Polymorphisms of *PTPN11* coding SHP-2 as biomarkers for ulcerative colitis susceptibility in the Japanese population

Yukiko Narumi, Hajime Isomoto, Mizuho Shiota, Kayoko Sato, Shinji Kondo, Haruhisa Machida, Katsunori Yanagihara, Yohei Mizuta, Shigeru Kohno, Kazuhiro Tsukamoto

Y. Narumi, M. Shiota, K. Sato, S. Kondo, K. Tsukamoto*

Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

H. Isomoto, H. Machida, K. Yanagihara, Y. Mizuta, S. Kohno

Second Department of Internal Medicine, Nagasaki

University School of Medicine, Sakamoto 1-7-1, Nagasaki 852-8501, Japan

K. Tsukamoto*

AVSS, Co., Ltd., 1-22 Wakaba-machi, Nagasaki 852-8137, Japan

*Corresponding author:

Kazuhiro Tsukamoto, M.D., Ph.D.

Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan. Tel: +81-95-819-2447, Fax: +81-95-819-2895

e-mail: ktsuka@nagasaki-u.ac.jp

Running Head: *PTPN11* polymorphisms and UC

susceptibility

Abstract

Objective   To identify genetic determinants of inflammatory bowel disease (IBD), we examined an association between polymorphisms of both the programmed cell death 1 gene (*PDCD1*) and the src homology 2 domain-containing tyrosine phosphatase 2 gene (*PTPN11*) and susceptibility to IBD.

Methods   Study subjects comprised 114 patients with ulcerative colitis (UC), 83 patients with Crohn's disease, and 200 healthy control subjects. Five single nucleotide polymorphisms (SNPs) in *PDCD1* and *PTPN11* were detected by PCR-restriction fragment length polymorphism. Subsequently, haplotypes composed of the two SNPs in *PTPN11* were constructed.

Results   The frequencies of the Hap 1 haplotype and its

homozygous Hap 1/Hap 1 diplotype of *PTPN11* were significantly increased in UC patients compared to control subjects ($P = 0.011$ and $P = 0.030$, respectively). While, no association was found for *PDCD1* for UC or CD and none for *PTPN11* for CD.

Conclusion   *PTPN11* is a genetic determinant for the pathogenesis of UC, and haplotyping of *PTPN11* may be useful as a genetic biomarker to identify high-risk individuals susceptible to UC.

Key words

PTPN11; polymorphisms; ulcerative colitis; candidate gene-based association study; Japanese population

Introduction

Chronic inflammatory bowel disease (IBD) is a multifactorial disorder characterized by inflammation specific to the gastrointestinal tract, resulting in intestinal malabsorption, immune system abnormalities, and an exaggerated T-cell response [1-3]. IBD consists of two main subtypes, ulcerative colitis (UC) and Crohn's disease (CD). Although the precise etiology of IBD remains unknown, several environmental factors, such as dietary components and microorganisms, and genetic factors may contribute to the occurrence of IBD [1-3]. Some genome-wide linkage analyses and candidate gene-based association studies with single nucleotide polymorphisms (SNPs), which focus on determining unknown genetic factors underlying the genetic determinants of the pathogenesis of IBD, have

identified possible IBD-susceptibility genes and

chromosomal loci [4-6].

IBD is involved in a complex interplay of immune and

inflammatory cells, including lymphocytes, macrophages,

and dendritic cells [7-9]. In addition, the interaction

between antigen-presenting cells and T-cells plays a crucial

role in the pathogenesis of IBD. We have already found an

association between a lack of susceptibility to UC in the

Japanese population and a polymorphism of the cytotoxic

T-lymphocyte antigen 4 gene (*CTLA4*) [10], which expresses

only on activated T-cells as an immunoreceptor. During

antigenic stimulation of T-cells, CTLA4 downregulates

T-cell activation after binding to B7-1 (CD80) and B7-2

(CD86) [11-14]. As another immunoreceptor of the

CD28/CTLA4 family, programmed cell death 1 (PD-1) is also

expressed on T-cells, as well as B-cells and myeloid cells.

PD-1 negatively signals T-cell activation and tolerance

through the interaction of its specific ligands, PD-L1 or

PD-L2 [13-15]. Moreover, polymorphisms of the PD-1 gene

(*PDCD1*) are associated with susceptibility to autoimmune

diseases, including systemic lupus erythematosus [16-18],

rheumatoid arthritis [19,20], and multiple sclerosis [21] in

the Caucasian and Chinese populations.

Conversely, the inhibitory signals of both CTLA4 and

PD-1 are mediated, at least in part, by the interaction with

src homology 2 domain-containing tyrosine phosphatase-2

(SHP-2), which plays a key role in the intracellular signaling

elicited by a number of growth factors, hormones, and

cytokines during development and hematopoiesis

[15,22-25]. In addition, germline mutations of *PTPN11*

encoding SHP-2 cause Noonan and LEOPARD syndromes

[26-28], as well as somatic mutations in acute myeloid

leukemia [29]. Moreover, an intronic polymorphism of

PTPN11, but not exonic mutations, is associated with gastric

atrophy in Japanese subjects infected with *cag*A-positive

Helicobacter pylori [30].

