ORIGINAL ARTICLE

Evaluation of *CARMA1/CARD11* and *Bob1* as Candidate Genes in Common Variable Immunodeficiency

G Tampella,* M Baronio,* M Vitali, A Soresina, R Badolato, S Giliani, A Plebani, V Lougaris

Department of Pediatrics and Institute of Molecular Medicine "A. Nocivelli", University of Brescia, Brescia, Italy

*These authors contributed equally to this paper.

Abstract

Background and Objective: The candidate gene approach has led to the detection of associations between common variable immunodeficiency (CVID) and mutations in the genes *TACI, ICOS, BAFF-R, CD19, CD20*, and *CD81*. Such mutations are present in less than 15% of cases, highlighting the complexity of the disease. Animal models for 2 genes involved in B-cell development, namely *CARMA1/CARD11* and Bob1, develop an immunological phenotype similar to that seen in CVID, with low immunoglobulin serum levels, defective responses to antigen, and defective B-cell activation.
The aim of this study was to evaluate *CARMA1/CARD11* and *Bob1* as candidate genes for the pathogenesis of CVID in a cohort of 66 patients with the disease.
Patients and Methods: We performed direct gene sequencing of *CARMA1/CARD11* and *Bob1* in 66 patients with CVID.
Results: Seven already reported genetic variants and 4 novel ones were found in the *CARMA1/CARD11* gene, while 1 already reported variant and 1 novel variant were found in the *Bob1* gene.
Conclusions: Although novel genetic variants were identified in both the *CARMA1/CARD11* and the *Bob1* gene, no disease-causing mutations were identified in our group of patients. However, 4 of the variants in *CARMA1* and 1 of those in *Bob1* were associated with the disease. Considering the heterogeneity and complexity of CVID, further studies are needed to better define the genetic mechanisms involved in the pathogenesis of the disease.

Key words: B cells. Common Variable Immunodeficiency. CVID. Candidate gene approach. Hypogammaglobulinemia.

Resumen

Antecedentes y objetivo: El método del gen candidato ha permitido detectar asociaciones entre la inmunodeficiencia común variable (IDCV) y las mutaciones en los genes *TACI, ICOS, BAFF-R, CD19, CD20* y *CD81*. Estas mutaciones están presentes en menos del 15% de los casos, lo que pone de manifiesto la complejidad de la enfermedad. Los modelos animales para 2 genes implicados en el desarrollo de linfocitos B, *CARMA1/CARD11* y *Bob1*, desarrollan un fenotipo inmunológico parecido al observado en la IDCV, con niveles séricos bajos de inmunoglobulina, respuestas deficientes frente a antígenos y activación defectuosa de linfocitos B.
El objetivo de este estudio fue evaluar los genes *CARMA1/CARD11* y *Bob1* como genes candidatos para la patogenia de la IDCV en una cohorte de 66 pacientes con esta enfermedad.
Pacientes y métodos: Se realizó una secuenciación directa de los genes *CARMA1/CARD11* y *Bob1* en 66 pacientes con IDCV.
Resultados: Para el gen *CARMA1/CARD11* se hallaron 7 variantes genéticas ya notificadas y 4 variantes nuevas, mientras que para el gen *Bob1* se hallaron 1 variante ya notificada y 1 variante nueva.
Conclusiones: A pesar de haber identificado nuevas variantes genéticas en ambos genes *CARMA1/CARD11* y *Bob1*, no se identificaron mutaciones causantes de enfermedad en nuestro grupo de pacientes. Sin embargo, 4 de las variantes del gen *CARMA1* y 1 del gen *Bob1* se asociaron a la enfermedad. Teniendo en cuenta la heterogeneidad y complejidad de la IDCV, se requieren más estudios para definir mejor los mecanismos genéticos implicados en la patogenia de la enfermedad.

Palabras clave: Linfocitos B. Inmunodeficiencia común variable. IDCV. Método del gen candidato. Hipogammaglobulinemia.

