CNR2 functional variant (Q63R) influences childhood immune thrombocytopenic purpura

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ABSTRACT

Immune thrombocytopenic purpura is an acquired autoimmune disorder that is the most common cause of thrombocytopenia in children. The endocannabinoid system is involved in immune regulation. We evaluated a common missense variant (CAA/CGG; Q63R) of the gene encoding the cannabinoid receptor *type 2* (GeneID 1269) in 190 children with immune thrombocytopenic purpura and 600 healthy controls. The allelic frequencies and genotype distribution of the polymorphism in the patients were significant compared to control samples (P=0.006 and P=0.0001, respectively). Interestingly, when acute and chronic immune thrombocytopenic purpura patients were analyzed separately with respect to controls, a significant overrepresentation of the RR genotype and of the R allele was observed only for the chronic form (P=0.00021 and P=0.011, respectively). The relative odds ratio suggested the risk of developing chronic form was more than double in immune thrombocytopenic purpura children homozygous for the variant (odds ratio=2.349, 95% CI: 1.544-3.573; P<0.001).

Key words: immune thrombocytopenic purpura, children, endocannabinoid system, cannabinoid receptor, CB2.

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Introduction

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by thrombocytopenia (peripheral blood platelet count < 150×10^9/L) due to autoantibody binding to platelet antigen(s) causing their premature destruction by the reticuloendothelial system, particularly in the spleen.[1] ITP diagnosis is based on low platelet number in the absence of other hematologic abnormalities or other causes of thrombocytopenia.[2] The annual incidence of pediatric ITP is about 4 to 6 cases per 100,000. About 50% of childhood ITP cases show an acute onset following a viral or bacterial infection that commonly resolves within weeks to months without treatment. Nevertheless, about one fourth of these patients go on to develop a chronic disease, defined by a platelet count less than 150×10^9/L at six months after diagnosis.[3,4]

Although the immunopathogenesis of ITP is autoantibody-mediated, the exact mechanism of immune dysfunction is not known. However, there is substantial evidence to suggest that T cells and their cytokines play a pivotal role in the control of antiplatelet autoantibodies.[5,6] A number of T-cell abnormalities have been demonstrated in patients with ITP and three main mechanisms have been hypothesized: i) a T-helper (Th)1 bias compared with Th2, particularly in chronic ITP; ii) the release of cytokines that interfere with megakaryocyte maturation and/or platelet release; and iii) a direct cytotoxic effect of T cells.[7]

T cells, as well as all other cellular components of the immune system, express cannabinoid receptors *type 1* and *2* (CB1 and CB2). The endocannabinoid system is also involved in immune regulation by suppressing cell activation, modulating Th1 and Th2 balance, and inhibiting pro-inflammatory cytokine production.[8-10] CB2 is encoded by the *CNR2* gene, mapping on 1p36.11 (GeneID 1269; GenBank: #NM_001841.2), and is expressed at 10- to 100-fold greater levels than the CB1 on immune cells, including T lymphocytes, B cells, macrophages and neutrophils.[10] Genome scan studies revealed a key role of the 1p36 region in different autoimmune diseases, such as rheumatoid arthritis,[11] systemic lupus erythematosus,[12] and type 1 diabetes.[13]

In this study, we show the *CNR2* gene variation rs35761398 (Q63R) is significantly associated with childhood chronic ITP.

Design and Methods

Patients

The study included 190 (99 females) unrelated Italian children (median age 7 years; range 0.3-15.5) with ITP referred from March 1995 to December 2009 to the Department of Pediatrics of the Second University of Naples. Diagnosis and treatment of ITP were made according to the guidelines of the American Society of Hematology (ASH)[14] and the Italian Association of Pediatric Hematology and Oncology (AIEOP).[15,16] Thrombocytopenia that resolved within six months of onset was defined as acute ITP; while persistence of throm-

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bocytopenia for longer than six months was defined as chronic ITP. Six hundred healthy Italian children (325 female, median age 9.7 years; range 1.8–13.8) served as controls. Controls, matched for age and sex, were recruited from the same geographical areas as ITP patients. These controls did not have a history of hematologic disorders of any kind. Clinical data are summarized in Table 1.

