

Title: A Polymorphism in *MAPKAPK3* Affects Response to Interferon Therapy for Chronic Hepatitis C

Short Title: MAPKAPK3 and IFN therapy for HCV infection

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Grant Support:

This study was supported by a grant from the Japanese Millennium Project, and in part by Dainippon Sumitomo Pharma Co., Ltd.

Abbreviations:

cDNA, complementary DNA;

GAS, IFN- γ activated site;

HCV, hepatitis C virus;

IFN, interferon;

IFNAR1, type I IFN receptor-1;

ISRE, IFN-stimulated response element;

JAK, Janus-activated kinase;

MAP, mitogen-activated protein;

MAPKAPK, MAP kinase-activated protein kinase;

MAPKK, MAP kinase kinases;

NR, Non-responders;

SNPs, single nucleotide polymorphisms;

SOCS1, suppressor of cytokine signaling 1;

SR, sustained responders;

STAT, signal transducer and activator of transcription.

Conflict of interest:

All authors certify that we have included on the title page of this manuscript any financial arrangements (e.g., consultancies, stock ownership, equity interests, patent-licensing arrangements, research support, major honoraria, etc.) that we have with a company whose product figures prominently in the submitted manuscript or with a company making a competing product. All funding sources supporting the work are acknowledged on this title page. None of the authors have any conflicts of interest associated with this study.

Word counts:

245 words (abstract)

5965 words (including abstract, figure and table legends, and references)

The number of figures and tables:

4 tables, 2 figures, and 2 supplementary tables (online only)

Abstract

Background & Aims: This study aimed to identify host single nucleotide polymorphisms (SNPs) that are associated with the efficacy of interferon (IFN) therapy in patients with chronic hepatitis C.

Methods: We examined whether 116 tagging-SNPs from 13 genes which are involved in Type I IFN signaling associate with the outcome of IFN therapy in Japanese case-control groups; the study included 468 sustained responders and 587 non-responders.

Results: We identified 2 SNPs (rs3792323 [A/T] and rs616589 [G/A]), located in intron 2 of *mitogen-activated protein kinase-activated protein kinase 3* (*MAPKAPK3*), that were associated with the outcome of IFN therapy in patients infected with hepatitis C virus (HCV) genotype 1b ($P=4.6\times 10^{-5}$ and 4.8×10^{-5} , respectively). The 2 SNPs were in strong linkage disequilibrium and multivariate logistic regression analysis showed that rs3792323 is an independent factor associated with the IFN efficacy (genotype 1b, $P=0.0011$). *MAPKAPK3* is a kinase involved in the mitogen and stress responses, but the biological significance of *MAPKAPK3* in IFN responses is poorly understood. Using an allele-specific transcript quantification assay in liver biopsy, we demonstrated that allele-specific expression of *MAPKAPK3* mRNA, corresponding to the risk allele for non-response, was significantly higher than that of the other allele. Luciferase reporter assay data indicated that overexpression of *MAPKAPK3* inhibits IFN- α -induced gene transcription via IFN-stimulated response element and IFN- γ activated site.

Conclusions: The SNP rs3792323 in *MAPKAPK3* associates with the outcome of IFN

therapy in patients with HCV genotype 1b. Our functional analyses indicate that MAPKAPK3 inhibits IFN- α -induced antiviral activity.

Introduction

Type I interferon (IFN), including IFN- α and IFN- β , has been widely used as an antiviral agent for chronic hepatitis C. However, even after the most effective combination therapy of pegylated-IFN- α plus ribavirin, more than 50% of patients infected with hepatitis C virus (HCV) genotype 1b and approximately 20% of patients with HCV genotype non-1b fail to eradicate the virus.¹⁻³

The mechanisms of modulating the responsiveness to IFN therapy have been studied extensively. Both viral and host factors have been implicated in the resistance to IFN therapy. Viral factors, such as HCV genotype, serum HCV RNA level, and the interferon sensitivity determining region, have been reported to be associated with the outcome of IFN therapy.^{3,4,5} On the other hand, host factors including age, sex, race, liver fibrosis, and obesity have been shown to associate with the outcome of IFN therapy.^{6,7} Furthermore, it has been reported that genetic polymorphisms of cytokines, chemokines, and IFN-stimulated genes are associated with the difference in response to IFN therapy.⁷⁻¹²

Recently, genetic polymorphism of type I IFN receptor-1 (IFNAR1) promoter region was reported to be associated with the outcome of IFN therapy in patients with HCV infection.¹³ Although the mechanisms of this polymorphism for the different responsiveness to IFN therapy are still unclear, polymorphism of IFNAR1 promoter region may influence the efficacy of IFN therapy, possibly through modulation of IFNAR1 expression level. Because type I IFN elicits antiviral activity by activation of signaling molecules downstream of type I IFN receptors, genetic polymorphisms in type I IFN signaling molecules could also potentially alter the responsiveness to IFN therapy. However, so far there has been no

evidence of associations between polymorphisms of genes involved in type I IFN signal transduction and the efficacy of IFN therapy in patients with chronic hepatitis C.

