/<7/<7/<7	<7	NA	+	6
1 (1)	15	1051 A>G	Arg 307 Gly	SH2 (11)	NA	NA	NA	42.2	—	4
1 (1)	23	IVS 13 (–2) a>t	Multiple truncated products	Kinase (13)	<20	<20	22	NA	—	4
2 (1)	[15]	1355 T>C	Leu 408 Pro	Kinase (14)	390/319	<7/<7	10/34	NA	+	3
1 (1)	12	1621 C>T	Gln 497 stop	Kinase (15)	190	<8	9	2.5	—	4
3 (1)	1.5/14/16	1694 A>T	Asp 521 Val	Kinase (15)	320/228/NA	16/95/NA	32/28/NA	2.0/NA/NA	+	Current report
1 (1)	27	1705 C>T	Arg 525 stop	Kinase (16)	346	16	8	8.1	—	4
5 (2)	32/[3.5]	1706 G>A	Arg 525 Glu	Kinase (16)	702/6–84	185/0	25/0–146	7.7/NA	–/+	3, 4
3 (1)	[14]	1899 A>G	Glu 589 Gly	Kinase (18)	20–60	0	9–26	NA	+	3
2 (2)	16/28	1924 T>G	Tyr 598 Asp	Kinase (18)	NA	NA	NA	4.7/ 9.3	+	4
1 (1)	24	1942–3 del AG	Fs, stop at 604	Kinase (18)	NA	0	1	36.1	+	4
2 (1)	[17]	1970 G>A	Gly 613 Asp	Kinase (18)	480/425	17/60	9/137	NA	+	3
1 (1)	16	2004–6 del GCA	Inframe	Kinase (19)	24	0	20	6.3	+	4
2 (1)	4/31	2008–40 del (33)	Inframe	Kinase (19)	397/527	<5/8	40/30	5.1/ 14.5	+	4
1 (1)	10	2061 del T	Fs, stop at 648	Kinase (19)	NA	16	13	47.0	—	4
<i>CD40L</i> Gene (Xq26.3–27) (Genebank No. NM 000074)										
1 (1)	8	52 C>T	Arg 11 stop	IC (1)	160	22	285	18	—	10,11
1 (1)	17	IVS2 + 2 t>a	E2 skip inframe del 44 aa Ins 19 bp (5' part of I2), Fs, stop at 118 wild-type cDNA	EC (2)	NA	NA	NA	14	—	10, 11
1 (1)	5	367 G>A	E3 skip (del 310–67) Fs, stop at 108 wild-type cDNA	EC (5)	267	71	518	NA	—	10, 11
3 (3)	3.5/15/24	782 C>T	Thr254Met	EC (5)	254/214/386	25/7/8	254/383/ 457	23	—	10, 11
1 (1)	22	782 C>T	Thr254Met	EC (5)	48	83	109	29.6	—	Current report
<i>SAP</i> Gene (Xq25) (Genebank No. NM 002351)										
1 (1)	14	415 G>T	Gly 39 Val	(1)	51/903	6	4	Absent	—	12
6 (1)	0.5–15	462 C>T	Arg 55 stop	(2)	55–779	0–253	10–114	NA	+	13
1 (1)	8	IVS3–28–32 del	Unstable mRNA	(3)	83/750	6	4	Absent	—	12
4 (1)	1–3	del 8 nt (548–55)	fs, stop at 100	(3)	90/120	0/3	4/5	NA	+	13
1 (1)	9	Not found	Not found		115/980	6	4	Absent	—	12
<i>ICOS</i> Gene (2q33) (Genebank No. AJ 277832)										
4 (2)	19–28	del 1815 bp	Del 443 bp, stop at 595 nt (only 28 aa left)	EC, TM, IC (1–4)	190/440/170/ 100	30/40/30/10	20/20/20/ 140	Absent	+	18
5 (2)	{20/18} {15/4/3}	del 1815 bp	Del 443 bp, stop at 595 nt (only 28 aa left)	EC, TM, IC (1–4)	{101/57} {255/213/54}	6/6/25/58/20	38/180/26/57/ 43	Absent	+	19
<i>TACI</i> Gene (17p11.2) (Genebank No. NM 012452)										
3 (1)	17/51/{18}a	310 T>C	Cys 104 Arg (homo)	EC (3)	662/497/420	13/38	105/153	Absent	{+}/–	20, 21
5 (3)	{52/50/ 22}b/65/ 73	310 T>C	Cys 104 Arg (heter)	EC (3)	630/954/543/ 240/40	NA	NA	NA	{+}/–	20
1 (1)	41	310 T>C; 204 ins A	Cys 104 Arg, Fs, stop at 80 (heter)	EC (3)	349	9	82	NA	—	21
2 (1)	68/63		Ser 144 stop (homo)	EC (3)	410/200	NA	NA	Absent	+	20
1 (2)	41/74	542 C>A	Ala 181 Glu (homo)	TM (4)	381/100	12/NA	62/NA	N/N	—	20, 21