Despite the importance of the regulatory functions of

PD-1 and SHP-2, no systemic studies have been reported on

the association of genetic polymorphisms of *PDCD1* and

PTPN11 with IBD in the Japanese population or in other

populations. Therefore, we investigated whether SNPs and

their combination polymorphisms, referred to as

haplotypes, in *PDCD1* and *PTPN11* are associated with IBD

susceptibility in the Japanese population, and whether SNPs

can be used as new genetic biomarkers for predicting the

onset of IBD.

Subjects and Methods

Subjects

The study subjects were all Japanese and unrelated,

consisting of 114 patients with UC, 83 patients with CD, and

200 gender-matched, healthy volunteers as control subjects.

The characteristics of the subjects are shown in Table I. IBD

patients were enrolled from eight general health clinics in

Nagasaki, Japan. The study protocol was approved by the

Committee for Ethical Issues dealing with the Human

Genome and Gene Analysis at Nagasaki University, and

written informed consent was obtained from each subject.

The diagnosis of IBD was made on the basis of

endoscopic, radiological, histological, and clinical criteria

of both the World Health Organization Council for

International Organizations of Medical Sciences and the

International Organization for the Study of Inflammatory

Bowel Disease [31,32]. Patients with indeterminate colitis,

multiple sclerosis, systemic lupus erythematosus, or other

diagnosed autoimmune diseases were excluded from the

study.

Preparation of genomic DNA

Genomic DNA was extracted from whole blood obtained

from each subject using a DNA Extractor WB-Rapid Kit (Wako,

Osaka, Japan) according to the manufacturer's protocol

[10].

Source of the genes and their polymorphisms

Three SNPs, nucleotide (*nt*) -538 G>A SNP (G-538A) in the promoter region, *nt* +7146 G>A SNP (G7146A) in intron 4, and *nt* +7785 C>T SNP (C7785T) in exon 5, in *PDCD1* (GenBank accession No.: NT_077995), located at 2q37.3, have been previously reported [17,19,21]. The adenine residue in the nucleotide sequence with respect to the transcriptional initiation site of this gene was numbered consecutively from +1.

Two SNPs, rs2301756 in intron 3 and rs3741983 in intron 13, in *PTPN11* (GenBank accession No.: NT_009775), located at 12q24.13, were selected using data available in the JSNP database (http://snp.ims.u-tokyo.ac.jp/) and the International HapMap database (http://www.hapmap.org/). The rs2301756 SNP in the International HapMap database is identical with JST057927 G-to-A in the JSNP database [30].

The structures and positions of these SNPs sites in *PDCD1* and *PTPN11* are shown in Figure 1.

Determination of three SNPs in PDCD1

The three SNPs, G-538A, G7146A, and C7785T, in *PDCD1* were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism. The polymorphic region was amplified by PCR with a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) using 20 ng of genomic DNA in a 25-µl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl$_2$, 200 µM dNTPs, 15 pmol each of forward primer: 5'-GTGTGAGGCCATCCACAAG-3' and reverse primer: 5'-ACCCCACTCCCATTCTGTC-3' for G-538A; forward primer: 5'-GCAGAGGTGGAAGGACAGG-3' and reverse primer:

5'-AGGCAGGCACACACATGG-3' for G7146A; forward primer:

5'-AGACGGAGTATGCCACCATTGTC-3' and reverse primer:

5'-AAATGCGCTGACCCGGGCTCAT-3' for C7785T, and 1 U

Taq DNA polymerase (Invitrogen, Carlsbad, CA). The

amplification protocol consisted of initial denaturation at

94°C for 2 min, followed by 35 cycles of denaturation at

94°C for 30 sec, annealing for 30 sec at 60°C for G-538A,

63°C for G7146A, and 62°C for C7785T, and extension at

72°C for 30 sec, and final extension at 72°C for 5 min. The

PCR products were digested with *Msp* I (New England

BioLabs Inc, Beverly, MA) for G-538A, *Pst* I (Invitrogen) for

G7146A, and *Pvu* II (Takara Bio Inc., Kyoto, Japan) for

C7785T. These digestion products were separated on a 6%

polyacrylamide gel electrophoresis (Nacalai Tesque, Kyoto,

Japan), and visualized with a UV transilluminator (Alpha

Innotech Co., San Leandro, CA) after ethidium bromide

(Nacalai Tesque) staining.

Determination of two SNPs in PTPN11

The two SNPs, rs2301756 and rs3741983, in *PTPN11* were

also analyzed by the same method. The polymorphic region

was amplified by PCR using 15 pmol each of forward primer:

5'-CTGGTCTTGAACTCCTGGC-3' and reverse primer:

5'-GACTTGCCGTCATTGCTCTC-3' for rs2301756; forward

primer: 5'-GGTGCCAGAGTCTTTTCCAG-3' and reverse

primer: 5'-GCTCTGATCTCCACTCGTC-3' for rs3741983. The

other constituents of the PCR mixture and the amplification

protocol were the same as described above except with the

annealing temperature at 60°C for rs2301756 and 62°C for

rs3741983. The PCR products were digested with *Ban* II

(Toyobo, Osaka, Japan) for rs2301756 and *Mnl* I (Fermentas

International Inc., Ontario, Canada) for rs3741983. The

digestion products were then separated by electrophoresis

on a 2% agarose gel, and visualized as described above.