In the present study, we examined whether single nucleotide polymorphisms (SNPs) in type I IFN signaling molecules are associated with the difference in response to IFN therapy in patients with chronic hepatitis C, using tagging-SNP approach in a large case-control study. The tagging-SNP serves as a marker to detect associations between a particular gene region and the outcome of IFN therapy. A small set of tagging-SNPs is sufficient to capture genetic variation, because polymorphisms which are physically close to each other have a tendency to be in linkage disequilibrium with each other.^{14,15} The HapMap online database (<http://www.hapmap.org>) allows the tagging-SNP approach to be applied readily to many genes or regions.¹⁶

As for type I IFN signaling molecules, we focused on two signaling cascades downstream of type I IFN receptors. First, we examined the Janus-activated kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, which is essential for type I IFN-induced antiviral activity.¹⁷ We selected tagging-SNPs for seven key genes in this pathway, including IFNAR1, IFNAR2, JAK1, tyrosine kinase 2, STAT1, STAT2, and IFN regulatory factor 9. Second, we also examined the p38 mitogen-activated protein (MAP) kinase pathway, which has been reported to cooperate with the JAK-STAT pathway in activation of type I IFN-induced antiviral activity.¹⁸⁻²³ We also selected tagging-SNPs for six key genes in this pathway, including ras-related C3 botulinum toxin substrate 1,¹⁸ MAP kinase kinases 3 (MAPKK3),¹⁹ MAPKK6,¹⁹ p38 MAP kinase,^{20,21} MAP kinase-activated protein kinase 2 (MAPKAPK2),²⁰⁻²³ and MAPKAPK3.²⁰⁻²²

Here, we provided genetic evidence suggesting that two SNPs in MAPKAPK3 are associated with the responsiveness to IFN therapy. The two SNPs may be useful as markers to predict the outcome of IFN therapy, which is very helpful clinically because IFN therapy is expensive and may cause serious adverse effects.²⁴ In addition, we provided functional evidences that suggest MAPKAPK3 influence IFN- α -induced antiviral activity.

Patients and methods

Study subjects and DNA preparation

We enrolled 1055 patients with chronic HCV infection who were treated with IFN monotherapy before 2001, at Toranomon Hospital, Hiroshima University Hospital, and Hiroshima University affiliated hospitals. Each patient was treated with 6×10^6 units of IFN intramuscularly every day for 8 weeks, followed by the same dose twice a week for 16 weeks, with a total dose of 528 million units. The characteristics of participating patients are described in Table 1. All patients had abnormal serum alanine transaminase levels for more than 6 months, and were positive for both anti-HCV antibody and serum HCV RNA. All patients were negative for HBs antigen, had no evidence of other liver diseases, and had not received immunosuppressive or antiviral therapy prior to enrolment in the study. Relapsed responders were excluded. Patients were classified into the following two groups: 1) sustained responders (SR). These patients had normal alanine transaminase levels and no evidence of viremia at 6 months after completion of IFN therapy; 2) Non-responders (NR). These patients never showed loss of viremia during treatment. HCV RNA levels were determined by Amplicor-monitor assay or branched-chain DNA assay, and stratified into two categories according to cut-off values which have been reported previously.^{25,26} It has been reported that having serum HCV RNA levels more than 1.0 MEQ/mL by branched-chain DNA assay or 100 KIU/mL by Amplicor-monitor assay is a predictor of poor responsiveness to IFN therapy. Histological staging was determined according to the previously described criteria using biopsy specimens of liver tissue.²⁷ All subjects in the present study were ethnically Japanese and gave written informed consent to participate in the study according to the process approved by the Ethical Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama. Genomic DNA samples were

obtained from peripheral blood of the participating patients. DNA extraction was performed according to a standard phenol-chloroform protocol.²⁸

Selection of tagging-SNPs and Genotyping

As shown in Supplementary Table 1, we selected 116 tagging-SNPs for a total set of 13 candidate genes related to type I IFN pathway, using the HapMap database (public release 21a; phase II of the January 2007 National Center for Biotechnology Information build 35 assembly; dbSNP build 125) and the Haploview program (<http://www.broad.mit.edu/mpg/haploview>). With the selection criteria of $r^2 > 0.8$ and minor allele frequency of > 0.05 for the Japanese population, tagging-SNPs were selected from all bins which cover the entire gene region from approximately 2000 bp upstream of the transcription start site to 1500 bp of the 3' untranslated region in each gene. The number of tagging-SNPs in each candidate gene was: IFNAR1, 3; IFNAR2, 6; JAK1, 8; tyrosine kinase 2, 3; STAT1, 23; STAT2, 1; IFN regulatory factor 9, 5; ras-related C3 botulinum toxin substrate 1, 7; MAPKK3, 5; MAPKK6, 35; p38 MAP kinase, 10; MAPKAPK2, 6; and MAPKAPK3, 4 (Supplementary Table 1). SNPs were genotyped by using the Invader assay²⁹ and the TaqMan assay³⁰ as described previously. The probe sets for the Invader assay were designed and synthesized by Third Wave Technologies (Madison, WI) and those for the TaqMan assay by Applied Biosystems (Foster City, CA).

SNP discovery

To identify genetic polymorphisms within the coding region of MAPKAPK3, we amplified appropriate fragments of genomic DNA from 48 patients by PCR and sequenced the products to identify SNPs using previously described methods.^{28,31}

Cells and cell culture

Human hepatoma cell line, Huh7, was purchased from RIKEN Cell Bank (Tsukuba, Japan). Huh7 cells were cultured in Dulbecco's modified minimal essential medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C under 5% CO₂.