Introduction

Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency disorder [1-4]. It is characterized by low serum immunoglobulin levels, which result in recurrent infections of the respiratory and gastrointestinal tract. The clinical history of patients with CVID may be further complicated by autoimmune phenomena, splenomegaly, and lymphomas [2,3,5]. Although the first case of CVID was described in 1953 [6], the identification of underlying genetic causes required a further 50 years. In fact, the first genetic defect in the *ICOS* gene was identified in 2004 in a small number of adult patients with CVID [7]. Using the candidate gene approach, at least 5 more mutated genes in CVID (*TACI*, *BAFF-R*, *CD19*, *CD20*, and *CD81*) have been identified since [8-13]. The identification of these genetic defects has contributed greatly to a better understanding of the disease. Nonetheless, mutations in the above-mentioned genes are found in less than 15% of CVID patients, strongly suggesting that other, not yet identified genes are involved in the pathogenesis of CVID.

In recent years, knock-out animal models for specific genes have been widely used to gain better understanding of the function of selected gene products. Such an approach has led to, among other things, the identification of the above-mentioned genetic defects in CVID. *CARMA1* (originally called *CARD11*) is a membrane-associated guanylate kinase family member that is required for T cell receptor (TCR)- and B cell receptor (BCR)-induced NF-kappa B activation [14]. *CARMA1* uses its N-terminal caspase activation and recruitment domain (CARD) to interact with the CARD in the downstream adaptor Bcl-10. Deletion of the entire *CARMA1* gene in mice has resulted in impaired T and B cell NF-kappa B activation associated with low immunoglobulin serum levels of all isotypes [15]. Deletion of the corresponding CARD domain in mice has also been found to result in altered B-cell development and severely compromised B-cell proliferation in response to both BCR and CD40 ligation [16]. In the same study, serum immunoglobulin levels were markedly reduced in the mutant mice.

Bob1 is a B cell–specific transcriptional co-activator which can stimulate transcription by interaction with Oct-1 or Oct-2 and the octamer sequence found in the promoter region and most of the enhancer regions of immunoglobulin genes. The role played by *Bob1* in B-cell maturation and function has been investigated in mice deficient for this gene [17-18], with results showing reduced numbers of mature B cells and severely reduced numbers of recirculating B cells. In addition, immunization with T-dependent or T-independent antigens has yielded reduced immune responses. In the absence of *Bob1*, germinal centers completely fail to develop after immunization with thymus-dependent antigen [17]. These mice are characterized by a reduction in mature B cells and serum titers of the immunoglobulin isotypes IgG1, IgG2a, IgG2b, and IgA [18].

The knock-out animal models for *CARMA1/CARD11* and *Bob1* both present significant similarities with the immunological phenotype of CVID, where hypogammaglobulinemia is associated with defects in B-cell maturation and activation. We therefore decided to evaluate these 2 genes for disease-associated mutations by direct gene sequencing in a large cohort of patients with CVID.

Materials and Methods

Patients and Methods

Sixty-six patients with CVID were included in the study. Diagnosis of CVID was made according to the criteria of the European Society for Immunodeficiencies (www.esid.org). All the patients are receiving regular immunoglobulin replacement therapy and under regular follow-up at the outpatient clinic of the Paediatric Immunology and Rheumatology Unit at the University of Brescia in Italy. Informed consent was obtained, from patients or patients' parents in the case of children, in all cases.

Primers, Polymerase Chain Reaction, and Genetic Sequencing

Primers were designed to include the flanking regions of the 23 exons encoding the *CARMA1/CARD11* gene and the 5 exons of *Bob1* (available upon request). Polymerase chain reaction products were then purified and direct gene sequencing of all exons of the *CARMA1/CARD11* and the *Bob1* gene was performed using an ABI PRISM 310 sequencer (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA). Sequences were analyzed using the Sequence Navigator software.

Antibodies

The primary antibodies used in this study were goat polyclonal anti-Bcl-10 (C-17), goat polyclonal anti-CARMA1 (L-20), goat polyclonal anti-MALT1 (C-16) (all from Santa Cruz Biotechnology, Heidelberg, Germany), and rabbit polyclonal anti-Actin (20-33) antibody (from Sigma Aldrich, St. Louis, Missouri,USA). Horseradish peroxidase-coupled donkey anti-goat or goat anti-rabbit antibodies were from Santa Cruz Biotechnology.