Haplotype structure of PTPN11

Subsequently, the two SNPs in *PTPN11* were used for

inference of the haplotype structure and for frequency

analysis using SNP Alyze 7.0 standard (Dynacom Inc.,

Yokohama, Japan) to emphasize the variability and to

enhance the power of detecting allelic association of rare

variants.

Statistical analysis

Differences in age and gender between patients and control

subjects were evaluated by an unpaired Student's *t*-test and a chi-square test, respectively, using SPSS 15 (SPSS Japan Inc., Tokyo, Japan) and Prism 4 (GraphPad Software Inc., San Diego, CA). The frequencies of the expected alleles were calculated from those of the observed genotypes according to the Hardy-Weinberg equilibrium. The frequencies of observed and expected alleles were compared by a chi-square test with Yates' correction using SNP Alyze 7.0 standard. The frequencies and distributions of alleles, genotypes, haplotypes, and diplotypes were compared between patients and control subjects by the chi-square test and logistic regression analysis using SPSS 15 and Prism 4. Odds ratios (OR) with 95% confidence intervals (CI) were calculated using SPSS 15. A *P* value of less than 0.05 was considered statistically significant.

Results

Association of SNPs in PDCD1 with susceptibility to IBD

The frequencies and distributions of alleles and genotypes

at the three SNP sites in *PDCD1* were identified and

compared between UC or CD patients and control subjects

(Tables II and III, respectively). G alleles at *nt* -538 and *nt*

+7146 and the C allele at *nt* +7785 are major alleles,

whereas the other alleles are minor alleles. The

distributions of these SNPs in *PDCD1* among IBD patients as

well as control subjects corresponded well to the

Hardy-Weinberg equilibrium, implying that our subject base

has homogeneous genetic backgrounds. With respect to

G7146A SNP, the A allele was not observed in the present

study (Table II), supporting previous results to show that

this allele has been identified in the Caucasian and African-descent populations, but not in the Japanese population [33]. Furthermore, there were no significant differences in the frequencies of alleles and genotypes at the three SNP sites in *PDCD1* between UC or CD patients and control subjects (Tables II and III).

Association of SNPs in PTPN11 with susceptibility to IBD

The frequencies and distributions of alleles and genotypes at the two SNP sites in *PTPN11* were identified and compared between UC or CD patients and control subjects (Tables II and III, respectively). The G allele at rs2301756 SNP and the T allele at rs3741983 SNP are major alleles, whereas the other alleles are minor alleles. The distributions of these SNPs in *PTPN11* among IBD patients as

well as control subjects corresponded well to the

Hardy-Weinberg equilibrium. A chi-square test revealed that

the frequencies of the G allele at rs2301756 and the T allele

at rs3741983 were significantly increased in patients with

UC as compared with those in control subjects (84.6% vs.

75.2%; *P* = 0.008 and 83.3% vs. 75.7%; *P* = 0.034,

respectively) (Table II).

In addition, logistic regression analysis indicated

that the frequencies of the G/G homozygous genotype at

rs2301756 SNP and the T/T homozygous genotype at

rs3741983 SNP were also significantly increased in patients

with UC as compared with those in control subjects (71.1%

vs. 57.5%; *P* = 0.018, OR = 1.814, 95% CI = 1.109 - 2.968 and

68.4% vs. 56.0%; *P* = 0.031, OR = 1.702, 95% CI = 1.050 -

2.761, respectively) (Table III). These results of the

genotype analyses regarding *PTPN11* polymorphisms

coincided well with those of the allele analyses between UC

patients and control subjects.

Association of haplotypes and diplotypes of PTPN11 with

susceptibility to IBD

Since a significant association was observed between allele

and genotype polymorphisms at rs2301756 and rs3741983

SNPs in *PTPN11* and susceptibility to UC, four haplotypes

composed of these two SNPs were constructed and

identified using SNP Alyze 7.0 standard (Table IV). Logistic

regression analyses revealed that the frequency of a

haplotype, Hap 1 (G allele at rs2301756 and T allele at

rs3741983), was significantly increased in patients with UC

as compared with that in control subjects (82.9% vs. 74.0%,

P = 0.011, OR = 1.703, 95% CI = 1.129 - 2.568) (Table IV).

Whereas, the frequency of another haplotype, Hap 2 (A

allele at rs2301756 and C allele at rs3741983), was

significantly decreased in UC patients as compared with

that in control subjects (14.9% vs. 23.0%, *P* = 0.016, OR =

0.587, 95% CI = 0.381 - 0.904) (Table IV).

Furthermore, 10 diplotypes composed of four

haplotypes were identified (Table V). Logistic regression

analysis showed that the frequency of the UC patients

possessing a Hap 1/Hap 1 diplotype was significantly

higher than that of control subjects (67.5% vs. 55.0%, *P* =

0.030, OR = 1.703, 95% CI = 1.053 - 2.754) (Table V).

However, a Hap 2/Hap 2 diplotype showed no statistically

significant lack of susceptibility to UC (Table V). These

results of the diplotype analysis regarding the Hap 1

haplotype of *PTPN11* coincided well with those of the

haplotype analysis between UC patients and control

subjects.