Allele-specific transcript quantification for MAPKAPK3

Allele-specific transcript quantification was performed as described previously,^{32,33} with some modifications. Liver biopsy samples were collected from five patients with informed consent before IFN therapy for chronic hepatitis C. Total RNA was isolated using RNeasy Micro kit (Qiagen, Hilden, Germany) and treated with 5 IU/mL RNase-Free DNase I (Qiagen). First strand complementary DNA (cDNA) was prepared using SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). Genomic DNA from peripheral blood of the five patients was prepared as described above. Both cDNA and genomic DNA were amplified with specific primers for 3' untranslated region of MAPKAPK3. The primers were: forward, 5'- CCTGTGAATGCTGAGTGAGCGAGTA -3'; reverse, 5'- AGTCACCCTTTGGGTCGGGAATAGT -3'.

For determination of allele-specific MAPKAPK3 mRNA expression, probes for SNP rs1385025 (A/G) were designed and synthesized by Third Wave Technologies. The invader assays were performed in a 5 µl reaction volume containing 1×signal buffer, 1×FRET Mix (FRET22/FTRE7), 30 ng cleavase[®] VIII enzyme, 0.3 µl probe mixture (all reagents from Third Wave Technologies), and 2 ng PCR product in 96-well plate format. The thermal profile was 95°C for 5 min, followed by 40 cycles at 63°C for 1 min, and a real-time intensity of fluorescence (FAM for G allele, and VIC for A of rs1385025) was measured by use of the Mx3000P Multiplex Quantitative PCR system (Stratagene, La Jolla, CA). For the

construction of standard curves for each allele, sequential dilution of an amplified product from genomic DNA of patients with double heterozygosity for rs1385025 and rs3792323 was used. Each experiment was performed in triplicate assay at least three times.

Luciferase reporter assay

One day before transfection, 7×10^3 of Huh7 cells were seeded in a 96-well culture plate. We used two types of firefly luciferase expression vector which contain promoter element IFN-stimulated response element (ISRE) or IFN- γ activated site (GAS). Huh7 cells were transfected with both 1 ng renilla luciferase expression vector pRL-TK (Promega, Madison, WI) and 10 ng firefly luciferase expression vector pISRE-TA-Luc or pGAS-TA-Luc (BD biosciences, San Jose, CA), in conjunction with 40 ng expression plasmid pDEST51/mock (empty vector), pDEST51/ MAPKAPK3, or pDEST51/suppressor of cytokine signaling 1 (SOCS1), by use of FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN). After 24 hr, cells were stimulated with IFN- α (Dainippon Sumitomo Pharma, Osaka, Japan) for 20 hr and followed by Dual-Luciferase Assay (Promega). Firefly luciferase activity was normalized by renilla luciferase activity. Each experiment was performed at least three times. Data are expressed as mean \pm SD in triplicate assay.

Statistical analysis

We calculated allele frequencies and tested fit to Hardy-Weinberg equilibrium by the chi-square test at each SNP, using Excel software (Microsoft, Redmond, WA).³⁴ Then, we compared differences in genotype distribution of each SNP between case and control with the chi-square test and Cochran-Armitage trend test (Excel).³⁵ LD coefficients (r^2) were calculated as described previously (Excel).³⁶ The age of SR and NR was compared by the Mann-Whitney U-test. Differences in categorical data of patients of the two groups were

analyzed by the chi-square test. To evaluate internal consistency of the results demonstrated by association study, two-stage replication design was simulated by Monte Carlo method. We assessed population stratification by analyzing the data from 116 tagging-SNPs in patients with HCV genotype 1b, using a genomic control method which has been reported previously.³⁷ Multivariate logistic regression with stepwise forward selection was performed with a significance level of 0.05 for including variables, by use of the StatFlex 5.0 software package (Artec Inc., Osaka, Japan). For case-control haplotype analysis, we estimated haplotype frequencies, and tested for association by chi-square analysis to detect differences in haplotype distribution between groups, by using Haploview 3.2 software. For allele-specific transcript quantification assay, statistical differences between allelic MAPKAPK3 mRNA expression corresponding to haplotype 1 and haplotype 2 were analyzed by Mann-Whitney U-test. For luciferase reporter assay, comparisons among the three groups were analyzed by the Kruskal-Wallis test, followed by Scheffe's test to evaluate statistical differences between the two groups (StatFlex 5.0 software package).

Results

Association between tagging-SNPs in MAPKAPK3 and the outcome of IFN therapy

We searched for the association between 116 tagging-SNPs for 13 candidate genes and the outcome of IFN therapy, employing 468 SR and 587 NR subjects. We were successful in genotyping all 116 tagging-SNPs (Supplementary Table 2). The mean call rate was 99.4% across all tagging-SNPs. None of the tagging-SNPs showed a significant deviation from Hardy-Weinberg equilibrium. Since HCV genotype 1b, which is the most common in Japan, is associated with poor response to IFN treatment, we divided the patients into two subgroups according to the genotypes of HCV with which they were infected (1b vs. non-1b), and performed the comparison separately.

We found that two SNPs rs3792323 (A/T) and rs616589 (G/A), located in intron 2 of MAPKAPK3, are associated with the outcome of IFN therapy in patients with HCV genotype 1b; the T allele for rs3792323 was significantly more frequent in NR than in SR (Table 2, 33.4% vs. 22.4%, $P=5.2\times 10^{-5}$, odds ratio = 0.57, 95% confidence interval = 0.44-0.75). Similarly, the A allele for rs616589 was significantly more frequent in NR than in SR (37.8% vs. 26.4%, $P=5.6\times 10^{-5}$, odds ratio = 0.59, 95% confidence interval = 0.45-0.76).

