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ORIGINAL REPORT

STAT3 Polymorphism Predicts Interferon-Alfa Response in Patients With Metastatic Renal Cell Carcinoma

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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ABSTRACT

Purpose
To clarify the effect of genetic polymorphisms on the response to interferon alfa (IFN-α) for metastatic renal cell carcinoma (MRCC), and to find a reliable molecular marker to select those patients with MRCC who would benefit from IFN-α immunotherapy.

Patients and Methods
We carried out an association study in which 463 single nucleotide polymorphisms (SNPs) in 33 candidate genes were genotyped in 75 Japanese patients who had received IFN-α for MRCC.

Results
After adjusting for lung metastasis, stepwise logistic regression analysis revealed that the SNPs in signal transducer and activator 3 (*STAT3*) were most significantly associated with better response to IFN-α. Linkage disequilibrium mapping revealed that the SNP in the 5′ region of STAT3, rs4796793, was the most significant predictor of IFN-α response (odds ratio [OR] = 2.73; 95% CI, 1.38 to 5.78). The highest OR was shown in the CC genotype at rs4796793 compared to the GG + GC genotypes (OR = 8.38, 95% CI, 1.63 to 42.96). Genotype-dependent expressions of STAT3 in B lymphocyte cell lines and the enhanced growth inhibitory effects of IFN-α by STAT3 suppression in an RCC cell line supported the results of the present association study.

Conclusion
The present study suggested that the STAT3 polymorphism is a useful diagnostic marker to predict the response to IFN-α therapy in patients with MRCC. An efficient response marker for IFN-α needs to be utilized to establish individual optimal treatment strategies, even when newer drug therapies are used as first line treatments for MRCC.

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INTRODUCTION

Renal cell carcinoma (RCC) accounts for approximately 3% of all malignancies. Its age-adjusted incidence rate per 100,000 individuals was 12.4 in the United States in 2002[1]; in Japan in 1997, the rate was 4.9 and 1.8 in the male and female populations, respectively.[2] Conventional chemotherapy is not effective in patients with metastatic RCC (MRCC); therefore, immunotherapy with interferon alfa (IFN-α) and/or interleukin-2 (IL-2) has been employed to improve survival. However, the response rate of MRCC to IFN-α therapy is 5% to 20%, and median survival is reported to be approximately 67.6 weeks.[3] Considering the low response rate and substantial adverse effects associated with IFN-α therapy, identification of reliable predictive markers for response to IFN-α is essential for establishing optimal treatment strategies for patients with MRCC.

The human genome sequence database and high throughput methods of single nucleotide polymorphism (SNP) typing have made it possible to quickly and easily analyze a large number of genomic polymorphisms. To identify a genetic marker to predict response to IFN-α therapy, we performed an association study of 463 SNPs on 33 candidate genes, and found that SNPs in the 5′-flanking region of signal transducer and activator 3 (*STAT3*) had the most significant association with responsiveness. We also showed that *STAT3* expression is reduced in the genotype responding to IFN-α, and suppression of *STAT3* by siRNA enhanced the inhibitory effect of IFN-α on cell growth in vitro.

PATIENTS AND METHODS

Case-Control Association Study

This study was approved by the ethics committees of the all clinical sites and Otsuka Pharmaceutical (Tokushima, Japan). All patients gave written informed consent. Patients were classified as complete response (CR), partial response (PR), no change, or progressive disease, on the basis of the percentage reduction of the tumor mass in response to three dosages of 3 to 6 million U per week of IFN-α treatment.[4] CR and PR with a sustained response for 4 weeks were pooled into the responders group and the others into the nonresponders group. To determine the sample size, it was assumed that the proportion of responders among patients with a minor allele (a) of a specific SNP was 50%, whereas that with a major allele (A) was 12.5%. The proportion of type a in patients was assumed to be 20% and that of type A was 80%. These assumptions led to sample sizes of 29 being required for both case and control groups if the power of the test for the association of the SNPs with the end point was to be 80%.

Polymorphism Genotyping

Genomic DNA was extracted from whole blood of RCC patients using PUREGENE Blood DNA Isolation Kit (Gentra Systems, Minneapolis, MN). The SNP genotyping was based on the invader assay (Third Wave Technologies, Madison, WI) combined with a polymerase chain reaction (PCR).[5] Genotyping procedures by invader assay were performed in 384-well plates using an automated robotic SNP typing system constructed with SAMI/Biomek FX robotics (Beckman Coulter, Fullerton, CA). The SNPs that failed to be genotyped by the invader assay were retyped using PCR-restriction fragment-length polymorphism analysis.