The study was approved by the Medical Ethics Committee of the Second University of Naples and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients' parents prior to participation in the study.

Molecular study

Genomic DNA was extracted from peripheral whole blood. The *CNR2* rs35761398 polymorphism was studied by polymerase chain reaction (PCR) followed by direct sequencing. The PCR conditions were: 94° C for 4 min followed by 31 cycles consisting of 94° C for 30 s, 60° C for 30 s, and 72° C for 30 s. Primers were chosen using *Primer3* software (Forward: 5'-GAGTGGTCCCCAGAAGACAG-3'; Reverse: 5'-CACAGAGGCTGTGAAGGTCA-3'). Amplimers were analyzed by direct sequencing using an ABI PRISM 310 automated sequencer (Applied Biosystem, Foster City, CA, USA) and the relative genotypes were assigned. The χ^2 test was used to assess differences in genotype and allelic frequencies. The odds ratio for genotype distributions was calculated using SAS software. *P* values less than 0.05 were considered significant.

Results and Discussion

In total, 190 ITP children were evaluated, divided into acute (n=86, female 45%) and chronic (n=104, female 58%) ITP (Table 1). The number of patients recruited with chronic ITP was greater because the hospital where the study was carried out is the childhood ITP referral center for the Campania region of southern Italy. All patients were genotyped for the *CNR2* rs35761398 variant, which changes the second and third adenosine at codon 63 (CAA) to guanosine (CGG) leading to the missense variant Q63R in the first intracellular signaling loop of the encoded CB2 protein. The allelic frequencies and the genotype distributions in controls and ITP patients are shown in Table 2. Whereas the allele frequencies in the controls were distributed according to Hardy-Weinberg (*P*=0.318) and were comparable to previously reported distributions,[17] this was not the case in the ITP patients ($P=7.069\times10^{-3}$). Indeed, there were significant differences in allelic frequencies and genotype distribution in ITP patients compared to control samples (*P*=0.006 and *P*=0.0001, respectively) (Table 2). In addition, the relative odds ratio (OR) suggested a double risk of developing ITP in RR homozygous children with respect to QR heterozygous and QQ homozygous children (OR=2.006 95% CI 1.441-2.795; $P<10^{-3}$) (Table 3).

Interestingly, when acute and chronic ITP patients were analyzed separately in comparison with controls, a significant overrepresentation of the RR genotype and of the R allele was observed only for the chronic form (*P*=0.00021 and *P*=0.011, respectively) (Table 2). Furthermore, the associated risk of developing chronic ITP increased more than two-fold for RR homozygous children (OR=2.349 95% CI 1.544-3.573; $P<10^{-3}$) (Table 3). The genotype and allele distribution in acute ITP patients was comparable to the control samples (Table 2). Patient's sex, the presence of autoimmune diseases, and platelet-associated antibodies were not significantly influenced by the *CNR2* rs35761398 variant (Table 2).

This case-control association study aimed to explore the molecular determinants that influence the susceptibility to ITP in childhood. For the first time, we showed an association between ITP and a functional variant of the *CNR2* gene, encoding for a protein known to affect immune function. The rationale for our study was based on: i) the linkage between the 1p36 locus, where *CNR2* maps, and sever-

Table 1. Clinical findings of 190 ITP children.

	ITP patients (n)	Controls (n)
Subjects	190	600
Female (%)	99 (52)	324 (54)
Median age (years, range)	7 (0.3-15.5)	9.7 (1.8–13.8)
Median age at diagnosis (years, range)	6.16 (0.3-13.5)	-
Acute ITP (%)	86 (45)	-
Acute ITP female (%)	39 (45)	-
Chronic ITP (%)	104 (55)	-
Chronic ITP female (%)	60 (58)	-
Autoimmune diseases^	21	-

^The autoimmune diseases have been reported only in patients with chronic ITP: thyroiditis, celiac disease, rheumatoid arthritis, and systemic lupus erythematosus.

Table 2. Case-control association study of CB2 Q63R polymorphism in 190 Italian ITP children: allelic frequencies and genotype distribution. Clinical characteristics in ITP patients according to the CB2 genotype distribution.