Cell Lysis, Immunoprecipitation, Electrophoresis, and Western Blotting

The cell pellets were lysed in ice-cold NP-40 lysis buffer made with 150 nM NaCl, 20 mM Tris-HCl pH 7.4, 1% NP-40, 10% glycerol, 2mM EDTA additioned with Complete Mini EDTA-Free Protease Inhibitor Cocktail (Roche, Milan, Italy), 10 mM sodium fluoride, and 20 μM sodium vanadate. After 30 minutes of incubation on ice, the pellets were centrifuged for 15 minutes at 13 000g, and the supernatant total protein extract was used for protein electrophoresis or immunoprecipitation experiments.

The immunoprecipitates were prepared by incubating 50 μL of magnetic DynabeadsR Protein G (Life Technologies, Carlsbad, California, USA) with 5 μg of anti-CARMA1 primary antibody for 30 minutes in agitation. The complexes were then incubated with 300 μg of total antigen for 30 minutes and immunoprecipitated through magnetic attraction. The immunoprecipitate was washed 3 times with washing buffer and eluted in 40 μL elution buffer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed after SDS sample buffer treatment and denaturation of immunoprecipitates and cell lysates at 95°C for 5 minutes. Proteins were electrotransferred onto a PVDF membrane (Amersham Biosciences, GE Europe GmbH, Munich, Germany). For immunodetection, the presence of the primary antibody was detected by secondary horseradish peroxidase conjugated antibodies using the enhanced chemiluminescence technique (LiteAblotR EXTEND, Euroclone, Milan, Italy).

Statistical Analysis

Allele frequencies of the genetic variants in CARMA1 and Bob1 in both patients with CVID and healthy controls were evaluated with the Fischer exact test and the χ^2 test.

Results

CARMA1/CARD11 Analysis

The direct genetic analysis of the *CARMA1/CARD11* gene in the 66 patients studied yielded interesting results, with the identification of 7 already reported single nucleotide polymorphisms (SNPs) and 4 novel ones (Figure 1).

Regarding the already reported genetic variants (Table 1), the silent G>C substitution (R68R) (rs10229368) in exon 2 was found in 3 patients in the heterozygous state. The comparison with database frequencies evidenced the presence of the minor allele in a small number of CVID patients (G:1/C:0 vs G:0.977/C:0.023, *P*<.001). The silent genetic variation in exon 7, C>T (D408D) (rs6945582) was found in 20 patients, 3 of whom presented the mutation in the homozygous state. Allele frequencies were similarly distributed in healthy controls and patients (C:0.8/T:0.2 vs C:0.83/T0:17, *P*=.585). In exon 10, the variation C>T, which results in a silent mutation (D526D) (rs1621509), was found in 27 patients, 3 of whom had the variation in the homozygous state. This variant was more common in patients than in controls (C:0.857/T:0.143 vs C:0.74/T:0.26, *P*<.001). The silent G>C substitution in exon 15 (T741T) (rs3735131) was found in 6 patients in the heterozygous state. The comparison with database frequencies showed a more frequent presence of the minor allele in CVID patients compared to healthy controls (G:0.955/C:0.045 vs G:0.991/C:0.009, *P*<.001). The silent A>G substitution in exon 18 (P867P) (rs3735124) was found in 6 CVID patients in the heterozygous state. Similarly to the genetic variant rs1621509, this variant was more common in patients than in controls (A:0.71/G:0.29 vs A:0.842/G:0.158, *P*<.001). Finally, the majority of CVID patients included in this study presented the silent nucleotide substitution A>G in exon 23 (R1085R) (rs1124581); 1 of the alleles was mutated in 31 patients while both were mutated in 19 patients. A similar distribution was observed between healthy controls and patients (A:0.440/G:0.560 vs A:0.48/G:0.52, *P*=.570).