Statistical Analysis

Association between response to IFN-α and genetic polymorphism were analyzed using Pearson's χ^2 test for the contingency table. Here, $P \leq .10$ was regarded as significant, instead of .05, as the power of the test was likely to decrease considerably, because of patient heterogeneity.[6] All P values were two sided. In the multivariate analysis, $P \leq .05$ was considered significant. Stepwise logistic regression analysis was performed, to evaluate the predictive significance of the SNPs after adjusting for clinical factors, until nonconvergence in the iteration algorithm for the maximum likelihood estimate was observed. This phenomenon indicated that it was highly correlated with those already in the model,[7] and thus should not be included. Statistical analyses were performed with SAS 8.2 (SAS Institute, Cary, NC).

Linkage Disequilibrium Analysis

Pairwise linkage disequilibrium (LD) was estimated using SNPAlyze v3.2 software (DYNACOM, Chiba, Japan) as $D = x_{11}\text{-}p_1q_1$, where x_{11} is the frequency of haplotype A_1B_1, and p_1 and q_1 are the frequencies of alleles A_1 and B_1 at loci A and B, respectively. A standardized LD coefficient, r, is given by $D/(p_1p_2q_1q_2)^{1/2}$, where p_2 and q_2 are the frequencies of the other alleles at loci A and B, respectively.[8] Lewontin's coefficient, D', is given by $D' = D/D_{max}$, where $D_{max} = min\ (p_1q_2, p_2q_1)$ when D is less than 0 or $D_{max} = min\ (p_1q_1, p_2q_2)$ when D is more than 0.[9]

Cell Lines

Thirty-two B-lymphocyte cell lines transformed by Epstein-Barr virus were established from healthy volunteers or were purchased from the Health Science Research Resources Bank at the Japan Health Sciences Foundation (Tokyo, Japan).

Real-Time PCR

Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using the oligo dT_{20} primer and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA). Synthesized cDNA was used for real-time PCR. Real-time PCR experiments were performed with ABI PRISM 7000 Sequence Detection System device (Applied BioSystems [ABI], Foster City, CA) using TaqMan Universal PCR Master Mix (ABI) and TaqMan Gene Expression Assays designed for *STAT3* (Hs00234174, ABI) or SOCS3 expression (Hs00269575, ABI). The correlation was tested by Pearson's correlation analysis using JMP 3.21J (SAS Institute).

RNA Interference

Validated Stealth RNAi DuoPak and Stealth RNAi Negative Control Duplexes (Invitrogen) were used in our RNAi experiments. Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. Cells were seeded again 6 hours after transfection and used for growth assay, Western blotting, and quantitative real-time PCR.

Interferon-Stimulated Response Element Luciferase Reporter Assay

The HEK293 cells were cotransfected with the *STAT3* siRNA reagent and the reporter vector, phosphorylated interferon-stimulated response element luciferase (pISRE-Luc; Stratagene, La Jolla, CA), or control vector, cytomegalovirus promoter-driven renilla luciferase (Promega, Madison, WI). These cotransfected cells were cultured in medium with or without interferon-α (100 U/mL) for 9 hours. The luciferase activities in each cell were measured and the relative activities were presented as described earlier herein.

RESULTS

Selection of Candidate Genes and Polymorphisms

Based on published papers on IFN-α function, 33 genes related to signal transduction via IFN-α receptors (11 genes), Th1/Th2 balance (10 genes), IFN-α induced gene expression (six genes), and pathogenesis of RCC were selected as candidate genes (Table 1). In the publicly available single nucleotide polymorphisms database (National Center for Biotechnology Information [NCBI]), 1,167 SNPs were registered in the 33 genes.

Genotyping and Association Analysis

DNA samples were extracted from 75 Japanese RCC patients who had received IFN-α therapy, and these were divided into two groups (29 responders and 46 nonresponders) using the criteria described in Patients and Methods. A total of 1,167 SNPs in the 33 genes were genotyped; only 463 SNPs turned out to be polymorphic, probably due to interracial genetic differences. Pearson's χ^2 test for the contingency table revealed that IFN-α response was associated with the genotypes of the 23 SNPs ($P < .1$). Because this is an exploratory study, no adjustments have been made for multiple comparisons. Redundant SNPs were eliminated on the basis of the result of Cramer's V statistics analysis, and multivariate logistic regression analysis was applied to the consequent 17 SNPs and 12 clinical factors.

The analysis indicated that the presence or absence of metastasis in the lung was the only factor that was significantly associated with IFN-α response. Next, to search for a set of SNPs that have mutually independent information for discriminating between the cases and controls after adjusting for metastases, stepwise logistic regression analysis was applied. The analysis revealed that *STAT3-2*, *STAT3-0*, *SOCS3-1*, *IL4R-34*, *PTGS1-4*, *PTGS1-5*, *ICSBP-38*, *PTGS1-3*, *PTGS2-12*, *TAP2-5*, and *IRF2-67* were significantly associated with the end point after adjusting for confounders; among these, *STAT3-2* was the most significant.