In Table 2, the Cochran-Armitage trend test (assuming an additive model for minor allele) revealed an allele dose-dependent association of rs3792323 with the outcome of IFN therapy ($P = 4.6\times 10^{-5}$), with decreased odds ratios of 0.58 and 0.30 for AT and TT genotypes, respectively (95% confidence interval = 0.41-0.83 for AT; 0.14-0.63 for TT). Under a dominant model for the T allele of rs3792323, a significant association was also seen in patients infected with HCV genotype 1b ($P = 1.9\times 10^{-4}$, odds ratio = 0.53, 95% confidence

interval = 0.38-0.74).

Similarly, an allele dose-dependent association of rs616589 with the responsiveness to IFN therapy was revealed in Table 2 ($P = 4.8 \times 10^{-5}$), with decreased odds ratios of 0.59 and 0.33 for GA and AA genotypes, respectively (95% confidence interval = 0.42-0.84 for GA; 0.17-0.62 for AA). Under a dominant model for the A allele of rs616589, a significant association was also seen in HCV genotype 1b-infected patients ($P = 2.3 \times 10^{-4}$, odds ratio = 0.53, 95% confidence interval = 0.38-0.75).

To adjust the P values for multiple testing, we applied a Bonferroni correction with each individual SNP as an independent variable (total 116 SNPs). Despite this conservative adjustment, our results for rs3792323 and rs616589 about patients with HCV genotype 1b remained highly significant ($p < 0.05$). On the other hand, the other tagging-SNPs did not show significant associations with the outcome of IFN therapy after Bonferroni corrections (Supplementary Table 2).

Internal validation of the observed associations

To evaluate internal consistency of the results demonstrated by association study, two-stage replication design was simulated by Monte Carlo method. A half of the cases and a half of the controls were randomly selected from HCV-1b infected patients in this study, and used for the first-stage test with a significance level α_1 in the allele-frequency model. Only the SNPs that were judged to be significantly associated with the phenotype are then submitted to the second-stage test. In the second stage, the remaining independent cases and controls are used to test the association between the selected SNPs and the phenotype with a significance level α_2 . We set α_1 at 0.01, 0.02, and 0.05, and calculated α_2 as $(0.05/116)/\alpha_1$,

since the global significance level after Bonferroni's correction is 0.05/116 (total 116 SNPs). The number of iterations was 100,000 for each condition. By this way, the results of the test in the first stage are validated by the test in the second stage.

In Table 3, the proportions of significant results of SNP rs3792323 were 0.563, 0.595, and 0.557 for $\alpha=0.01$, 0.02, and 0.05, respectively. Similarly, those of SNP rs616589 were 0.554, 0.579, and 0.540 for $\alpha=0.01$, 0.02, and 0.05, respectively. These results suggested that the results of the first-stage test could be replicated in many cases if two halves of the patients were independently tested for the association.

Population stratification analysis

We assessed population stratification by analyzing the data from 116 tagging-SNPs in patients with HCV genotype 1b, using genomic control method. We estimated the inflation factor, which can effectively adjust for the confounding effect of population stratification regardless of its extent (inflation factor = 1.18, 95% confidence interval = 0.87-1.65). The corrected P values for rs3792323 and rs616589 in MAPKAPK3 were 0.00017 and 0.00018, respectively. After a Bonferroni correction, the results for the two SNPs remained highly significant ($P<0.05$). These results suggest that population stratification in our patients is negligible.

Haplotype analysis

We examined whether MAPKAPK3 haplotypes show more significant associations with the effect of IFN therapy than single marker analysis. Since the two SNPs rs3792323 and rs616589 were in strong linkage disequilibrium with an r-squared value of 0.82 in our genotype data of 1055 patients, we constructed MAPKAPK3 haplotypes from three

tagging-SNPs (rs3792323 A>T, rs3804628 G>A, rs2040397 C>T), using the haploview 3.2 software. As results, four haplotypes with frequencies above 5% were deduced in patients with HCV genotype 1b: AGC 43.9%, TGC 29.8%, AGT 20.6%, and AAC 5.7%. Although haplotype TGC was the most significantly associated with the outcome of IFN therapy in four haplotypes ($P = 0.000051$), this P value was comparable to that for single marker analysis ($P = 0.000046$ for rs3792323 in Cochran-Armitage trend test).

Results of multivariate logistic regression analysis

To determine independent factors on the outcome of IFN therapy in patients infected with HCV genotype 1b, we used multivariate logistic regression analysis with stepwise forward selection. We evaluated the following six factors: SNP rs3792323 (A allele vs. T allele), rs616589 (G allele vs. A allele), age (per year increase), sex (male vs. female), fibrosis stage (F0 to F1 vs. F2 to F4), and HCV RNA level before treatment (low vs. high).

We found that SNP rs3792323 is an independent factor associated with the IFN efficacy (Table 4, $P = 0.0011$, odds ratio = 0.29, 95% confidence interval = 0.14-0.61). On the other hand, SNP rs616589 was removed from this model, suggesting that the two SNPs are not independently associated with the outcome of IFN therapy. This is consistent with the result that the two SNPs were in strong linkage disequilibrium in our genotype data.

Next, to eliminate the effect of confounding factors, we also tried running models by forcing in above mentioned four factors (age, sex, HCV RNA level, and fibrosis stage) and SNP rs3792323 into multivariate logistic regression analysis in patients with HCV genotype 1b. We identified that SNP rs3792323 is associated with the outcome of IFN therapy ($P = 0.0014$, odds ratio = 0.30, 95% confidence interval = 0.14-0.63).