Regarding the novel genetic variants, 4 nucleotide substitutions that have not yet been described were identified (Figure 1 and Table 2). One of these was a synonymous variant C>T, I222I in exon 4, found in the heterozygous state in 1 patient. A second novel variant was identified in exon 4, namely a C>G mutation resulting in an amino acid change from leucine to valine in

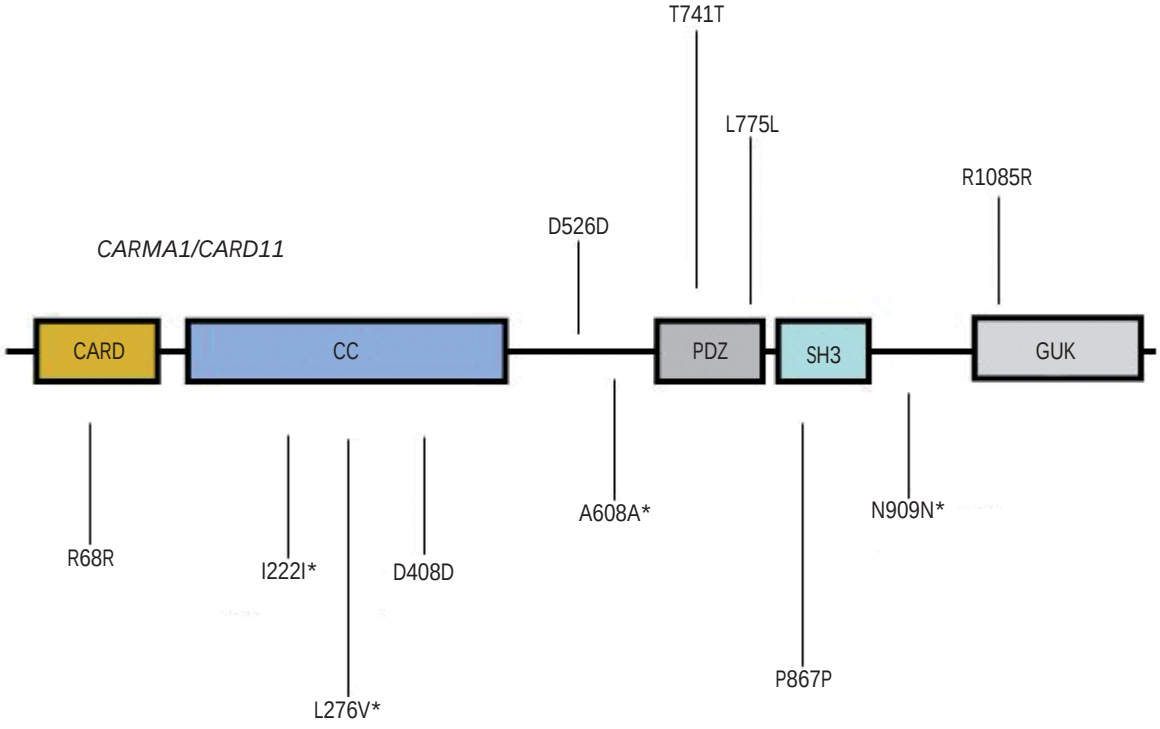

Figure 1. Mutations of *CARMA1* in Common Variable Immunodeficiency (CVID). Genetic variants identified in the *CARMA1/CARD11* gene in 66 patients with CVID. The asterisk (*) indicates genetic variants not previously identified.

Table 1. Allele Frequencies of Previously Reported Genetic Variants of the *CARMA1/CARD11* and *Bob1* Genes

CARMA1/CARD11

SNP	Mutation Effect	Allele Frequency		*P* (χ^2 Test)	*P* (Fisher Test)
		Healthy Controls	Patients With CVID		
rs10229368	R68R	G:0.977/C:0.023	G:1.000/C:0.000	<.001	<.001
rs6945582	D408D	C:0.800/T:0.200	C:0.830/T:0.170	.585	.358
rs1621509	D526D	C:0.857/T:0.143	C:0.740/T:0.260	<.001	<.001
rs3735131	T741T	G:0.991/C:0.009	G:0.955/C:0.045	<.001	<.001
rs3735124	P867P	A:0.842/G:0.158	A:0.710/G:0.158	<.001	<.001

Bob 1

SNP	Mutation Effect	Allele Frequency		*P* (χ^2 Test)	*P* (Fisher Test)
		Healthy Controls	Patients With CVID		
rs11213848	D243D	C:0.980/T:0.020	C:0.967/T:0.033	<.001	<.001

Abbreviations: CVID, common variable immunodeficiency; SNP, single nucleotide polymorphism.