Discussion

The present study is the first report to demonstrate an

association between *PTPN11* polymorphisms and

susceptibility to UC in the Japanese population. The

presence of the G allele and its homozygous G/G genotype

at rs2301756 SNP, the T allele and its homozygous T/T

genotype at rs3741983 SNP, the Hap 1 haplotype, and its

homozygous Hap 1/Hap 1 diplotype in *PTPN11* conferred

susceptibility to UC. Inversely, the Hap 2 haplotype showed

the statistically significant lack of susceptibility to UC.

These findings imply that *PTPN11* is a genetic determinant

for predisposition to the onset of UC in Japanese individuals. However, the current study population was relatively small, and further study on a larger number of the Japanese population and on other ethnic populations is necessary to confirm the association between *PTPN11* and UC.

SHP-2, coded by *PTPN11*, is a key downstream molecule that downregulates T-cell and B-cell activation through interaction with CTLA4 and PD-1 [13-15,22-25]. SHP-2 is recruited to the phosphorylated tyrosine residue in the immunoreceptor tyrosine-based switch motif of the cytoplasmic tail of PD-1, and in the immunoreceptor tyrosine-based inhibitory motif of the cytoplasmic tail of CTLA4 [13-15,34,35]. Although the mechanisms of the *PTPN11* polymorphisms identified in this study are

unknown, *PTPN11* polymorphisms may affect the

expression, affinity to immunoreceptors, and inhibitory

activities of SHP-2, leading to the reduction in the SHP-2

downregulatory effect on T-cell and B-cell activation.

Another mechanism of inflammatory suppression is

the downregulation of SHP-2 on the interleukin 6

(IL-6)/signal transducer and activator of transcription 3

(STAT3) signaling pathway through binding of SHP-2 to the

receptor complex, glycoprotein 130 and IL-6 receptor alpha

[36,37]. An increase in serum IL-6 concentrations and

hyperactivation of STAT3 signaling have been reported in

patients with IBD and experimental colitis *in vivo* [38-40].

Although the IL-6/STAT3 signaling is implicated in the

pathogenesis of IBD, IL-6 expression and polymorphisms

are associated with the progression and severity of IBD, but

not the onset [40-42]. These pathophysiological and genetic

finings of IBD imply that the vicious pathway of T-cell

activation driven by IL-6/STAT3 signaling can contribute to

the perpetuation of chronic intestinal inflammatory process,

probably resulting in the development of IBD. Thus, the

reduction in the SHP-2 downregulatory effect against

IL-6/STAT3 signaling due to its genetic polymorphisms may

explain the progressing mechanism of IL-6-induced STAT3

hyperactivation in IBD patients.

Germline mutations of *PTPN11* have been reported in

patients with the autosomal dominant-inherited Noonan

and LEOPARD syndromes [26-28], as well as somatic

mutations in leukemogenesis [29]. These mutations are

exclusively heterozygous missense mutations in exons

containing the interacting portions of the amino terminal

src homology 2 and protein tyrosine phosphatase domains, which introduce amino acid substitutions, leading to the gain-of-function of SHP-2 [43,44]. On the other hand, an intronic polymorphism (rs2301756 SNP in intron 3 of *PTPN11*), but not an exonic mutation, is associated with gastric atrophy in Japanese individuals infected with *cag*A-positive *H. pylori* [30], indicating persistent chronic inflammation in epithelial cells of the stomach in the individuals possessing the G/A genotype at the rs2301756 SNP site in *PTPN11*. Atrophy of the gastric mucosae is the endpoint of chronic diseases, and is often found in patients with chronic gastritis with *H. pylori* infection. Likewise, since persistent chronic inflammation in epithelial cells of the colon contributes to the pathogenesis of UC, chronic inflammation due to *PTPN11* polymorphisms in the gastric

and colon mucosae may be explained by the same mechanism. Thus, from the pathophysiological perspective, we speculate that intronic polymorphisms, especially possessing the Hap 1 haplotype and its homozygous Hap 1/Hap 1 diplotype, may interfere with gene expression. This causes a reduction of SHP-2 mRNA and protein levels, subsequently leading to the loss-of-function of SHP-2, which is in contrast to the gain-of-function of SHP-2 caused by exonic missense mutations of *PTPN11*. Therefore, the accumulation of altering the loss-of-function of SHP-2 caused by *PTPN11* polymorphisms may lead to the reduction in the inhibitory effects against T-cell and B-cell activation, resulting in persistent chronic intestinal inflammation and the onset of UC.

Conclusions

As *PTPN11* is a genetic determinant for susceptibility to UC in the Japanese population, intronic polymorphisms of *PTPN11* may be useful as new DNA-based biomarkers for identifying individuals at high-risk for UC. Moreover, SHP-2 may be a good target molecule for novel drugs discovery.

Acknowledgments

References

1.   Fiocchi C: Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 115: 182-205, 1998.

2.   Farrell RJ, Peppercorn MA: Ulcerative colitis. Lancet 359: 331-340, 2002.

3.   Yang H, Taylor KD, Rotter JI: Inflammatory bowel disease, I. genetic epidemiology. Mol. Genet. Metab. 74: 1-21, 2001.

