# Glutathione S-transferase gene polymorphisms in Japanese patients with rheumatoid arthritis

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# Abstract Objective

To investigate the role of polymorphisms of the glutathione S-transferase M1 (GSTM1), GSTT1, and GSTP1 genes in determining susceptibility to rheumatoid arthritis (RA) and association with the clinical features.

# Methods

Polymorphisms of the GSTM1, GSTT1, and GSTP1 genes in 108 Japanese patients with RA and in 143 healthy controls were analyzed by polymerase chain reaction (PCR) or PCR-restriction fragment length polymorphism.

# Results

The frequency of the GSTM1 null genotype was significantly higher among RA patients than among control subjects (60.2% and 44.1%, respectively. P = 0.011). Moreover, the female patients with GSTM1 homozygous null genotype showed significantly higher serum MMP-3 level than the female patients with non-null genotype (P = 0.030). Frequencies of the GSTT1 and GSTP1 gene polymorphism were not different between RA patients and controls.

Conclusion

The GSTM1 homozygous null genotype could be a genetic factor that determines susceptibility to RA and may have influence on the disease process.

Key words

Rheumatoid arthritis, glutathione S-transferase, polymorphism, matlix metalloproteinase-3.

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## Introduction

Rheumatoid arthritis (RA) is a chronic disease characterized by symmetrical inflammation of the peripheral joints, potentially resulting in progressive destruction of cartilage and bones. The exact etiology of RA, including genetic factor, is still unclear, although it is the most common inflammatory arthritis worldwide.

Oxygen free radicals, causing tissue oxidative stress, have been implicated as mediators of tissue damage in several human diseases such as RA and other inflammatory diseases (1). For example, reactive oxygen species (ROS) produced during hypoxia-reperfusion injury in the inflamed joints can mediate persistent synovial inflammation (2). The impaired pro-oxidant and antioxidant system in RA patients may contribute to the disease process in RA (3, 4). Superoxide and superoxide dismutase system has been shown to be involved in pathogenesis of RA(5, 6). The glutathione S-transferase (GST) superfamily of enzymes are involved in the detoxification of a variety of reactive intermediates and in the protection from oxidative damage (7, 8). Polymorphisms have been identified in some GST genes, such as mu (GSTM), theta (GSTT), pi (GSTP), and others. A large percentage of individuals display a homozygous deletion in the GSTM1 and GSTT1 genes, and these genes lead to the absence of enzymatic activity(9). A single nucleotide substitution (A-G) at position 313 of the GSTP1 gene, which causes an Iso-to-Val substitution, substantially diminishes GSTP1 enzyme activity (10).

A large number of studies have shown the association between GST polymorphisms and various types of diseases, most of which are cancers, including tobacco-associated cancers, bladder cancer, and gastrointestinal cancer. However, data from different studies are conflicting (11-13). Although the properties of GST suggest that they may have anti-inflammatory effects, there have been few reports about the association between GST polymorphisms and inflammatory diseases. In order to clarify the possible involvement of GST genes in disease susceptibility to RA and severity of RA in Japanese population, we examined the polymorphisms of these genes in Japanese patients with RA.

## **Patients and methods**

#### Patients and controls

One hundred and eight RA patients (86 female and 22 male) who attended Kobe University Hospital were included in the study. (Median age was 45.0 years at the disease onset.) Control population consisted of 143 healthy volunteers (78 female and 65 male, median age 43.8 years.) Sample DNA were isolated when informed consent was obtained. All patients and controls were Japanese. All patients met the American Collage of Rheumatology (formerly, American Rheumatism Association) 1987 criteria for RA (14). The characteristics of these patients are shown in Table I.

#### Analysis of GST polymorphisms

Sample DNA was isolated from peripheral blood mononuclear cells. GSTM1 and GSTT1 genotypes were determined using polymerase chain reaction (PCR)based methods as described earlier (15). Briefly, the GSTM1, GSTT1 and  $\beta$ -globin genes were simultaneously amplified by PCR with mixed primers for each genes. PCR products were separated by agarose gel electrophoreses, and visualized under ultraviolet trans-illuminator. The presence or absence of the GSTM1 (219 basepairs) and GSTT1 (480 bp) was determined by identifying the positive or negative bands for each size. The positive bands meant that sample was homo- or heterozygous. The allele distribution for GSTP1 was determined by PCR-restriction fragment length polymorphism (RFLP) (16). Briefly, PCR reaction was conducted with GSTP1 primers and the 176 bp amplified fragment of GSTP1 gene was subjected to restriction digestion with BsmAI. The digested products were run on an agarose gel, and the genotype was determined by analysis of the bands on the gel.