Continued

Table 5. (Continued)

Patient No. (family No.)	Age at diagnosis, y	Genomic DNA mutation (nucleotide, nt)	Predicted effect on protein	Affected domain (exon)	IgG, mg/dL	IgA, mg/dL	IgM, mg/dL	Protein expression, %	Family history	Reference(s)
8 (6)	{79/46/43}/31/34/41/41/65	542 C>A	Ala 181 Glu (heter)	TM (4)	NA/33/1870/250/70/230/560/50	NA	NA	NA	{+}/–	20
1 (1)	37	581 C>A	Ser 194 stop (heter)	TM (4)	150	NA	NA		–	20
1 (1)	8	602 G>A	Arg 202 His (homo)	IC (4)	498	32	133	N	–	21
1 (1)	8	602 G>A	Arg 202 His (homo)	IC (4)	498	32	133	N	–	21
1 (1)	33	602 G>A	Arg 202 His (heter)	IC (4)	2520	NA	NA		–	20
BAFFR Gene (22q13.2) (Genebank No. NM 052945)										
1 (1)	60	Del 24	Unreleased	TM (2)	600	530	600	Absent	–	1, 22, 23, 26
CD19 Gene (16p11.2) (Genebank No. NM 001770)										
1 (1)	10	972 ins A	Fs, stop at 328	TM (6)	325	292	25	Absent	–	25
3 (1)	35/33/49	Del 1384 GA	Fs, stop at 465	IC (11)	204/198/256	18/7/19	47/30/63	Absent	+	23–26

Abbreviations: *BAFFR*, B-cell-activating factor of the tumor necrosis family receptor; *Btk*, Bruton tyrosine kinase; cDNA, complementary DNA; Del, deletion; EC, extracellular; Fs, frameshift; IC, intracytoplasmic; *ICOS*, inducible costimulator molecule; N, normal; NA, not available; PH, pelckstrin homology; *SAP*, signaling lymphocyte activation molecule-associated protein; SH, Src homology; *TACI*, transmembrane activator and calcium-modulator and cytophilin ligand interactor; TH, Tec homology; TM, transmembrane.

Symbols: –, negative; +, positive.

^a Data in brackets ([]) indicates index cases. Data in braces ({} means the same family; a and b were from the same family. Absent means below 2% expression. The expressions of candidate proteins were evaluated by flow cytometry (*Btk*, *CD40L*, *ICOS*, *TACI*, *BAFFR*, and *CD19*) or Western blot (*SAP*). To date, mutations of *BAFFR* were found in 4 patients.²² Clinical presentations of 1 patient with mutation of *BAFFR* from 3 patients (from the same family) have been partly released.^{22,26}

expressions translated from these candidate genes, significantly and equally detectable by flow cytometry or Western blot, conflict with this hypothesis. We constructed a comprehensive molecular and genetic approach to screen a larger number of CVID patients in the near future. Identifying the molecular and genetic basis for CVID will help more clearly define its clinical course. We suggest that mutations of *Btk*, *CD40L*, and *SAP* in males and *ICOS*, *TACI*, *BAFFR*, and *CD19* in both males and females should be considered in CVID patients, although mutations of the *ICOS*, *TACI*, *BAFFR*, and *CD19* genes occur in less than 15% of CVID patients.^{1,23}

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