LD Mapping of STAT3

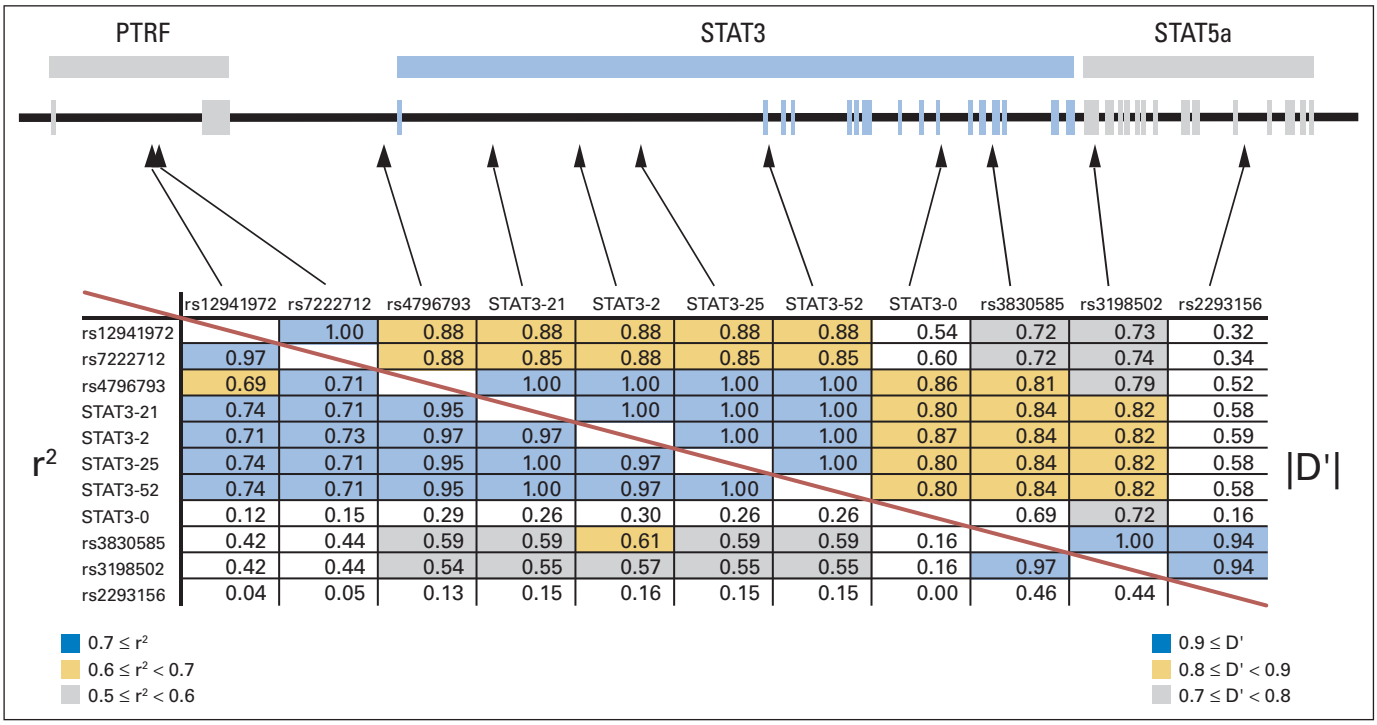
STAT3-2, which was located in the first intron and in LD with *STAT3-21*, *STAT3-25*, and *STAT3-52*, was the most significant genetic factor associated with response to IFN-α; therefore, we analyzed the *STAT3* gene more in detail: an attempt to locate a polymorphism that affected *STAT3* expression or function. In addition to searching in publicly available databases, we carried out extensive direct sequencing of *STAT3* in 12 individuals that covered the entire 24 exons, along

Table 1. 463 SNPs on 33 Candidate Genes Genotyped in 75 Patients

Gene Symbol	Gene Name	No. of SNPs
Related to interferon receptor and signal transduction		
IFNAR1	Interferon receptor 1	31
IFNAR2	Interferon receptor 2	22
JAK1	Janus kinase 1 (a protein tyrosine kinase)	56
STAT1	Signal transducer and activator of transcription 1, 91 kDa	15
STAT2	Signal transducer and activator of transcription 2, 113 kDa	0
STAT3	Signal transducer and activator of transcription 3	12
ISGF3G	Interferon-stimulated transcription factor 3	2
SOCS1	Suppressor of cytokine signaling 1/SSI-1	1
SOCS2	Suppressor of cytokine signaling 2/STATI2	6
SOCS3	Suppressor of cytokine signaling 3/SSI-3	2
PTPN11	Protein tyrosine phosphatase, nonreceptor type 11 (Noonan syndrome 1)	3
Related to Th1/Th2 balance		
STAT4	Signal transducer and activator of transcription 4	12
IL2	Interleukin-2	3
IFNG	Interferon gamma	7
TNF	Tumor necrosis factor	4
LTA	Lymphotoxin alpha	9
IL4	Interleukin-4	18
IL4R	Interleukin-4 receptor	21
IL5	Interleukin-5	1
IL6	Interleukin-6	5
IL13	Interleukin-13	2
Affected with interferon stimulation		
PRKR	Protein kinase, interferon-inducible double stranded RNA dependent	6
IRF2	Interferon regulatory factor 2	39
ICSBP	Interferon consensus sequence binding protein 1	30
PTGS1	Prostaglandin-endoperoxide synthase 1	6
PTGS2	Prostaglandin-endoperoxide synthase 2	11
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	57
Others		
TAP1	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	14
TAP2	Transporter 2, ATP-binding cassette, subfamily B (MDR/TAP)	47
PSMB8	Proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional protease 7)	7
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	4
GSTT1	Glutathione *S*-transferase theta 1	5
VEGF	Vascular endothelial growth factor	5
Total		463