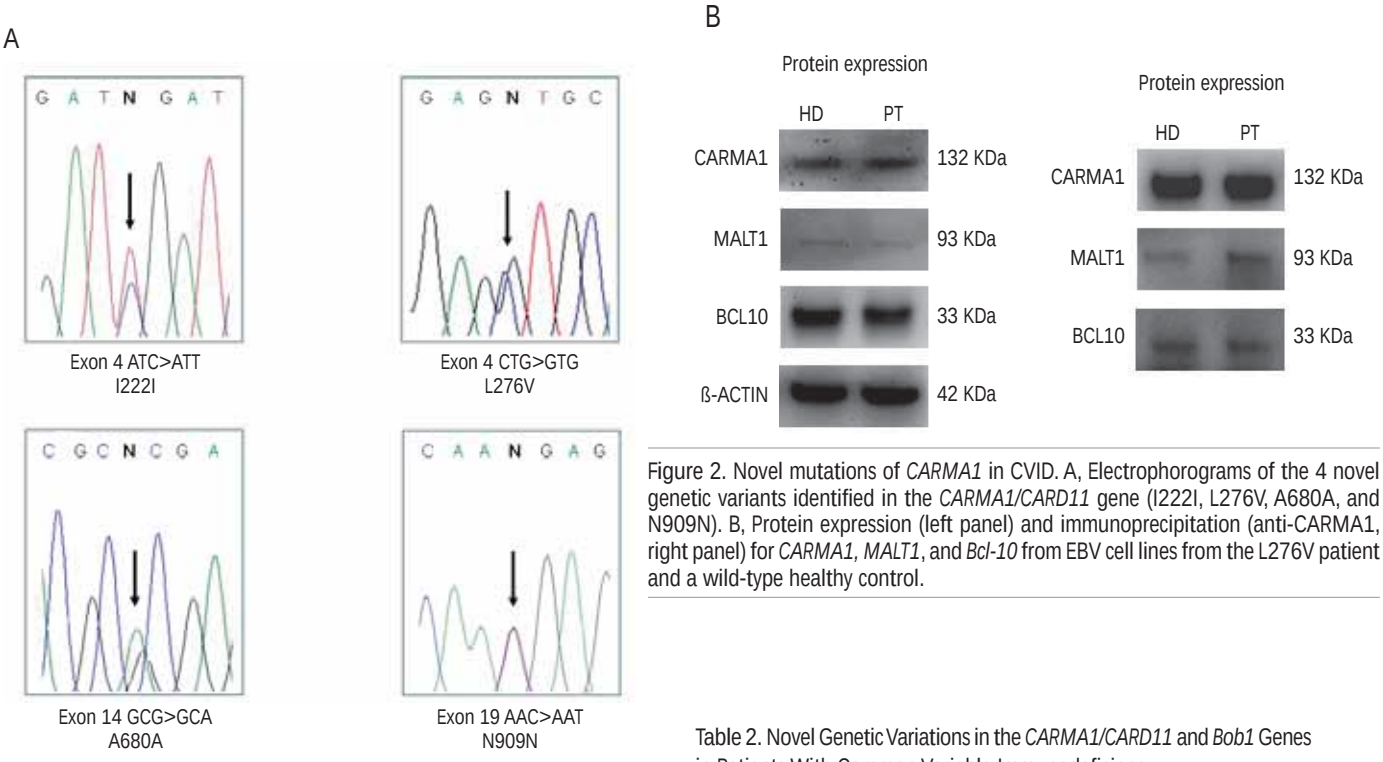

Figure 2. Novel mutations of *CARMA1* in CVID. A, Electrophorograms of the 4 novel genetic variants identified in the *CARMA1/CARD11* gene (I222I, L276V, A680A, and N909N). B, Protein expression (left panel) and immunoprecipitation (anti-CARMA1, right panel) for *CARMA1*, *MALT1*, and *Bcl-10* from EBV cell lines from the L276V patient and a wild-type healthy control.

position 276 (C>G, L276V). The same mutation was found in a healthy parent of the same patient. The third novel variant was identified in exon 14: a G>A substitution that does not cause amino acid changes (A680A). Finally, the fourth novel genetic variant was identified in exon 19: a C>T substitution that does not cause amino acid changes (N909N).Considering that 3 of the 4 novel genetic variants were synonymous, we decided to focus our attention on the missense L276V mutation found in 1 patient. We therefore performed protein expression experiments on EBV cell lines, and found that the protein was normally expressed in the presence of 1 mutated