#### Laboratory test

Rheumatoid factor (RF) and C-reactive protein level (CRP), matrix metallopro-

 Table I. Characteristics of the 108 patients with rheumatoid arthritis and 143 healthy controls.

	RA patients		controls	
Female/male (%Female)	86/22	(79.6)	78/65 (54.5)	
Age, years	45.0ª	(19-84)	43.8 (22-67)	
Disease duration, years	7	(1-32)		
RF positive patients $(n = 108)$	73	(66.2)		
CRP positive patients $(n = 108)$	81	(74.3)		
MMP-3 positive patients $(n = 90)$	59	(65.6)		
Sharp score (hand) $(n = 92)$	26	(0-231)		
Larsen score (hand) $(n = 92)$	33	(0-99)		
Tender joint count $(n = 108)$	5	(0-27)		
Swollen joint count ( $n = 108$ )	2	(0-24)		

Values are given as medians (range) or numbers (percentage).

<sup>a</sup> Age at disease onset.

teinase 3 (MMP-3), IgG-RF, antiagalactosyl IgG antibody (CA-RF), anti-citrullinated peptide antibody (anti-CCP) were measured at the first visit or the first diagnosis of RA. MMP-3 and anti-CCP were not measured for some patients because those tests were not available at the first visit or the diagnosis. Levels of serum MMP-3 was determined by a 1-step sandwich EIA system (Daiichi Pure Chemicals, Tokyo, Japan) (17). The normal values of serum MMP-3 are 36.9-121 ng/ml for male and 17.3-59.7 ng/ml for female. Levels of anti-CCP were determined by ELISA (Medical & Biological Laboratories, Aichi, Japan), and more than 5 U/ml were considered positive. IgG-RF, and CA-RF levels were determined by ELISA and electrochemiluminescence immunoassay, respectively. Normal values for IgG-RF and CA-RF are < 2 (index) and <6.00 IU/ml, respectively. RF and CRP were determined by routine laboratory techniques, with normal values of 10 IU/ml and 0.3mg/dl, respectively.

## Radiographic assessment

All radiographs are scored by the same investigator according to the modified Sharp/van der Heijde method and Larsen method (18-20). One hundred sixty nine radiographs of 92 patients were scored. For 52 patients, radiographs were taken more than once during the follow up period. For 27 out of 52 patients, the first radiograph was taken within 5 years since the disease onset. The radiographs of these 27 patients were analyzed in the study.

### Statistical analysis

Chi-square analysis on a 2 x 2 tables or Fisher's exact probability tests were used to compare frequencies of the homozygous null genotype of the GSTM1 and GSTT1 genes, distribution of GSTP1 genotypes, positivity of the CRP, MMP-3, RF, IgG-RF, CA-RF, and anti-CCP when indicated. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated when 2 by 2 tables were indicated. Serum concentration of MMP-3 and CRP were compared by Mann-Whitney's U test.

#### Results

# Frequencies of the homozygous null genotypes of GSTM1 and GSTT1, and distribution of GSTP1 genotypes in RA patients and controls

The frequency of the GSTM1 null genotype was significantly higher among RA patients than among control subjects (60.2% and 44.1%, respectively. OR 1.92, 95%CI 1.14-2.69, P = 0.011). Because there was a gap in the ratio of male and female between patients and controls, we separated both patients and controls into male and female, and determined GST genotype distribution in each gender. In the female patients, the frequency of the GSTM1 null genotype was also significantly higher than that of female controls (58.1% and 39.7%, respectively. OR 2.11, 95%CI 1.27-2.94, P = 0.019).

On the other hand, there was no significant difference between male patients and male controls (68.2% and 49.2%, respectively. OR 2.21, 95%CI 0.94-3.48, P = 0.123) (Table II).

The frequencies of the GSTT1 null genotype among all, female, and male patients with RA did not differ from those among all, female and male controls. Also, no significant difference was observed in the frequency of the GSTM1 and GSTT1 double null genotype between RA patients and controls (data not shown). Statistical analysis did not disclose a significant difference in GSTP1 genotype distribution between RA patients and controls (Table II).

