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(Citation)
Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics,29(1):11-23

(Issue Date)
2021-05-04

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/90009320

Oncology Research, Vol. 29, pp. 11–23
Printed in the USA.

0965-0407/22 $90.00 + .00
DOI: https://doi.org/10.3727/096504022X16418911579334
E-ISSN 1555-3906
www.cognizantcommunication.com

STAT3 Polymorphism Associates With mTOR Inhibitor-Induced Interstitial Lung Disease in Patients With Renal Cell Carcinoma

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We evaluated the association of signal transducer and activator of transcription 3 (*STAT3*) polymorphisms with the incidence of mammalian target of rapamycin (mTOR) inhibitor-induced interstitial lung disease (ILD) in patients with renal cell carcinoma (RCC). We also used lung-derived cell lines to investigate the mechanisms of this association. Japanese patients with metastatic RCC who were treated with mTOR inhibitors were genotyped for the *STAT3* polymorphism, rs4796793 (−1697C/G). We evaluated the association of the *STAT3* genotype with the incidence of ILD and therapeutic outcome. In the 57 patients included in the primary analysis, the ILD rate within 140 days was significantly higher in patients with the GG genotype compared with those with other genotypes (77.8% vs. 23.1%, odds ratio = 11.67, 95% confidential interval = 3.06–44.46). There were no significant differences in progression-free survival or time-to-treatment failure between the patients with the GG genotype and those with other genotypes. An in vitro study demonstrated that some lung-derived cell lines carrying the GG genotype exhibited an increase in the expression of mesenchymal markers, such as fibronectin, N-cadherin, and vimentin, and decreases in E-cadherin, which is an epithelial marker associated with exposure to everolimus, although STAT3 expression and activity were not related to the genotype. In conclusion, the GG genotype of the *STAT3* rs4796793 polymorphism increases the risk of mTOR inhibitor-induced ILD, supporting its use as a predictive marker for RCC.

Key words: mTOR inhibitor; Interstitial lung disease; STAT3; Polymorphism; Epithelial–mesenchymal transition (EMT)

INTRODUCTION

Mammalian target of rapamycin (mTOR) inhibitors are used as immunosuppressants after organ transplantation and as anticancer agents for metastatic renal cell carcinoma (mRCC), breast cancer, and tuberous sclerosis complex. They play an important role as a treatment option for renal cell carcinoma (RCC) because of their unique mechanisms of action, other than vascular endothelial growth factor receptor inhibition[1].

Interstitial lung disease (ILD) is a serious adverse reaction caused by mTOR inhibitors. The most common symptoms are breathlessness and respiratory distress, which worsen over time and can be fatal[2]. The incidence of mTOR inhibitor-induced ILD in patients with mRCC is 14%–29% for temsirolimus[2–5] and 14%–49% for everolimus[5–8]. A previous study reported that 10% of patients required interruption and/or dose reduction during everolimus therapy because of ILD[9]. Thus, mTOR inhibitor-induced ILD can cause treatment interruption, although it

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is responsible for less mortality than epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI)-induced ILD[10,11]. A screening method or a suitable biomarker is needed to detect patients at high risk for mTOR inhibitor-induced ILD. The risk factors for EGFR-TKI-induced ILD include age, worse performance status, smoking habit, and preexisting ILD[12]; however, complete information on the risk factors for mTOR inhibitor-induced ILD is limited[13]. Krebs von den Lungen-6 antigen (KL-6) is well known as a serum circulating ILD marker[14]. Patients with mTOR inhibitor-induced ILD were also reported to exhibit high KL-6 levels at the onset of disease[15]. Because circulating KL-6 levels in patients with drug-induced ILD is only elevated in severe cases[16,17], predictive markers with high sensitivity and specificity are needed to prevent an increase in ILD severity.