Table 2. Novel Genetic Variations in the *CARMA1/CARD11* and *Bob1* Genes in Patients With Common Variable Immunodeficiency

Novel Mutations	Heterozygous	Homozygous
CARMA1		
C>T, I222I	1	–
G>C, L276V	1	–
G>A, A680A	1	–
C>T, N909N	6	–
Bob1		
G>A, Q171Q	1	–

allele (Figure 2B). Since *CARMA1/CARD11* interacts with bcl10 and MALT1 in the CBM complex [19], we investigated whether this genetic variation might influence this interaction. Immunoprecipitation experiments using EBV cell lines showed that interactions within the CBM complex remained intact in the presence of the L276V mutation (Figure 2B).

Bob1 Analysis

The direct genetic analysis of the *Bob1* gene in 66 patients with CVID also yielded interesting results. Three patients were found to carry the synonymous SNP (documented on Pubmed as rs11213848) consisting of a C>T substitution in the heterozygous state (D243D) (Figure 3A). The allele frequency analysis showed that the minor allele was significantly more common in patients than in controls (C:0.980/T:0.020 vs C:0.967/T:0.033, *P*<.001) (Table 1). A novel synonymous SNP (not previously reported) consisting of a G>A substitution (Q171Q) was identified in 1 patient in the heterozygous state (Figure 3B).

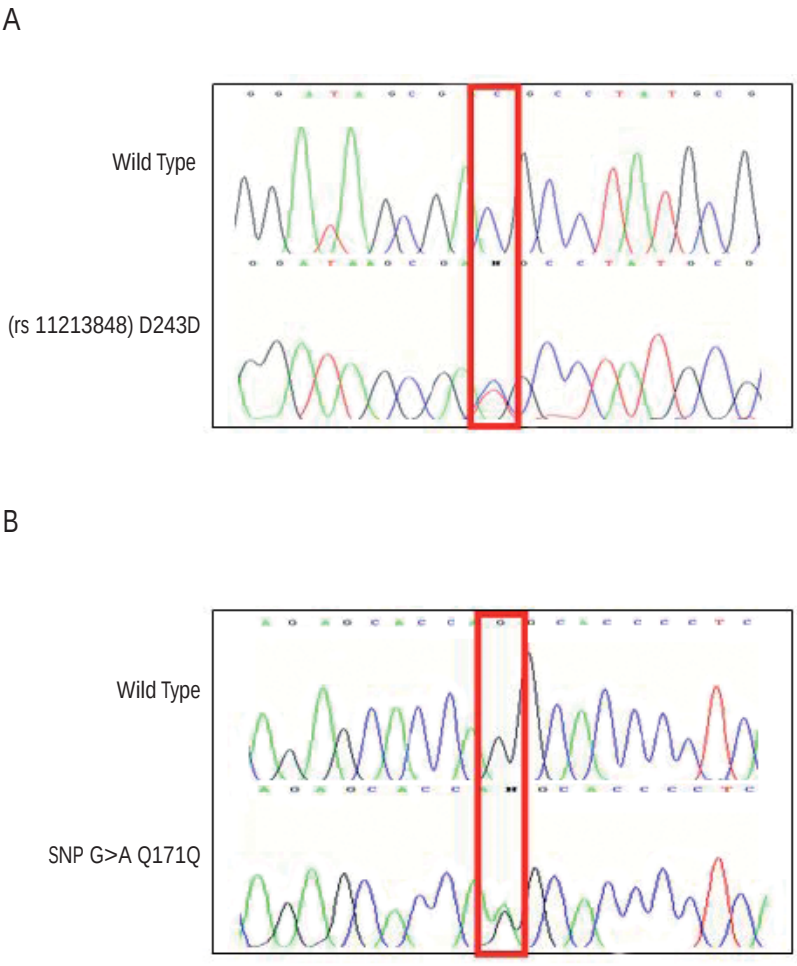

Figure 3. Mutations of *Bob1* in Common Variable Immunodeficiency (CVID). A, Electrophorogram of the already reported mutation rs11213848 (D243D). B, The novel synonymous genetic variant in *Bob1*.