# Association of GST genotypes with the markers of disease activity and laboratory findings

Next we analyzed association between GST genotypes and disease activity markers in the female patients. Tender joint counts and swollen joint counts did not associate with GST genotypes. Positivity of CRP, MMP-3, RF, IgG-RF, CA-RF, and anti-CCP did not associate with GST genotypes (Table III). However, when we compared the serum levels of disease markers with the genotypes, the female patients with GSTM1 homozygous null genotype showed significantly higher serum MMP-3 levels (P = 0.030) (Fig. 1) and higher serum CRP levels (P=0.056, data not shown) than those with nonnull genotype.

These disease activity markers and disease markers were not associated with GST genotypes in all and male patients (data not shown). There was no significant association between GSTP1 genotype distribution and the laboratory findings in all, male and female patients (data not shown).

# Association of GST genotypes and laboratory tests with the radiographic score of hands

We determined the radiographic scores using the modified Sharp/van der Heijde method and Larsen method. Twenty seven patients whose first radiographs were taken within 5 years after disease onset were divided into the 3 groups according to the radiographic scores of 
 Table II. Frequencies of the GSTM1 null and GSTT1 null homozygotes and distribution of the GSTP1 genotypes in controls and patients with rheumatoid arthritis (RA).

	GSTM1	GSTT1		GSTP1		
	null	null	Ile/Ile	Ile/Val	Val/Val	
All subjects						
normal subjects $(n = 143)$	63 (44.1)	62 (43.3)	116 (81.1)	25 (17.5)	2 (1.4)	
RA patients $(n = 108)$	65 (60.2) <sup>a</sup>	45 (41.7)	89 (82.4)	18 (16.8	1 (0.9)	
Female subjects						
normal subjects (n=78)	31 (39.7)	32 (41.0)	65 (83.3)	12 (15.4)	1 (1.3)	
RA patients $(n = 86)$	50 (58.1) <sup>b</sup>	36 (41.9)	70 (81.4)	15 (17.4)	1 (1.2)	
Male subjects						
normal subjects $(n = 65)$	32 (49.2)	30 (46.2)	51 (78.5)	13 (20.0)	1 (1.5)	
RA patients $(n = 22)$	15 (68.2)	9 (40.9)	19 (86.4)	3 (13.6)	0 (0.0)	

Null means homozygous null genotype. Values are the number (%) of subjects. GST = glutathione S-transferase. <sup>a</sup> Significant difference versus normal subjects (p = 0.011) <sup>b</sup> Significant difference versus normal subjects (p = 0.019).

hands at  $5.0 \pm 0.5$  years after disease onset: groups with the score more than 60 (n = 6), between 20 to 60 (n = 13)and less than 20 (n = 8). We examined the association of radiographic scores with GST genotype distribution and disease activity markers in these patients.

We found that serum MMP-3 levels associate with the radiographic score and GSTM1 null genotype. The group with the scores more than 60 showed significantly increased levels of serum MMP-3 and CRP compared with other groups (P = 0.03 and P = 0.004, respectively, by Mann-Whitney's U test). We have not found direct association between radiographic scores and the GST genotypes by simple and multiple regression analysis (data not shown), although the serum MMP-3 level was significantly higher in the patients with GSTM1 homozygous null genotype compared with those with the non-null genotype among these 27 patients (P = 0.001, data not shown).

## Discussion

There have been a few reports of an increased risk of RA due to the GSTM1 null genotype, whereas a number of studies have shown the association between GST polymorphisms and various types of diseases. The data presented suggests that polymorphism in the GSTM1 gene is associated with disease susceptibility to RA in the Japanese population. Recently, a study has reported that smoking was associated with the most severe disease in patients who carried the GSTM1-null polymorphism (21) and the functional allele for GSTM1 may reduce risk of RA (22) in Caucasian population. Among Asians, it has been shown that GSTM1-null polymorphism is associated with increased susceptibility to RA and risk of severe RA in Korea (23). The results of our report are consistent with this study. We also showed the association between GSTM1 null genotype and increased serum MMP-3 levels.

As previously reported, the frequency of the GSTM1 null genotype is lower in Japanese than in the Caucasians and other Asians. And the GSTT1 null genotype and the GSTP1 genotype distribution are also different from those of the Caucasians (24). Our data on the frequencies of GSTM1 null and GSTT1 null genotypes and GSTP1 genotype distribution in healthy con-



 
 Table III. GSTM1 and GSTT1 polymorphisms and laboratory manifestations in female patients with rheumatoid arthritis.