SNP discovery within the coding region of MAPKAPK3

To investigate whether there is any genetic polymorphism in MAPKAPK3 that results in amino acid substitution, we sequenced the coding region of MAPKAPK3 from genomic DNA isolated from 48 patients. We did not find any non-synonymous allelic variants in MAPKAPK3.

Allele-specific transcript quantification of MAPKAPK3

Next, we examined the possibility that SNP rs3792323 associates with MAPKAPK3 expression in liver biopsy specimens from patients with chronic hepatitis C. Because rs3792323 in MAPKAPK3 intron 2 was not present in mRNA, we selected SNP rs1385025 (A/G) in 3' untranslated region as a marker SNP. We confirmed that rs1385025 showed complete linkage disequilibrium to rs3792323 (D' values = 1), using HapMap data and Haploview program. We selected five patients who were doubly heterozygous with genotype rs1385025 A/G and rs3792323 A/T for this assay. Haplotype pairs of these patients were theoretically specified to be haplotype 1 (rs1385025A rs3792323A) and haplotype 2 (rs1385025G rs3792323T).

We measured the relative contribution of each haplotype to MAPKAPK3 transcription in these patients, using probes that detect each allele of rs1385025. As shown in Figure 1, allele-specific MAPKAPK3 mRNA expression corresponding to haplotype 2 was 1.15- to 1.76-fold higher than that of haplotype 1 ($P = 0.009$). This result indicated that SNP rs1385025 and rs3792323 associate with the expression level of MAPKAPK3.

Effects of MAPKAPK3 on IFN- α -induced gene transcription via ISRE and GAS

elements

We tested whether transient overexpression of MAPKAPK3 influences IFN- α -induced gene transcription via ISRE and GAS elements which are essential promoter elements for type I IFN-induced antiviral activity, by use of luciferase reporter assay. When MAPKAPK3 was overexpressed in Huh7 cells, IFN- α -induced luciferase activities via ISRE and GAS elements were significantly suppressed by various doses of IFN- α , compared with the negative control (Fig. 2). Similar results were obtained in SOCS1, which has been reported to suppress IFN- α -induced gene expressions. In addition, we also overexpressed beta-galactosidase gene as a negative control. In comparison with this control, MAPKAPK3 also significantly inhibited IFN- α -induced luciferase activities via ISRE and GAS elements (data not shown). These results suggest that MAPKAPK3 can inhibit IFN- α -induced gene transcription via ISRE and GAS elements.

Discussion

We identified that SNP rs3792323 (A/T) and rs616589 (G/A), located in MAPKAPK3, are associated with the outcome of IFN therapy in patients infected with HCV genotype 1b (Table 2). In our genotype data of 1055 patients, the two SNPs were in strong linkage disequilibrium with an r-squared value of 0.82. Multivariate logistic regression analysis showed that rs3792323 is an independent factor associated with the IFN efficacy (Table 4).

MAPKAPK3 is expressed in every human tissue.³⁸ MAPKAPK3 encodes a serine/threonine specific protein kinase and functions as a mitogen-activated protein kinase-activated protein kinase in both mitogen and stress responses.³⁹ MAPKAPK3 shares 72% nucleotide and 75% amino acid identity with MAPKAPK2. MAPKAPK3 and MAPKAPK2 act as downstream kinases of p38 MAP kinase under type I IFN stimulation.²² It has been shown that disruption of p38 α MAP kinase gene results in defective transcription of genes that are regulated by ISRE and GAS elements.²² It was also reported that pharmacological inhibition of p38 MAP kinase partially inhibits type I IFN-induced antiviral activity.²¹ In mouse embryonic fibroblasts with targeted disruption of MAPKAPK2, it was indicated that type I IFN-induced antiviral activity was decreased.²² On the other hand, little is known about the role of MAPKAPK3 in the responses to type I IFN.

In the present study, we hypothesized that enhanced expression of MAPKAPK3 is associated with resistance to IFN therapy, for the following three reasons. First, carriers of the T allele for rs3792323, rather than the A allele, were more likely to show NR to IFN therapy (Tables 2 and 4). Thus, the T allele for rs3792323 was considered as a risk allele for non-response. Similarly, the A allele for rs616589 was also considered as a risk allele for non-response.

Second, allele-specific MAPKAPK3 mRNA expression corresponding to the T allele for rs3792323 (risk allele for non-response) was significantly higher than that of the A allele for rs3792323 in liver biopsy specimens of participating patients (Fig. 1). Third, we did not find any non-synonymous allelic variants in MAPKAPK3 from the analysis of genomic DNA from 48 patients.

To examine our hypothesis, we examined whether enhanced MAPKAPK3 expression influences IFN- α -induced gene transcription. In reporter gene assay, overexpression of MAPKAPK3 inhibited IFN- α -induced gene transcription via ISRE and GAS elements (Fig. 2), suggesting that MAPKAPK3 play an important role in inhibition of IFN- α -induced antiviral activity. Several downstream effectors of MAPKAPK3 have been reported, including actin-binding protein, such as heat shock protein 27,³⁹ and transcription factors, such as basic helix-loop-helix transcription factor E47⁴⁰ and cyclic AMP responsive element binding protein.⁴¹ However, the mechanisms by which MAPKAPK3 inhibits IFN- α -induced gene transcription are still unclear, and further investigations are required. It is also interesting to examine the allele-specific MAPKAPK3 mRNA levels during interferon therapy. Although we have no direct information, MAPKAPK3 mRNA expression is not inducible by IFN- α stimulation in human hepatoma cell line Huh-7 and HepG2 (data not shown).