4.   Taylor KD, Yang H, Rotter JI: Inflammatory bowel disease, II. gene mapping. Mol. Genet. Metab. 74: 22-44, 2001.

5.   Bonen DK, Cho JH: The genetics of inflammatory bowel disease. Gastroenterology 124: 521-536, 2003.

6.   Ahmad T, Tamboli CP, Jewell D, Colombel J-F: Clinical relevance of advances in genetics and

pharmacogenetics of IBD. Gastroenterology 126:

1533-1549, 2004.

7.   Powrie F: T cells in inflammatory bowel disease:

protective and pathogenic role. Immunity 3: 171-174,

1995.

8.   Blumberg RS, Saubermann LJ, Strober W. Animal

models of mucosal inflammation and their relation to

human inflammatory bowel disease. Curr. Opin.

Immunol. 11: 648-656, 1999.

9.   Mizoguchi A, Mizoguchi E, Bhan AK: Immune networks

in animal models of inflammatory bowel disease.

Inflamm. Bowel Dis. 9: 246-259, 2003.

10.   Machida H, Tsukamoto K, Wen C-Y, Narumi Y, Shikuwa

S, Isomoto H, Takeshima F, Mizuta Y, Niikawa N,

Murata I, Kohno S: Association of polymorphic alleles

of *CTLA4* with inflammatory bowel disease in the Japanese. World J. Gastroenterol. 11: 4188-4193, 2005.

11.   Karandikar NJ, Vanderlugt CL, Walunas TL, Miller SD, Bluestone JA: CTLA-4: a negative regulator of autoimmune disease. J. Exp. Med. 184: 783-788, 1996.

12.   Salomon B, Bluestone JA: Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu. Rev. Immunol. 19: 225-252, 2001.

13.   Khoury SJ, Sayegh MH: The roles of the new negative T cell costimulatory pathways in regulating autoimmunity. Immunity 20: 529-538, 2004.

14.   Zhang X, Schwartz JCD, Guo X, Bhatia S, Cao E, Chen L, Zhang Z-Y, Edidin MA, Nathenson SG, Almo SC:

Structural and functional analysis of the costimulatory receptor programmed death-1. Immunity 20: 337-347, 2004.

15.   Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, Greenfield EA, Bourque K, Boussiotis VA, Carter LL, Carreno BM, Malenkovich N, Nishimura H, Okazaki T, Honjo T, Sharpe AH, Freeman GJ: PD-L2 is a second ligand for PD-1 and inhibit T cell activation. Nat. Immunol. 2: 261-268, 2001.

16.   Prokunina L, Castillejo-López C, Öberg F, Gunnarsson I, Berg L, Magnusson V, Brookes AJ, Tentler D, Kristjansdóttir H, Gröndal G, Bolstad AI, Svenungsson E, Lundberg I, Sturfelt G, Jönssen A, Truedsson L, Lima G, Alcocer-Varela J, Jonsson R, Gyllensten UB, Harley

JB, Alarcón-Segovia D, Steinsson K, Alarcón-Riquelme ME: A regulatory polymorphism in *PDCD1* is associated with susceptibility to systemic lupus erythematosus in humans. Nat. Genet. 32: 666-669, 2002.

17. Ferreiros-Vidal I, Gomez-Reino JJ, Barros F, Carracedo A, Carreira P, Gonzalez-Escribano F, Liz M, Martin J, Ordi J, Vicario JL, Gonzalez A: Association of PDCD1 with susceptibility to systemic lupus erythematosus: evidence of population-specific effects. Arthritis Rheum. 50: 2590-2597, 2004.

18. Johansson M, Ärlestig L, Möller B, Rantapää-Dahlqvist S: Association of a PDCD1 polymorphism with renal manifestations in systemic lupus erythematosus. Arthritis Rheum. 52: 1665-1669, 2005.

19. Lin S-C, Yen J-H, Tsai J-J, Tsai W-C, Ou T-T, Liu H-W, Chen C-J: Association of a programmed death 1 gene polymorphism with the development of rheumatoid arthritis, but not systemic lupus erythematosus. Arthritis Rheum. 50: 770-775, 2004.

20. Kong EKP, Prokunina-Olsson L, Wong WHS., Lau C-S, Chan T-M, Alarcón-Riquelme M, Lau YL: A new haplotype of *PDCD1* is associated with rheumatoid arthritis in Hong Kong Chinese. Arthritis Rheum. 52: 1058-1062, 2005.

21. Kroner A, Mehling M, Hemmer B, Riechmann P, Toyka KV, Mäurer M, Wiendl H: A PD-1 polymorphism is associated with disease progression in multiple sclerosis. Ann. Neurol. 58: 50-57, 2005.

22. Chuang E, Lee K-M, Robbins MD, Duerr JM, Alegre M-L,

Hambor JE, Neveu MJ, Bluestone JA, Thompson CB: Regulation of cytotoxic T lymphocyte-associated molecule-4 by src kinases. J. Immunol. 162: 1270-1277, 1999.

23. Schneider H, Rudd CE: Tyrosine phosphatase SHP-2 binding to CTLA-4: absence of direct YVKM/YFIP motif recognition. Biochem. Biophys. Res. Commun. 269: 279-283, 2000.

24. Okazaki T, Maeda A, Nishimura H, Kurosaki T, Honjo T: PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. Proc. Natl. Acad. Sci. U.S.A. 98: 13866-13871, 2001.

25. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL:

SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. J. Immunol. 173: 945-954, 2004.