Abbreviations: SNP, single nucleotide polymorphisms; ATP, adenosine 5′-triphosphate; MDR, multidrug resistance; TAP, transporter.

with their 100-base-pair flanking intron sequences, and the regions spanning 2.2 kb upstream of exon 1, and found an additional 59 SNPs. Among these SNPs, only two had a minor allele frequency more than 0.1, which was our criterion for use in LD block analysis. To estimate the LD block in the *STAT3* gene, pairwise LD was calculated among SNPs with a minor allele frequency more than 0.1 in the *STAT3* genes and the genes next to *STAT3* [polymerase I and transcript release factor (*PTRF*) and *STAT5a*]. Although the LD block appeared to cover the neighboring genes, D' and r^2 values among SNPs in *STAT3* were much higher than those in the neighboring genes (Fig 1). Moreover, SNPs in *PTRF* and *STAT5a* showed only a weak association with response to IFN-α. Therefore, we concluded that *STAT3* is a candidate gene for acting as a determinant factor for IFN-α response. Among the SNPs in tight LD ($|D'| = 1.0, r^2 > 0.9$) with *STAT3-2*, rs4796793, which was located in the 5′-flanking region, showed the most significant association with IFN-α response. The minor allele frequency of rs4796793 was 32.6% for nonresponders and 56.9% for responders (nonresponders *v* responders; $\chi^2 = 8.61$, $P = .0033$). The homozygous carriers of the minor allele also showed significant association (nonresponders *v* responders; $\chi^2 = 8.31$; $P = .0039$) and the highest odds ratio (OR; OR = 8.38, 95% CI, 1.63 to 42.96; Fig 2). According to the NCBI Database (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=4796793), in Japanese, Chinese, and white populations, the proportions of CC of rs4796793 were 0.159, 0.133, and 0.034; CG were 0.500, 0,400, and 0.390; and GG were 0.341, 0.467, and 0.576, respectively. The proportion in the general Japanese population was similar to those in the RCC cases in this study (where the frequencies of CC, CG, and GG were 0.133, 0.573, and 0.293, respectively).



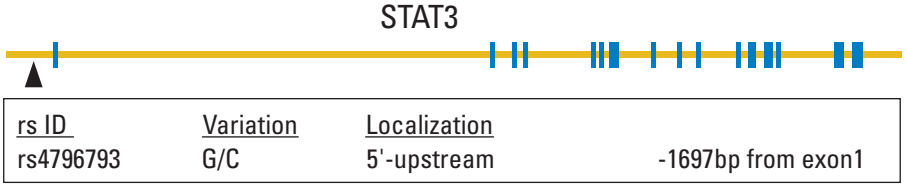
	rs12941972	rs7222712	rs4796793	STAT3-21	STAT3-2	STAT3-25	STAT3-52	STAT3-0	rs3830585	rs3198502	rs2293156
rs12941972		1.00	0.88	0.88	0.88	0.88	0.88	0.54	0.72	0.73	0.32
rs7222712	0.97		0.88	0.85	0.88	0.85	0.85	0.60	0.72	0.74	0.34
rs4796793	0.69	0.71		1.00	1.00	1.00	1.00	0.86	0.81	0.79	0.52
STAT3-21	0.74	0.71	0.95		1.00	1.00	1.00	0.80	0.84	0.82	0.58
STAT3-2	0.71	0.73	0.97	0.97		1.00	1.00	0.87	0.84	0.82	0.59
STAT3-25	0.74	0.71	0.95	1.00	0.97		1.00	0.80	0.84	0.82	0.58
STAT3-52	0.74	0.71	0.95	1.00	0.97	1.00		0.80	0.84	0.82	0.58
STAT3-0	0.12	0.15	0.29	0.26	0.30	0.26	0.26		0.69	0.72	0.16
rs3830585	0.42	0.44	0.59	0.59	0.61	0.59	0.59	0.16		1.00	0.94
rs3198502	0.42	0.44	0.54	0.55	0.57	0.55	0.55	0.16	0.97		0.94
rs2293156	0.04	0.05	0.13	0.15	0.16	0.15	0.15	0.00	0.46	0.44	

Fig 1. Linkage disequilibrium (LD) pattern of the *STAT3* gene. The upper section shows the genomic region, including *STAT3* and its neighboring genes, and the locations of the SNPs with allele frequencies more than 0.1. The lower section shows pairwise LD in the region. Rs4796793, located in the 5′ region upstream of the gene, was in tight LD ($|D'| = 1.0$, $r^2 > 0.9$) with *STAT3-21* (rs1026916), *STAT3-2* (rs1905341), *STAT3-25* (rs744166), and *STAT3-52* (rs2306581) associated with the IFN-α response in the association study.