In this study, the association between the MAPKAPK3 polymorphisms and the efficacy of IFN therapy was observed in HCV genotype 1b, but not found in genotype non-1b. The reason for this difference between the two groups is yet to be seen. As one possible reason, the high susceptibility of genotype non-1b to IFN treatment may make the fine effect of SNPs obscure. As another possible explanation, the effect of MAPKAPK3 on IFN efficacy may

vary among different HCV genotypes. It was reported that associations between SNPs in the osteopontin gene and the efficacy of IFN therapy were particularly evident in patients with genotype 1b and a high virus titer, rather than in patients with genotype non-1b.²⁶ Until now, in various HCV genotypes including 1a, 1b, and 2a, HCV subgenomic replicon cell lines, which exhibit autonomous HCV RNA replication in human hepatoma cell line, have been established. In the case that MAPKAPK3 is overexpressed in these HCV replicon cell lines, it is interesting to examine whether the effect of MAPKAPK3 on IFN efficacy is different or not among these HCV genotypes. It is also important to test associations between the MAPKAPK3 SNPs and the IFN efficacy in each subgroup of patients infected with each HCV genotype including 1a, 2a, and 2b.

Recently, it has been reported that the combination treatment of IFN- α plus ribavirin results in higher rates of sustained response than IFN monotherapy.^{2,3} Various mechanisms of ribavirin activity against HCV have been proposed.⁴² However, it is notable that treatment with ribavirin alone has no effect on serum HCV RNA level.^{43,44} On the other hand, the addition of ribavirin to IFN- α monotherapy leads to marked reduction of serum HCV RNA level. These facts suggest that IFN- α signaling is important for the induction of antiviral activity not only in IFN- α monotherapy but also in IFN- α combination therapy with ribavirin. Therefore, it is likely that the inhibitory effect of MAPKAPK3 on IFN signaling influences the efficacy of IFN- α combination therapy as well as IFN- α monotherapy. At present, about 50% of patients infected with HCV genotype 1b fail to eradicate the virus even after combination therapy of IFN- α plus ribavirin.¹⁻³ It is important to examine whether the two SNPs are associated with the responsiveness to combination therapy of IFN- α plus ribavirin.

It remains to be seen whether or not rs3792323 and rs616589 are functional cis-acting

polymorphisms affecting MAPKAPK3 expression. Even if the two SNPs do not have functional effects, it is expected that the two SNPs can serve as marker SNPs in linkage disequilibrium with functional cis-acting polymorphisms.⁴⁵ Furthermore, the two SNPs may be useful as genetic markers to predict the efficacy of IFN therapy. In our patients with IFN- α monotherapy, patients with risk alleles for non-response (T allele for rs3792323, A allele for rs616589) were more likely to be NR compared with risk allele-negative patients (Tables 2 and 4). Although the results of our internal validation suggested that the observed association between the two SNPs in MAPKAPK3 and the IFN efficacy is internally consistent, further replication with an independent cohort is needed to confirm the association. It is also interesting to test associations between the two SNPs and the phenotypes in relapsed patients. At present, we do not enroll enough number of these patients to examine the association. In future, we will enroll enough number of patients and test the association.

It was reported that polymorphism of GT-repeat length in IFNAR1 promoter region associated with the outcome of IFN therapy for chronic HCV infection, in a study of 157 Japanese patients (HCV genotype total, $P=0.008$).¹³ In our study, we could not find a similar association for analyzed tagging-SNPs in IFNAR1 (Supplementary Table 2). The reason for discrepancy between the two studies is not clear at present. Unfortunately, the GT-repeat polymorphism in IFNAR1 is not included in the HapMap database. Therefore, it is not clear whether tagging-SNPs have strong linkage disequilibrium with the GT-repeat polymorphism in IFNAR1. Possibly, tagging-SNPs may not capture the GT-repeat polymorphism in IFNAR1. To explain the discrepancy between the present study and the previous one, it is desirable to genotype the GT-repeat polymorphism in IFNAR1 by direct sequencing.

In conclusion, we identified that SNP rs3792323 in MAPKAPK3 is strongly associated with the outcome of IFN therapy in patients infected with HCV genotype 1b. In addition, we demonstrated that SNP rs3792323 associates with the expression level of MAPKAPK3 and MAPKAPK3 inhibits IFN- α -induced gene transcription via ISRE and GAS elements. Therefore, MAPKAPK3 may play an important role in inhibition of IFN- α -induced antiviral activity.

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Tables

Table 1. Characteristics of patients with chronic hepatitis C.

| | Sustained responders | Non-responders | <i>P</i> value |
|---------------------|----------------------|----------------|----------------|
| Number of patients | 468 | 587 | - |
| Age (years) mean±SD | 54.6±11.8 | 55.9±10.3 | 0.1* |
| Sex | | | 0.002 |
| Male | 325 | 354 | |
| Female | 143 | 233 | |
| HCV genotype | | | <0.0001† |
| 1a | 3 | 3 | |
| 1b | 208 | 434 | |
| 2a | 209 | 101 | |
| 2b | 48 | 49 | |
| HCV RNA level¶ | | | <0.0001‡ |
| High | 177 | 420 | |
| Low | 216 | 69 | |
| No data | 75 | 98 | |
| Fibrosis stage | | | 0.19§ |
| F0 | 5 | 5 | |
| F1 | 213 | 254 | |
| F2 | 130 | 173 | |
| F3 | 24 | 43 | |
| F4 | 17 | 28 | |
| No biopsy | 79 | 84 | |

*By Mann-Whitney U test. †‡§By chi-square test.