26. Tartaglia M, Mehler EL, Goldberg R, Zampino G, Brunner HG, Kremer H, van der Burgt I, Crosby AH, Ion A, Jeffery S, Kalidas K, Patton MA, Kucherlapati RS, Gelb BD: Mutations in *PTPN11*, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat. Genet. 29: 465-468, 2001.

27. Digilio MC, Conti E, Sarkozy A, Migarelli R, Dottorini T, Marino B, Pizzuti A, Dallapiccola B: Grouping of multiple-lentigines/LEOPARD and Noonan syndromes on the *PTPN11* gene. Am. J. Hum. Genet. 71: 389-394,

2002.

28. Sarkozy A, Conti E, Seripa D, Digilio MC, Grifone N,

    Tandoi C, Fazio VM, Ciommo VD, Marino B, Pizzuti A,

    Dallapiccola B: Correlation between *PTPN11* gene

    mutations and congenital heart defects in Noonan and

    LEOPARD syndromes. J. Med. Genet. 40: 704-708,

    2003.

29. Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner

    J, Jung A, Hählen K, Hasle H, Licht JD, Gelb BD: Somatic

    mutations in *PTPN11* in juvenile myelomonocyte

    leukemia, myelodysplastic syndromes and acute

    myeloid leukemia. Nat. Genet. 34: 148-150, 2003.

30. Goto Y, Ando T, Yamamoto K, Tamakoshi A, El-Omar E,

    Goto H, Hamajima N: Association between serum

    pepsinogens and polymorphism of *PTPN11* encoding

SHP-2 among *Helicobacter pylori* seropositive

Japanese. Int. J. Cancer 118: 203-208, 2006.

31. Podolsky DK: Inflammatory bowel disease (1). N. Eng. J.

Med. 325: 928-937, 1991.

32. Podolsky DK: Inflammatory bowel disease (2). N. Eng. J.

Med. 325: 1008-1016, 1991.

33. Mori M, Yamada R, Kobayashi K, Kawaida R, Yamamoto

K: Ethnic difference in allele frequency of

autoimmune-disease-associated SNPs. J. Hum. Genet.

50: 264-266, 2005.

34. Greenwald RJ, Freeman GJ, Sharpe AH: The B7 family

revisited. Annu. Rev. Immunol. 23: 515-548, 2005.

35. Okazaki T, Honjo T: PD-1 and PD-1 ligands: from

discovery to clinical application. Int. Immunol. 19:

813-824, 2007.

36. Heinrich PC, Behrmann I, Müller-Newen G, Schaper F, Graeve L: Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem. J. 334: 297-314, 1998.

37. Kamimura D, Ishihara K, Hirano T: IL-6 signal transduction and its physiological roles: the signal orchestration model. Rev. Physiol. Biochem. Pharmacol. 149: 1-38, 2003.

38. Holtkamp W, Stollberg T, Reis HE: Serum interleukin-6 is related to disease activity but not disease specificity in inflammatory bowel disease. J. Clin. Gastroenterol. 20: 123-126, 1995.

39. Mudter J, Weigmann B, Bartsch B, Kiesslich R, Strand D, Galle PR, Lehr HA, Schmidt J, Neurath MF: Activation pattern of signal transducers and activators of

transcription (STAT) factors in inflammatory bowel diseases. Am. J. Gastroenterol. 100: 64-72, 2005.

40. Carey R, Jurickova I, Ballard E, Bonkowski E, Han X, Xu H, Denson LA: Activation of an IL-6:STAT3-dependent transcriptome in pediatric-onset inflammatory bowel disease. Inflamm. Bowel Dis. 14: 446-457, 2008.

41. Balding J, Livingstone WJ, Conroy J, Mynett-Johnson L, Weir DG, Mahmund N, Smith OP: Inflammatory bowel disease: the role of inflammatory cytokine gene polymorphisms. Mediators Inflamm. 13: 181-187, 2004.

42. Kwon KH, Murakami A, Hayashi R, Ohigashi H: Interleuikin-1β targets interleukin-6 in progressing dextran sulfate sodium-induced experimental colitis. Biochem. Biophys. Res. Commun. 337: 647-654, 2005.

43. Tartaglia M, Gelb BD: Germ-line and somatic *PTPN11*

mutations in human disease. Eur. J. Med. Genet. 48:

81-96, 2005.

44. Oishi K, Gaengel K, Krishnamoorthy S, Kamiya K, Kim

I-K, Ying H, Weber U, Perkins LA, Tartaglia M, Mlodzik

M, Pick L, Gelb BD: Transgenic *Drosophila* models of

Noonan syndrome causing *PTPN11* gain-of-function

mutations. Hum. Mol. Genet. 15: 543-553, 2006.

Figure Legends

Figure 1: Locations of the SNP sites in *PDCD1* (A) and *PTPN11*

(B). The horizontal bars indicate the genomic sequence of

PDCD1 (A) and of *PTPN11* (B). Rectangles represent exons in

each gene and exon numbers are indicated above each exon.

Arrows indicate the positions of the SNP sites.

ATG: transcriptional initiation site.

TGA: stop codon site.