Effect of rs4796793 on STAT3 *Transcription*

Because rs4796793 was located in the 5′-flanking region of the gene, we next examined the direct effect of rs4796793 on *STAT3* promoter activity by luciferase reporter assay. Although both of the inserted sequences containing the SNP site enhanced the transcription of the reporter gene, no significant difference in the reporter activity between the alleles was observed in either unstimulated (Fig A1, online only) or IL-6–stimulated conditions (data not shown). This result suggested that rs4796793 itself had no direct influence on *STAT3* promoter activity.

Under the hypothesis that unidentified SNPs, or a combination of SNPs, in LD with rs4796793 might affect *STAT3* mRNA level, we genotyped 32 B lymphocyte cell lines. We then compared *STAT3* mRNA expression levels assessed by real-time PCR among the three groups; namely, major homozygote (G/G), heterozygote (G/C) and minor homozygote (C/C). Pearson's correlation analysis revealed a significant correlation between *STAT3* expression and rs4796793 SNP genotype ($R^2 = 0.14$; $P = .0375$; Fig 3). The C allele, which was observed more frequently in IFN-α responders, had a tendency for lower *STAT3* expression. These results suggested that unidentified



rs ID	Variation	Localization	
rs4796793	G/C	5'-upstream	-1697bp from exon1

Allele/ Genotype	Count NON	Count RES	Frequency NON	Frequency RES		χ^2	*P*	Odds	95% CI
G	62	25	0.67	0.43	G *v* C	8.614	.0033*	2.73	1.38 to 5.38
C	30	33	0.33	0.57					
GG	18	4	0.39	0.14	GG *v* GC *v* CC	11.110	.0039*		
GC	26	17	0.57	0.59	GC + CC *v* GG	5.509	.0189	4.02	1.20 to 13.48
CC	2	8	0.04	0.28	CC *v* GG + GC	8.312	.0039*	8.38	1.63 to 42.96

* $P < .01$

Fig 2. Association of rs4796793 with interferon alfa (IFN-α) response. The upper figure shows the location of rs4796793 in the *STAT3* gene. The lower figure shows the significant association between rs4796793 and IFN-α response. χ^2 testing was performed for the alleles, or the genotypes of rs4796793. NON, nonresponder; RES, responder.

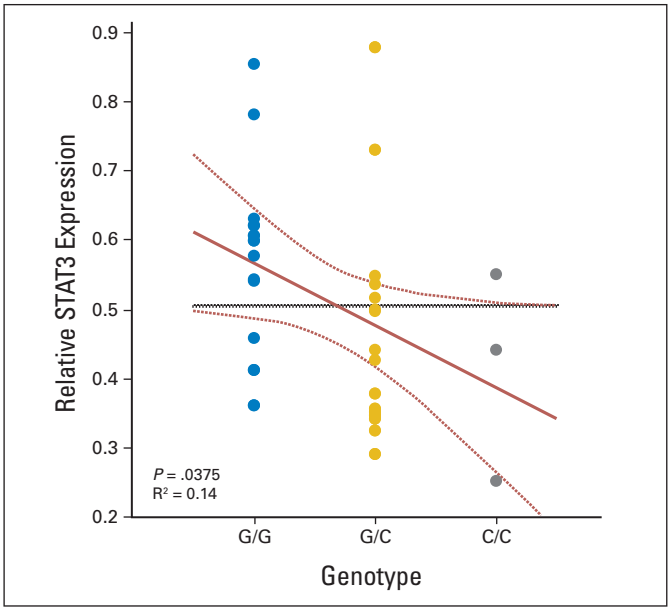


Fig 3. Endogenous *STAT3* expression in human B-cell lines. *STAT3* mRNA expression was measured in 32 B-lymphocyte cell lines, transformed by Epstein Barr virus, using real-time polymerase chain reaction. The *STAT3* expression of each sample was described by the ratio with the *GAPDH* housekeeping gene expression level in the same sample. Simple linear regression analysis revealed a significant correlation between *STAT3* expression and rs4796793 genotype (P = .0375, R^2 = 0.14). The dotted curving lines show 95% CIs.

SNPs or a combination of SNPs in LD with rs4796793 might affect *STAT3* expression.