Discussion

CVID is the most common symptomatic primary immunodeficiency disorder. Although it was described more than 5 decades ago, underlying genetic mechanisms were only recently identified in a small number of patients. The candidate gene approach based on knowledge derived from single gene knock-out animal models has been very helpful in gaining insight into these mechanisms. Nonetheless, mutations in the genes encoding for ICOS, TACI, BAFF-R, CD19, CD20, and CD81 account for less than 15% of cases of CVID [8-13]. The remaining 85% of patients do not have a definitive genetic diagnosis, and it is likely that genes besides the ones already mentioned may be involved in the pathogenesis of the disease.

CVID is characterized by low immunoglobulin serum levels and variable degrees of defects in B-cell function (majority of cases) and T-cell function (minority of cases). An important gene for Nf-kB activation after TCR or BCR cross-linking is *CARMA1/CARD11*. The CARMA1/CARD11 protein interacts with other proteins such as bcl10 and MALT1 in the CBM complex and mediates T- and B-cell activation [14]. Interestingly, different groups have been able to study the importance of *CARMA1/CARD11* by deleting either the entire gene [15] or just the CARD domain [16]. In both cases, the animal models showed variable degrees of defective B- and T-cell activation, always accompanied by low immunoglobulin serum levels.

B-cell activation and differentiation depends largely on the correct function of diverse transcription factors, including *Bob1*. Mice lacking *Bob1* have been shown to have reduced numbers of mature B cells and a severe reduction in the number of recirculating B cells associated with hypogammaglobulinemia, defective immune responses to antigens, and impaired germinal center formation after immunization [17,18]. The immunological phenotype of the knock-out animal models for *CARMA1/CARD11* and *Bob1* is similar to the one usually observed in CVID. We therefore decided to investigate whether mutations in *CARMA1/CARD11* may be associated with CVID.

The genetic analysis of 66 patients with CVID evidenced 7 known SNPs and 4 novel ones in *CARMA1/CARD11*. The previously reported genetic variants were all synonymous. However, analysis of the allele frequencies of the 4 novel *CARMA1/CARD11* variants (rs10229368, rs1621509, rs3735131 and rs3735124) and the 1 novel *Bob1* variant (rs11213848) showed that the minor alleles were more common in patients than in controls. As previously mentioned, none of these variants cause amino acid changes, although it cannot be excluded that post-translational modifications, such as methylation, may be disturbed. Considering the presence of these variants in healthy controls as well, a larger number of

patients and healthy controls should be evaluated to confirm these findings and better define the significance, if any, of these silent mutations. Regarding the novel genetic variants, 3 were synonymous and 1 led to the amino acid change leucine to valine in the heterozygous state. The same genetic variant was present in a healthy member of the patient's family, suggesting the benign nature of this variation. In order to exclude a low-penetrance trait, we investigated the levels of protein expression for *Bob1* and the interaction in the CBM complex between MALT1-CARMA1-Bcl10. Both the expression levels and protein-protein interactions were found to be normal. *Bob1*, in contrast, appeared to be less heterogeneous. One of the previously reported synonymous genetic variants was found in a limited number of CVID patients, with the same allele frequencies as in healthy controls, and a novel synonymous variation consisting of a G>A substitution (Q171Q) was identified in 1 patient in the heterozygous state.

In conclusion, the genetic analysis of the *CARMA1/CARD11* and *Bob1* genes in CVID did not evidence any disease-causing mutations, at least in our cohort of patients. Four genetic variants of *CARMA1/CARD11*, a highly polymorphic gene, and 1 variant in *Bob1* appear to be associated with the disease. Further studies with larger numbers of patients are needed to further verify the findings of this study.

Acknowledgments

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement no 201549. (EURO-PADnet HEALTH-F2-2008-201549). It has also received funding from the Fondazione C. Golgi in Brescia, Italy. We would like to thank the patients, the patients' families, and the nurses for all their efforts.

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Manuscript received August 16, 2010; accepted for publication, December 29, 2010.

Vassilios Lougaris

Department of Pediatrics and Institute of Molecular Medicine "A. Nocivelli"
University of Brescia
Piazzale Spedali Civili 1
25123, Brescia, Italy