	GSTM1		GSTT1		
Female patients $(n = 86)$	non-null	null	non-null	null	
Tender joint count $(n = 86)$	4.5 (0-27)	5.0 (0-25)	5 (0-25)	4.5 (0-27)	
Swollen joint count $(n = 86)$	2 (0-24)	2 (0-23)	2 (0-22)	1.5 (0-24)	
CRP (n = 86)	23/13 (63.9)	39/11 (78.0)	36/14 (72.0)	26/10 (72.2)	
MMP-3 $(n = 71)$	17/12 (58.6)	30/12 (71.4)	29/14 (67.4)	18/10 (64.3)	
RF(n = 86)	25/11 (69.4)	33/17 (66.0)	35/15 (70.0)	23/13 (63.9)	
IgG-RF ( $n = 64$ )	5/23 (17.9)	8/28 (22.2)	7/30 (18.9)	6/21 (22.2)	
CA-RF(n = 64)	20/7 (74.1)	29/8 (78.4)	30/8 (78.9)	19/7 (73.1)	
Anti-CCP $(n = 65)$	23/5 (82.1)	31/6 (83.8)	33/6 (84.6)	21/5 (80.8)	

Values indicate median (range) for tender and swollen joint count, and positive/negative (%positive) for CRP, MMP-3, RF, IgG-RF, CA-RF and anti-CCP.

Fig. 1. Serum MMP-3 levels in female patients with GSTM1 null and non-null genotypes. Bars indicate medians of each group. P = 0.03 by Mann-Whitney's U test.

## GST polymorphisms in RA / S. Morinobu et al.

trols are consistent with findings in previous reports on the GST gene polymorphism frequency in Japanese population (24-26). Our data on Japanese population suggest that the importance of the GST genotype differ among ethnic group and among GST polymorphisms.

In the present study, we found that the GSTM1 null genotype is associated with disease susceptibility in female patients, but not in male patients. This may due to small population of the male patients in the study. Larger numbers of patients would be needed to confirm whether the GSTM1 null genotype is associated with disease susceptibility in Japanese male patients with RA. Another possible explanation is that the GST genes may have different effect on disease susceptibility in male and female patients because of the polygenic nature and the sex-related predisposing factors of the disease.

We also found that serum MMP-3 levels were significantly increased in the female patients with GSTM1 homozygous null genotype compared with those with the non-null genotype. Serum CRP levels were correlated with serum MMP-3 levels (R = 0.41, P <0.001), compatible with a previous report (27). The serum CRP levels were higher in GSTM1 null female patients, but the difference was not statistically significant, indicating that GSTM1 polymorphism might have stronger influence on MMP-3 levels. MMP-3 is abundant in the synovium and synovial fluid of RA joints, and it can degrade proteoglycans and collagens, and activate other metalloproteinases, such as MMP-2 and MMP-9, suggesting a role for MMP-3 in destruction of cartilage and bone in RA joints (28). Clinically, MMP-3 is considered to be a useful predictor of the degree of joint destruction in early RA, and a useful marker of local joint inflammation (29-32). Thus our data may indicate that GSTM1 play a role in the pathology of RA inflammation, as well as disease susceptibility. It has been reported that the rate of radiographic progression is fast in the early years of the disease and becomes decreased over time (33, 34). Also other studies reported that high disease activity, as measured by CRP, erythrocyte sedimentation, and other markers at the first year or the first five years, correlates to radiographic progression (35, 36). There is no single model to predict radiological progression of RA. We examined radiographic progression of hands of 52 patients, and for 46 patients, their course of radiographic changes fell into 3 groups. Our preliminary result was consistent with a report that demonstrated that the course of joint destruction of RA patients showed three patterns, and the increases of number of joint erosions during first five years were different among these subsets (35). Thus we divided twenty seven patients into three groups according to radiographic score at five years since onset in this study. Our finding that radiographic damage were associated with serum MMP-3 and CRP level is consistent with previous reports (31, 32). We have not found direct association between GST genotype distribution and radiographic damage probably due to the limited number of patients. A further study with a sufficient number of patients with early RA may be necessary to examine the association between GST genotypes and radiographic outcomes. In summary, we have shown that the GSTM1 gene polymorphism is one of the genetic factors to determine susceptibility to RA. In order to further clarify the role of GSTM1 null genotype, a prospective study to examine the effects of GSTM1 polymorphism on the responses to treatment with a larger patient population might be needed.

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#### GST polymorphisms in RA / S. Morinobu et al.

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