Enhancement of IFN-α–Induced Growth Inhibition by STAT3 *Suppression*

Because rs4796793 genotype was correlated with *STAT3* expression, we next studied the effect of *STAT3* expression level on IFN-α–induced growth inhibition in ACHN and HEK293 cell lines. *STAT3* mRNA, STAT3 protein and a phosphorylated form of STAT3 (pSTAT3) were apparently decreased by *STAT3* siRNA (Fig A2, online only). In this condition, *STAT3* siRNA enhanced IFN-α -induced growth inhibition in ACHN and HEK293 cells, especially at a concentration of 10^5 and 10^4 U/mL IFN-α (Fig 4). These results suggest that reduced *STAT3* expression enhanced the sensitivity of ACHN and HEK293 cells to IFN-α.

Enhancement of IFN-α–Induced ISRE Activity by STAT3 *Suppression*

Although suppression on the IFN-α pathway, such as phosphorylation of *STAT1* and expression of IFN-α inducible genes, was not observed by *STAT3* suppression, the enhancement of IFN-α -induced ISRE activity was observed under a *STAT3* suppressed condition by *STAT3* siRNA using ISRE-luc reporter gene assay (Fig 5).

DISCUSSION

We carried out a comprehensive association study of polymorphisms in 33 candidate genes for response to IFN-α therapy in Japanese patients with metastatic RCC. We found 11 candidate SNPs that could be predictive genetic markers for the efficacy of IFN-α in RCC. Among these, SNPs in *STAT3* showed the most significant association with response to IFN-α. An extensive search for SNPs in *STAT3* and LD analysis revealed that rs4796793, which was located in the 5′-flanking region of *STAT3*, was the most remarkable candidate marker for the outcome of IFN-α therapy in RCC patients. Moreover, judging from the equal frequency of this SNP in *STAT3* in our patients and the general population, the SNP seems to have an effect not on susceptibility to RCC but on the outcome of IFN-α therapy in established RCC. Although the direct effect of the SNP on the promoter activity of *STAT3* was not determined in the reporter assays, genotype-dependent *STAT3* mRNA expression was observed in B-lymphocyte cell lines. This implies that an unidentified SNP or a combination of SNPs in tight LD with rs4796793 affects *STAT3* promoter activity or the stability of *STAT3* mRNA. As well, the enhanced growth inhibitory effects of IFN-α and ISRE activity by *STAT3* suppression in an RCC cell line supported that *STAT3* is a key molecule regulating IFN-α response.

STATs are ligand-induced transcriptional factors[10] that are activated in response to growth factors and cytokines such as IFN-α.[10-11] Among the *STAT* family, *STAT3* is recognized as a primordial STAT protein because it is activated by a wide variety of cytokines, growth factors, and other stimuli,[12-13] and participates in a wide variety of physiological processes. Therefore, to discuss the involvement of *STAT3* in IFN-α sensitivity, we need to consider the two different aspects of *STAT3*: its roles in tumor cells and in the immune system.

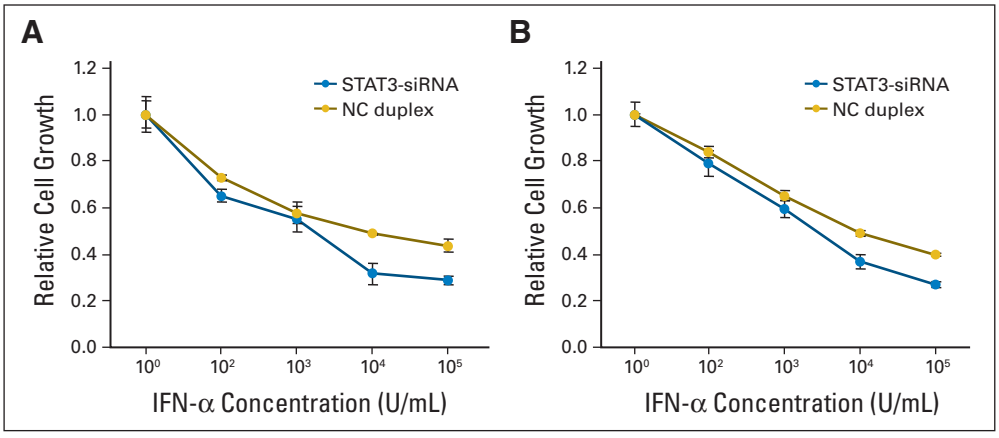


Fig 4. Enhancement of interferon alfa (IFN-α) -induced cell growth inhibition by *STAT3* siRNA. IFN-α–induced cell growth inhibition was enhanced by *STAT3* siRNA in ACHN and HEK293 cells. The open circles show the growth of cells transfected with *STAT3* siRNA and the closed circles show that with negative control (NC) siRNA. Each experiment was done in triplicate, and the error bars represent standard deviation.