Table I   Clinical characteristics of study subjects

| Characteristics | Patients with | | Control subjects |
	UC (n = 114)	CD (n = 83)	(n = 200)
Age, mean ± SD (years)	44.2 ± 16.7*	34.3 ± 12.5	32.5 ± 11.2
Age range (years)	14-83	17-75	20-60
Male/female (%)	59/55 (51.7/48.3)	50/33 (60.2/39.8)	126/74 (62.5/37.5)

*: $P<0.01$ as compared with control subjects

SD: standard deviation

Table II Distributions of alleles at the SNP sites in each gene among study subjects

Gene	SNP	Allele	Number (%) of alleles		Allele comparison*	Number (%) of alleles		Allele comparison*
			UC	Control	P value	CD	Control	P value
PDCD1	G-538A	G	121 (53.1)	206 (51.5)	0.308	84 (50.6)	206 (51.5)	0.717
		A	107 (46.9)	194 (48.5)		82 (49.4)	194 (48.5)	
	G7146A	G	228 (100)	400 (100)	–	166 (100)	400 (100)	–
		A	0	0		0	0	
	C7785T	C	152 (66.7)	264 (66.0)	0.935	152 (66.7)	264 (66.0)	0.512
		T	76 (33.3)	136 (34.0)		76 (33.3)	136 (34.0)	
PTPN11	rs2301756	A	35 (15.4)	99 (24.8)	0.008	36 (21.7)	99 (24.8)	0.503
		G	193 (84.6)	301 (75.2)		130 (78.3)	301 (75.2)	
	rs3741983	C	38 (16.7)	97 (24.3)	0.034	36 (21.7)	97 (24.3)	0.585
		T	190 (83.3)	303 (75.7)		130 (78.3)	303 (75.7)	
Total number of alleles			228	400		166	400	

*Each allele was compared with another allele by chi-square test.

Table III   Distributions of genotypes at the SNP sites in each gene among study subjects

Gene	SNP	Genotype	Number (%) of genotype		Genotype comparison*		Number (%) of genotype		Genotype comparison*	
			UC	Control	OR (95% CI)	P value	CD	Control	OR (95% CI)	P value
PDCD1	G-538A	G/G	33 (28.9)	55 (27.5)	1.074 (0.645 - 1.789)	0.784	18 (21.7)	55 (27.5)	0.730 (0.398 - 1.340)	0.310
		G/A	55 (48.2)	96 (48.0)	1.010 (0.637 - 1.600)	0.967	48 (57.8)	96 (48.0)	1.486 (0.886 - 2.490)	0.133
		A/A	26 (22.8)	49 (24.5)	0.910 (0.529 - 1.568)	0.735	33 (39.8)	49 (24.5)	0.794 (0.426 - 1.480)	0.467
	G+7146A	G/G	114 (100)	200 (100)	–	–	83 (100)	200 (100)	–	–
		G/A	0	0	–	–	0	0	–	–
		A/A	0	0	–	–	0	0	–	–
	C+7785T	C/C	54 (47.4)	87 (43.5)	1.169 (0.737 - 1.855)	0.508	39 (47.0)	87 (43.5)	1.151 (0.689 - 1.924)	0.591
		C/T	44 (38.6)	89 (44.5)	0.768 (0.481 - 1.228)	0.270	37 (44.6)	89 (44.5)	0.983 (0.588 - 1.645)	0.948
		T/T	16 (14.0)	23 (11.5)	1.256 (0.634 - 2.490)	0.513	7 (8.4)	23 (11.5)	0.709 (0.292 - 1.722)	0.447
PTPN11	rs2301756	A/A	2 (1.8)	14 (7.0)	0.237 (0.053 - 1.063)	0.060	7 (8.4)	14 (7.0)	1.224 (0.475 - 3.151)	0.676
		A/G	31 (27.2)	71 (35.5)	0.679 (0.410 - 1.123)	0.132	22 (26.5)	71 (35.5)	0.655 (0.372 - 1.155)	0.144
		G/G	81 (71.1)	115 (57.5)	1.814 (1.109 - 2.968)	0.018	54 (65.1)	115 (57.5)	1.376 (0.809 - 2.341)	0.239
	rs374198	C/C	2 (1.8)	9 (4.5)	0.379 (0.080 - 1.785)	0.220	6 (7.2)	9 (4.5)	1.654 (0.569 - 4.803)	0.355
		C/T	34 (29.8)	79 (39.5)	0.651 (0.398 - 1.064)	0.087	24 (28.9)	79 (39.5)	0.623 (0.358 - 1.083)	0.093
		T/T	78 (68.4)	112 (56.0)	1.702 (1.050 - 2.761)	0.031	53 (63.9)	112 (56.0)	1.388 (0.819 - 2.353)	0.223
Total number of subjects			114	200			83	200		

*Each genotype was compared with other genotypes combined by logistic regression analysis.