	Patients		Controls		
Allelic frequencies (%)	**Q**	**R**	**Q**	**R**	***P***
ITP *vs.* CTRL	30	70	42	58	**0.006** (χ^2=7.689; df=1)
Acute ITP *vs.* CTRL	34	66			0.197 (χ^2=1.663; df=1)
Chronic ITP *vs.* CTRL	28	72			**0.011** (χ^2=6.442; df=1)

Genotype distribution (n)	**QQ**	**QR**	**RR**	**QQ**	**QR**	**RR**	***P***
ITP *vs.* CTRL (%)	18 (9.5)	79 (41.5)	93 (49)				**0.0001** (χ^2=18.206; df=2)
Acute ITP *vs.* CTRL (%)	9 (10.5)	40 (46.5)	37 (43)	96 (16)	310 (52)	194 (32)	0.108 (χ^2=4.45; df=2)
Chronic ITP *vs.* CTRL (%)	9 (8.5)	40 (38.5)	55 (53)				**0.00021** (χ^2=16.900; df=2)

Demographic and clinical findings (n)	**QQ**	**QR**	**RR**	***P***
Sex				
Female	12	38	49	0.728
Male	8	38	45	(χ^2=0.634; df=2)
Platelet-associated antibodies				
no	3	27	26	0.884
yes	3	25	20	(χ^2=0.246; df=2)
Presence of autoimmune diseases				
no	10	43	60	0.110
yes	2	13	6	(χ^2=0.458; df=2)

CTRL: controls; P values less than 0.05 (in bold) were considered significant.

Table 3. Case-control association study of CB2 Q63R polymorphism in 190 Italian ITP children: odds ratios.

ITP *vs.* CTRL	Odds Ratio	95% CI	*P*
RR *vs.* QQ	2.557	1.466-4.455	**0.001**
RR *vs.* (QQ+QR)	2.006	1.441-2.795	**0.000**
(RR+QR) *vs.* QQ	1.820	1.074-3.083	**0.025**
Acute ITP *vs.* CTRL			
RR *vs.* QQ	2.034	0.956-4.319	0.086
RR *vs.* (QQ+QR)	1.580	1.000-2.497	0.052
(RR+QR) *vs.* QQ	1.630	0.800-3.315	0.203
Chronic ITP *vs.* CTRL			
RR *vs.* QQ	3.024	1.453-6.283	**0.002**
RR *vs.* (QQ+QR)	2.349	1.544-3.573	**0.000**
(RR+QR) *vs.* QQ	2.011	0.994-4064	0.053

CTRL: controls; P values less than 0.05 (in bold) were considered significant.

al autoimmune diseases; ii) the immunomodulating effect of CB2; and (iii) the evidence of abnormal autoreactive T-cell activation in chronic ITP. Cannabinoid ligands, acting on CB2 receptors expressed by immune cells, can inhibit cytokine production, decrease antigen presentation, modulate cell migration, and mediate suppressive effects on effectors.[18,19] Furthermore, immunomodulation by cannabinoids is totally absent in mice lacking the CB2 receptor.[20]

The Q63R CB2 variant results in the amino acid substitution of a polar, uncharged, glutamine with a positively charged arginine. This change could affect the CB2 tertiary structure, altering the immunomodulating properties of CB2. It has been shown that human T cells from CB2 R63 homozygotes show a two-fold reduction in endocannabinoid-induced inhibition of proliferation with respect to cells from CB2 Q63 homozygotes.[21] Although the immunopathogenic cause of ITP has not yet been clarified, there is overwhelming evidence to suggest that a generalized dysfunction of autoreactive T cells could represent the critical immunopathological factor in chronic ITP.

Data presented in this study confirm the role of CB2 in autoimmunity susceptibility and reveal a significant and previously unknown association between CB2 and childhood ITP. However, other studies are needed before the CB2 receptor, localized on immune effector cells, is considered an eligible molecular target to modulate autoreactive, innate, and adaptive immune responses in the chronic form of ITP.

Authorship and Disclosures

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