†between HCV genotype 1b and non-1b, ‡between HCV RNA level High and Low, §between F0-1 and F2-4.

¶Low HCV RNA level: <100 KIU/mL by Amplicor-monitor assay and <1.0 MEQ/mL by branched-chain DNA assay.

HCV, hepatitis C virus.

Table 2. Associations between the two SNPs in MAPKAPK3 and the outcome of IFN therapy.

| dbSNP ID | Allele 1/2 | HCV genotype | Number of patients | Allele frequency, % | | P value | OR (95% CI) | Genotype (%) | | | Cochran-Armitage trend test. P value | OR (95% CI) | | Dominant model for allele 2 (11 vs. 12, 22) | | Recessive model for allele 2 (11, 12 vs. 22) | | |
|-------------|---------------|-----------------|-----------------------|---------------------|---------------------|---------------------|---------------------|---------------|---------------------|---------------|--|---------------------|---------------------|--|---------------------|---|---------------------|---------------------|
| | | | | 1 | 2 | | | 11 | 12 | 22 | | 11 VS. 12 | 11 VS. 22 | P value | OR (95% CI) | P value | OR (95% CI) | |
| | | | | | | | | | | | | | | | | | | |
| rs3792323 | A/T | total | SR (n=468) | 71.8 | 28.2 | 0.088 | 0.85 (0.70-1.02) | 242 (51.8) | 187 (40.0) | 38 (8.1) | 0.089 | 0.83 (0.65-1.08) | 0.74 (0.47-1.15) | 0.10 | 0.82 (0.64-1.04) | 0.32 | 0.80 (0.52-1.23) | |
| | | | NR (n=587) | 68.4 | 31.6 | | 273 (46.7) | 253 (43.3) | 58 (9.9) | | | | | | | | | |
| | | | 1b | SR (n=208) | 77.6 | | 22.4 | 0.000052 | 0.57 (0.44-0.75) | 124 (59.6) | | 75 (36.1) | 9 (4.3) | | 0.000046 | | 0.58 (0.41-0.83) | 0.30 (0.14-0.63) |
| | NR (n=434) | 66.6 | 33.4 | 189 (43.9) | 196 (45.5) | 46 (10.7) | | | | | | | | | | | | |
| | non-1b | SR (n=260) | 67.2 | 32.8 | 0.056 | 1.36 (0.99-1.86) | 118 (45.6) | | 112 (43.2) | 29 (11.2) | 0.06 | 1.40 (0.92-2.14) | 1.72 (0.83-3.57) | 0.067 | | 1.45 (0.97-2.17) | 0.27 | 1.48 (0.73-2.99) |
| | NR (n=153) | 73.5 | 26.5 | 84 (54.9) | | 57 (37.3) | 12 (7.8) | | | | | | | | | | | |
| | rs616589 | G/A | total | SR (n=468) | | 68.0 | 32.0 | 0.034 | 0.82 (0.68-0.99) | 215 (46.0) | | 205 (43.9) | 47 (10.1) | | 0.033 | 0.83 (0.64-1.07) | | 0.67 (0.44-1.00) |
| | NR (n=587) | | | 63.6 | 36.4 | 235 (40.3) | 271 (46.5) | | 77 (13.2) | | | | | | | | | |
| | 1b | | | SR (n=208) | 73.6 | 26.4 | 0.000056 | | 0.59 (0.45-0.76) | 111 (53.4) | 84 (40.4) | 13 (6.3) | 0.000048 | 0.59 (0.42-0.84) | | 0.33 (0.17-0.62) | 0.00023 | 0.53 (0.38-0.75) |
| NR (n=434) | 62.2 | 37.8 | 164 (38.0) | 209 (48.4) | 59 (13.7) | | | | | | | | | | | | | |
| non-1b | SR (n=260) | 63.5 | 36.5 | 0.24 | 1.20 (0.89-1.61) | 104 (40.2) | | 121 (46.7) | 34 (13.1) | 0.25 | 1.33 (0.87-2.05) | 1.29 (0.68-2.46) | | 0.18 | 1.32 (0.88-1.98) | 0.72 | | 1.12 (0.61-2.06) |
| NR (n=153) | 67.5 | 32.5 | 71 (47.0) | | 62 (41.1) | 18 (11.9) | | | | | | | | | | | | |

P values were calculated from case-control analysis by chi-square test and unadjusted for multiple testing. ORs of having a sustained response to IFN therapy were calculated. Allele 1 and allele 2 denote a major and a minor allele, respectively.

SR, sustained responder; NR, non-responder; OR, odds ratio; CI , confidence interval.

Table 3. Internal validation analysis of the observed associations between the two SNPs in MAPKAPK3 and the IFN efficacy in patients with HCV genotype 1b.

| SNP | Alpha1 | Proportion of significant results | 95% CI |
|-----------|--------|-----------------------------------|---------------|
| rs3792323 | 0.01 | 0.563 | 0.560 - 0.566 |
| | 0.02 | 0.595 | 0.591 - 0.598 |
| | 0.05 | 0.557 | 0.554 - 0.560 |
| rs616589 | 0.01 | 0.554 | 0.551 - 0.557 |
| | 0.02 | 0.579 | 0.576 - 0.582 |
| | 0.05 | 0.540 | 0.537 - 0.543 |

Alpha1, a significance level for the first-stage test; CI, confidence interval.