OR: odds ratio; CI: confidence interval

Table IV   Distributions of haplotypes in *PTPN11* among study subjects

Haplotype	SNP		Number (%) of haplotypes		Haplotype comparison*		Number (%) of haplotypes		Haplotype comparison*	
	rs2301756	rs3741983	UC	Control	OR (95% CI)	*P* value	CD	Control	OR (95% CI)	*P* value
Hap 1	G	T	189 (82.9)	296 (74.0)	1.703 (1.129 - 2.568)	0.011	125 (75.3)	296 (74.0)	1.071 (0.706 - 1.626)	0.747
Hap 2	A	C	34 (14.9)	92 (23.0)	0.587 (0.381 - 0.904)	0.016	31 (18.7)	92 (23.0)	0.769 (0.488 - 1.211)	0.257
Hap 3	G	C	4 (1.8)	5 (1.3)	1.411 (0.375 - 5.307)	0.611	5 (3.0)	5 (1.3)	2.453 (0.701 - 8.590)	0.160
Hap 4	A	T	1 (0.4)	7 (1.8)	0.247 (0.030 - 2.023)	0.193	5 (3.0)	7 (1.8)	1.744 (0.545 - 5.574)	0.348
Total number of haplotypes			228	400			166	400		

*Each haplotype was compared with other haplotypes combined by logistic regression analysis.
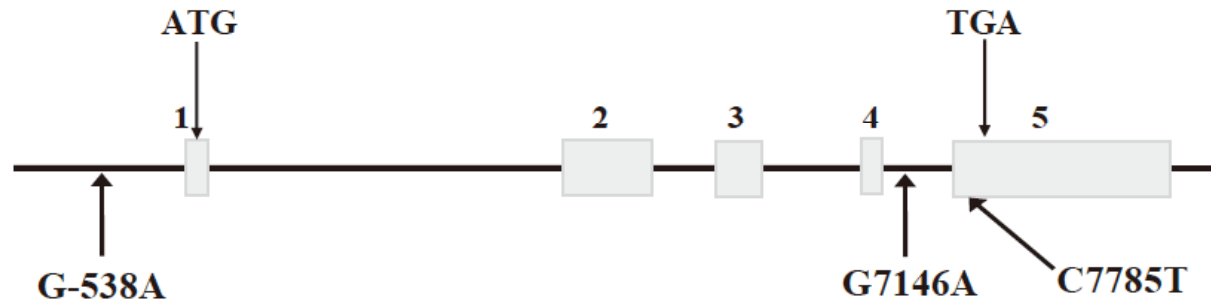
OR: odds ratio; CI: confidence interval

Table V   Distributions of diplotypes of *PTPN11* among study subjects

Diplotype	Number (%) of diplotypes		Diplotype comparison*		Number (%) of diplotypes		Diplotype comparison*	
	UC	Control	OR (95% CI)	*P* value	CD	Control	OR (95% CI)	*P* value
Hap 1 / Hap 1	77 (67.5)	110 (55.0)	1.703 (1.053 - 2.754)	0.030	50 (60.2)	110 (55.0)	1.24 (0.737 - 2.086)	0.418
Hap 1 / Hap 2	30 (26.3)	69 (34.5)	0.678 (0.408 - 1.128)	0.134	21 (25.3)	69 (34.5)	0.643 (0.362 - 1.142)	0.132
Hap 1 / Hap 3	4 (3.5)	5 (2.5)	1.418 (0.373 - 5.391)	0.608	3 (3.6)	5 (2.5)	1.463 (0.341 - 6.265)	0.609
Hap 1 / Hap 4	1 (0.9)	2 (1.0)	0.876 (0.079 - 9.770)	0.914	1 (1.2)	2 (1.0)	1.207 (0.108 - 13.499)	0.878
Hap 2 / Hap 2	2 (1.8)	9 (4.5)	0.379 (0.080 - 1.785)	0.220	5 (6.0)	9 (4.5)	1.360 (0.442 - 4.188)	0.592
Hap 2 / Hap 3	0	0	-	-	0	0	-	-
Hap 2 / Hap 4	0	5 (2.5)	-	-	0	5 (2.5)	-	-
Hap 3 / Hap 3	0	0	-	-	1 (1.2)	0	-	-
Hap 3 / Hap 4	0	0	-	-	0	0	-	-
Hap 4 / Hap 4	0	0	-	-	2 (2.4)	0	-	-
Total number of subjects	114	200			83	200		

*Each diplotype was compared with other diplotypes combined by logistic regression analysis.
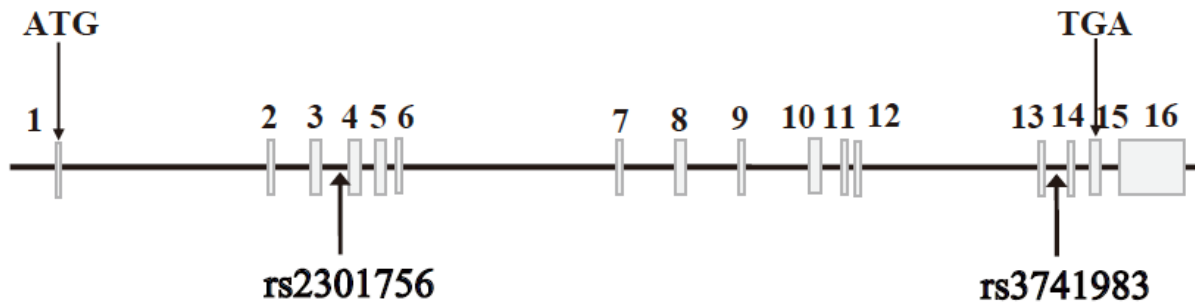
OR: odds ratio; CI: confidence interval

Figure 1: Locations of the SNP sites in *PDCD1* and *PTPN11*