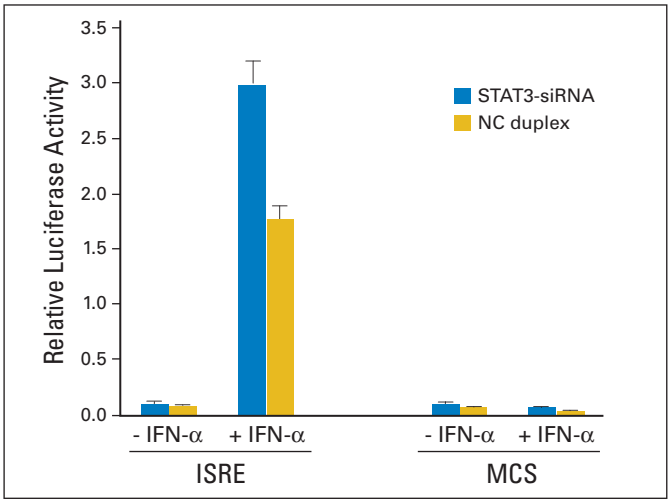


Fig 5. Effect of *STAT3* siRNA on interferon-stimulated response element (ISRE) -luciferase activity. Interferon alfa (IFN-α) -induced ISRE activity in HEK293 was enhanced by *STAT3* siRNA. The closed columns show the relative luciferase activity transfected with *STAT3* siRNA and the open columns show the relative luciferase activity transfected with negative control (NC) siRNA. Experiments were performed in triplicate, and the relative luciferase activity was presented as the mean ± SE, after normalizing with *Renilla* luciferase activity. MCS, Firefly luciferase vector without ISRE sequence, pLuc-MCS.

STAT3 has been reported to be frequently overexpressed in various cancers[14] and has, therefore, been recognized as a type of oncogene. Additionally, laboratory-induced mutation, resulting in constitutive *STAT3* activation, can transform normal cells,[15] whereas no naturally occurring mutations of *STAT3* have been reported.[16] In previous studies on RCC, the most frequently activated *STAT* was reported to be *STAT3*,[17] and the expression of pSTAT3 was associated with clinical outcome.[18] These findings suggest that inherited *STAT3* polymorphisms, which correlate with *STAT3* expression, might have a substantial effect on the progression or survival of cancer cells. In this context, *SOCS3*, a negative regulator of the Janus kinase (Jak)-STAT signaling pathways may have some role in the response because *SOCS3* expression was decreased by *STAT3* suppression (Fig A3, online only). For example, constitutive SOCS3 expression in cutaneous T-cell lymphoma cell lines,[19,20] as well as in several chronic myelogenous leukemia (CML) cell lines and blast cells of CML patients,[21] seems to be involved in the respective resistances to IFN-α treatment.

Research on *STAT3* in respect to tumor immunity has been limited because ablating *STAT3* leads to embryonic lethality. However, a recent report showed that conditional *STAT3* knockout in hematopoietic cells results in enhanced intrinsic antitumor immunity in vivo.[22] This report demonstrates that *STAT3* activation in tumor-enhancing immune cells is an important contributor to impaired antitumor immunity. Also, that marked activation of dendritic cells (DCs), natural killer (NK) cells and neutrophils in tumor-bearing mice with $STAT3^{-/-}$ hematopoietic cells is responsible for enhanced antitumor immunity.[22] Because IFN-α is thought to elicit its therapeutic effect via enhancing antitumor immunity mediated by NK and T lymph cells, it is probable that patients with a minor rs4796793 allele have better intrinsic tumor immune surveillance, which can be activated by IFN-α administration, and so respond well to IFN-α therapy.

A relationship between *STAT3* activation in tumor cells and antitumor immunity has also been reported.[23] Activation of *STAT3* in tumor cells promotes the expression of factors that inhibit the functional differentiation and mutation of DCs, resulting in suppression of proinflammatory cytokines and the chemokines necessary for antitumor immunity.[23] Collectively, these reports support the idea that the inherited genetic polymorphism in *STAT3*, which affects gene transcription, might influence the outcome of IFN-α therapy for RCC via antitumor immunity.

This study is exploratory in nature because the number of samples was limited and no statistical adjustment was done to avoid false-positive results caused by multiple testing. However, the SNPs in *IL4R* were shown to be associated with the response to IFN-α in RCC in this study, which is consistent with our previous study that showed an association between *IL4R* polymorphisms and long survival in RCC patients.[24] Because *IL4* is widely used for DC differentiation,[25] SNPs in *IL4R* may be correlated with DC differentiation in patients.

We have now launched a prospective cohort study in Japan to confirm the association with the SNP in *STAT3* and the response to IFN-α therapy; however, confirmatory results are expected to be a few years away. We also hope that the association with the SNP in *STAT3* and the response to IFN-α therapy will be tested in other case-control studies in different races.