Table 4. Predictive factors independently associated with the response to IFN therapy in patients with HCV genotype 1b by multivariate logistic regression analysis.

| Variable | HCV genotype 1b | | |
|--------------------------------|-----------------|------|------------|
| | <i>P</i> value | OR | 95% CI |
| rs3792323 (T allele/ A allele) | 0.0011 | 0.29 | 0.14-0.61 |
| Age (per year increase) | 0.0096 | 0.97 | 0.95-0.99 |
| Fibrosis stage (F0-1/F2-4) | 0.035 | 1.66 | 1.04-2.66 |
| HCV RNA level (low/high) * | <0.00001 | 8.25 | 5.05-13.50 |

*Low HCV RNA level: <100 KIU/mL by Amplicor-monitor assay and <1.0 MEQ/mL by branched-chain DNA assay. ORs of having a sustained response to IFN therapy were calculated.

OR, odds ratio; CI, confidence interval.

Figure legends

Fig. 1. Allele-specific transcript quantification of MAPKAPK3. Allele-specific MAPKAPK3 mRNA expression ratio for haplotype 2 to haplotype 1 are shown. Individual data from five patients liver biopsy are indicated. Each experiment was performed in triplicate assay at least three times. Data represent mean \pm SD.

Fig. 2. Effects of MAPKAPK3 on IFN- α -induced gene transcription via ISRE and GAS elements. Huh7 cells were transfected with both 1 ng renilla luciferase expression vector pRL-TK (internal control) and 10 ng firefly luciferase expression vector pISRE-TA-Luc (A) or pGAS-TA-Luc (B), in conjunction with 40 ng expression plasmid pDEST51/mock (negative control, open bar), pDEST51/SOCS1 (positive control, shaded bar), or pDEST51/MAPKAPK3 (hatched bar). After 24 hr, cells were stimulated with IFN- α for another 20 hr, and luciferase induction was measured. Firefly luciferase activity was normalized by renilla luciferase activity. Data represent mean \pm SD of triplicate assay. (* P <0.05 for comparison with mock). ISRE, interferon-stimulated response element; GAS, interferon γ -activated site; SOCS1, suppressor of cytokine signaling 1.

FOOTNOTE*Acknowledgment:*

The authors thank the patients who generously agreed to participate in this study. We also thank the team members at Toranomon Hospital, Hiroshima University Hospital, and Hiroshima Liver Study Group for clinical sample collection. We acknowledge Dr. N. Osawa and Dr. Y. Nagasaka for helpful discussion; Y. Kikuchi, M. Habata, M. Yahata, and T. Hirooka for technical assistance; and other members of the SNP Research Center for assistance with various aspects of this study.

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Figure

Fig. 1.

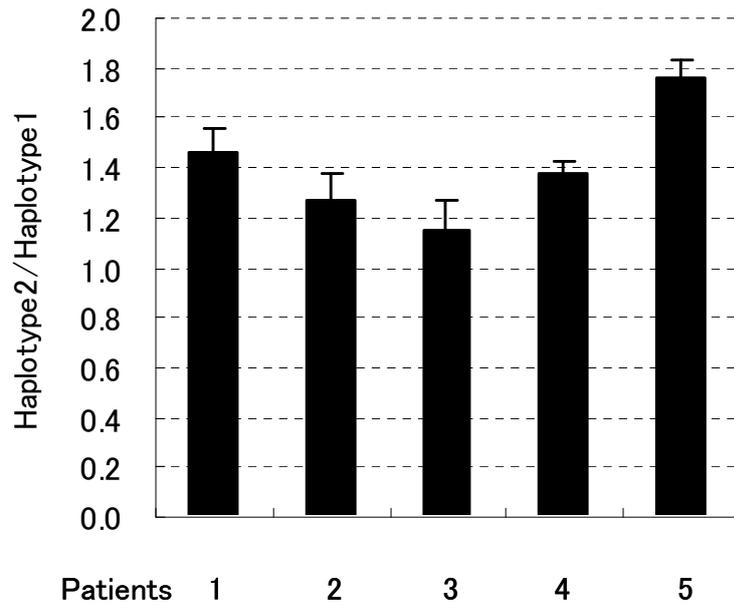


Fig. 2A.

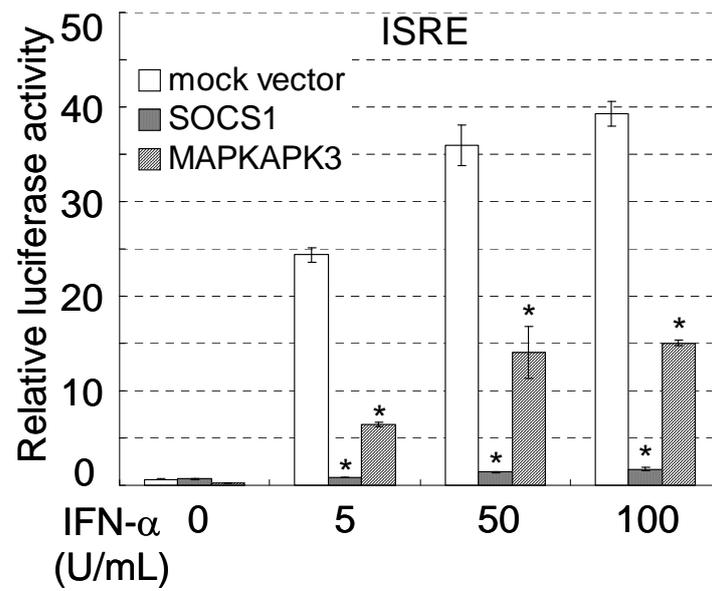


Fig. 2B.