mTOR inhibitor-induced ILD develops in patients with any cancer regardless of the mTOR inhibitor used. Hence, the factors associated with mTOR or related signal pathways are likely associated with the biological mechanism of ILD. We considered the possibility that signal transducer and activator of transcription 3 (STAT3) is involved in mTOR inhibitor-induced ILD. STAT3 is a mediator of cell signaling for metastasis, migration, and various cellular responses to cytokines[18]. STAT3 plays an important role in the epithelial–mesenchymal transition (EMT), a process of tissue fibrosis, by positively regulating transcription of zinc finger E-box-binding homeobox 1 (ZEB1) and Snail, which are transcriptional repressors of the epithelial marker E-cadherin[19]. STAT3 activation results in EMT in alveolar epithelial cells, and this response is associated with bleomycin-induced ILD[20]. Interestingly, single nucleotide polymorphisms in the gene coding STAT3 affect the efficacy of interferon therapy in patients with RCC[21]. Moreover, recent reports suggest that mTOR inhibitor-induced ILD is associated with clinical outcomes such as overall survival, time-to-treatment failure, and disease control rate[6,22]. We previously found an association between therapeutic response to vascular endothelial growth factor receptor-TKI in patients with mRCC with *STAT3* genotype[23]. Thus, we hypothesize that the functional polymorphisms of *STAT3* are also predictive of mTOR inhibitor-induced ILD.

In this study, we evaluated the association of *STAT3* polymorphisms with the incidence of mTOR inhibitor-induced ILD in patients with RCC. A preliminary in vitro study was also conducted to verify this association in various lung-derived cell lines.

MATERIALS AND METHODS

Study Design

This study was a retrospective multicenter cohort study conducted at five Japanese hospitals (Kobe University Hospital, Kyoto University Hospital, Mie University Hospital, Kumamoto University Hospital, and Nagoya University Hospital). The representative center at Kobe University Hospital collected the anonymized case reports and the frozen whole-blood samples from cooperative centers after the ethics committee at each center approved the research plan.

Patients

We retrospectively examined patients with mRCC who were treated with an mTOR inhibitor (everolimus or temsirolimus) between April 2010 and March 2017. The eligibility criteria included age >20 years, an expected survival of at least 12 weeks after mTOR inhibitor therapy, and an Eastern Cooperative Oncology Group Performance Status ≥2. The exclusion criteria included intolerance to everolimus or temsirolimus and serious complications. The exclusion criterion for the primary analysis was discontinuation of everolimus or temsirolimus treatment within 140 days for any reason other than ILD. All patients provided written informed consent. Some of the patients were also included in a previous pharmacokinetic study[24]. The study was approved by each institutional ethics committee at all participating centers (No. 160020 in a representative research institution) and complied with the Declaration of Helsinki and its later amendments or comparable ethical standards.

Blood Sampling and SNP Genotyping Assay

All frozen blood samples were sent to the representative site. Blood-derived DNA was isolated using a NucleoSpin Blood kit (MACHEREY-NAGEL, GmbH, Düren, Germany) according to the manufacturer's protocol. Cell line-derived DNA was isolated using a NucleoSpin Tissue kit (MACHEREY-NAGEL). The *STAT3* polymorphism, rs4796793, was detected using a TaqMan SNP Genotyping Assay (assay ID: C_27977213_10; Thermo Fisher Scientific, Waltham, MA, USA). Reactions were conducted using an ABI PRISM 7900HT Sequence Detection System according to the following protocol: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The post-polymerase chain reaction (PCR) plates were read on a 7900HT system and used to determine the genotype.

Evaluation of Clinical Outcomes

ILD induced by everolimus or temsirolimus was defined as pulmonary fibrosis or pneumonitis of grade 1 or higher severity as defined by the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 and assessed by the treating physician. Additionally, the physician comprehensively determined whether the symptoms were caused by mTOR inhibitor-induced ILD or other pneumonitis as routine care

referring to the practical guide[25]. The prognosis risk classification for mRCC was performed using the Memorial Sloan Kettering Cancer Center score[26]. Responses were assessed by the treating physician based on the Response Evaluation Criteria in Solid Tumors version 1.1. The objective response rate was defined as the percentage of patients who had a best response of complete response or partial response. The disease control rate was defined as the percentage of patients who had a best response of complete response, partial response, or stable disease. Other clinical data were collected from medical records by the research coordinator at each center.