In conclusion, we showed that rs4796793 in *STAT3* is a strong candidate genetic marker for predicting response to IFN-α therapy in RCC. *STAT3* expression was correlated with the SNP genotype and suppression of *STAT3* led to enhanced sensitivity to IFN-α in vitro. These findings, together with recent research on *STAT3*, suggested that SNPs might influence the sensitivity of tumor cells to IFN-α and/or the activation status of antitumor immunity. Although further studies using other validation sets will be necessary to confirm the involvement of SNPs in the response to IFN-α, the present study provides important evidence for recognizing *STAT3* as a diagnostic marker for IFN-α sensitivity. Several novel agents that are believed to improve RCC treatments are now being developed.[26] As well, the simultaneous or sequential combination of IFN-α with tyrosine kinase inhibitors needs to be the subject of future investigation. However, even when these new-type drugs such as TKIs are used as first-line treatments, the identification of reliable predictive markers of response to IFN-α is essential for establishing optimal treatment strategies for patients with MRCC.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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REFERENCES

1. Surveillance, Epidemiology, and End Results: http://seer.cancer.gov/csr/1975_2002/results_merged/sect_11_kidney_pelvis.pdf

2. Marumo K, Satomi Y, Miyao N, et al: The prevalence of renal cell carcinoma: A nation-wide survey in Japan in 1997. Int J Urol 8:359-365, 2001

3. Pyrhonen S, Salminen E, Ruutu M, et al: Prospective randomized trial of interferon alfa-2a plus vinblastine versus vinblastine alone in patients with advanced renal cell cancer. J Clin Oncol 17: 2859-2867, 1999

4. WHO Handbook for Reporting Results of Cancer Treatment. WHO offset publication 48, Geneva, Switzerland, WHO, 1979

5. Hsu TM, Law SM, Duan S, et al: Genotyping single-nucleotide polymorphisms by the invader assay with dual-color fluorescence polarization detection. Clin Chem 47:1373-1377, 2001

6. Akazawa K, Nakamura T, Palesch Y: Power of logrank test and Cox regression model in clinical trials with heterogeneous samples. Stat Med 16: 583-597, 1997

7. Bryson MC, Johnson ME: The incidence of monotone likelihood in the Cox model. Technometrics 23:381-383, 1981

8. Hill WG, Robertson A: Linkage disequilibrium in finite populations. Theor Appl Genet 38:226-231, 1968

9. Lewontin RC: The interaction of selection and linkage: I, General considerations—Heterotic models. Genetics 49:49-67, 1964

10. Darnell JE Jr: STATs & gene regulation. Science 277:1630-1635, 1997

11. Bowman T, Garcia R, Turkson J, et al: STATs in oncogenesis. Oncogene 19:2474-2488, 2002

12. Takeda K, Akira S: STAT family of transcription factors in cytokine-mediated biological responses. Cytokine Growth Factor Rev 11:199-207, 2000

13. Hirano T, Ishihara K, Hibi M: Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene 19:2548-2556, 2000

14. Yu H, Jove R: The STATs of cancer–new molecular targets come of age. Nat Rev Cancer 4:97-105, 2004

15. Bromberg JF, Wrzeszczynska MH, Devgan G, et al: Stat3 as an oncogene. Cell 98:295-303, 1999

16. Darnell JE: Validating Stat3 in cancer therapy. Nat Med 11:595-596, 2005

17. Bromberg J: Stat proteins and oncogenesis. J Clin Invest 109:1139-1142, 2002

18. Horiguchi A, Oya M, Shimada T, et al: Activation of signal transducer and activator of transcription 3 in renal cell carcinoma: A study of incidence and its association with pathological features and clinical outcome. J Urol 168:762-765, 2002

19. Brender C, Lovato P, Sommer VH, et al: Constitutive SOCS-3 expression protects T-cell lymphoma against growth inhibition by IFNalpha. Leukemia 19:209-213, 2005

20. Brender C, Nielsen M, Kaltoft K, et al: STAT3-mediated constitutive expression of SOCS-3 in cutaneous T-cell lymphoma. Blood 97:1056-1062, 2001

21. Sakai I, Takeuchi K, Yamauchi H, et al: Constitutive expression of SOCS3 confers resistance to IFN-alpha in chronic myelogenous leukemia cells. Blood 100:2926-2931, 2002

22. Kortylewski M, Kujawski M, Wang T, et al: Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med 11:1314-1321, 2005

23. Wang T, Niu G, Kortylewski M, et al: Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. Nat Med 10:48-54, 2004

24. Nakamura E, Megumi Y, Kobayashi T, et al: Genetic polymorphisms of the interleukin-4 receptor alpha gene are associated with an increasing risk and a poor prognosis of sporadic renal cell carcinoma in a Japanese population. Clin Cancer Res 8:2620-2625, 2002

25. Tatsugami K, Eto M, Harano M, et al: Dendritic-cell therapy after non-myeloablative stem-cell transplantation for renal-cell cancer. Lancet Oncol 5:750-752, 2004

26. Amato RJ: Renal cell carcinoma: Review of novel single-agent therapeutics and combination regimens. Ann Oncol 16:7-15, 2005

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).