The primary outcome was the incidence of ILD within 140 days after the initiation of everolimus or temsirolimus therapy. The secondary outcomes included the incidence of ILD throughout the entire course of mTOR inhibitor therapy, objective response rate, disease control rate, discontinuation rate attributable to progressive disease or ILD, cumulative incidence of ILD, progression-free survival, and time-to-treatment failure. Time-to-event analysis was performed for all entered patients because the exclusion criteria for the primary analysis were selected to definitely evaluate the incidence of drug-induced ILD. The response rate was evaluated in patients whose data for overall best response was available.

The incidence of ILD was confirmed by a sensitivity analysis using a data-spitting method to assess inter-institutional bias, because patients from four individual institutes were included in the study. Four analytical subgroups were established by deleting data from one institution per subgroup. The incidence of ILD within 140 days and throughout the entire course of mTOR inhibitor therapy was compared by *STAT3* genotype for each analytical subgroup.

Sample Size Estimation

Based on the results of our previous study, we assumed a prevalence of 0.40 for the GG genotype designated rs4796793[27]. For a total sample size of 60 patients, the incidence of ILD with a 0.05 two-sided significance level has 80% power to detect the difference between the GG genotype (expected ILD incidence rate = 0.65) as well as other genotypes (expected ILD incidence rate = 0.25) of rs4796793. Therefore, the recruiting goal for this study was set to 80 patients to account for excluded patients.

Chemicals and Antibodies

Everolimus was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Anti-phosphorylated (p)-STAT3 at tyrosine 705, STAT3, N-cadherin, and vimentin antibodies as well as anti-rabbit and mouse horseradish peroxidase conjugate immunoglobulin G were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-fibronectin and β-actin antibodies were purchased from Sigma-Aldrich. Anti-E-cadherin antibody was obtained from BD Transduction Laboratories (Lexington, KY, USA).

Cells and Cell Culture

A549, LK-2, EBC-1, ABC-1, SK-MES-1, and COR-L23 cells were the gifts from Dr. Kohji Takara (Himeji Dokkyo University, Hyogo, Japan). A549, EBC-1, ABC-1, and SK-MES-1 cells were maintained in Eagle's minimal essential medium (Sigma-Aldrich) supplemented with nonessential amino acids (FUJIFILM Wako Chemical, Tokyo, Japan) and subcultured with 0.125% trypsin and 0.01% ethylenediaminetetraacetic acid. COR-L23 and LK-2 cells were maintained in RPMI-1640 (Sigma-Aldrich) supplemented with nonessential amino acids. Thereafter, the cells were seeded into culture flasks and grown in a humidified atmosphere of 5% CO_2 at 37°C.

Western Blot Analysis

Proteins in the total cell lysate were extracted from cells treated with 0.7% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate-based cell lysis buffer containing dithiothreitol, phenylmethylsulfonyl fluoride, and leupeptin. The proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane (Amersham Protran NC; GE Healthcare, Buckinghamshire, UK). The membranes were blocked in wash buffer solution containing 5% skim milk. The membranes were incubated overnight at 4°C in wash buffer containing specific primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Antibody-bound proteins were visualized by treating the membrane with Amersham ECL prime (GE Healthcare). Images were acquired using ChemiStage CC-16 (KURABO, Osaka, Japan). Wherever indicated, the membranes were stripped and reprobed with different primary antibodies. The intensities of the protein bands in the densitometric assay were determined using ImageJ software (National Institutes of Health)[28].

Statistical Analysis

Categorical variables across groups were compared using Fisher's exact test. Ordinal variables across groups were compared using the Cochran–Armitage trend test. Continuous variables across groups were compared using the Mann–Whitney *U*-test. In addition, the cumulative incidence rate and probabilities of progression-free survival and time-to-treatment failure were calculated using the Kaplan–Meier method, and significant differences were determined using the log-rank test. Differences between three or more groups were analyzed using a one-way analysis of variance followed by Dunnett's test.

A value of $p < 0.05$ (two-tailed) was considered statistically significant. In the case of multiple comparison, the p value divided by the number of comparisons was considered statistically significant by applying the Bonferroni correction. All statistical analyses were performed using SPSS software version 24.

RESULTS

Patient Characteristics in Primary Analysis

We closed the study after enrolling 75 patients despite not meeting the recruiting goal, because we did not expect to identify additional suitable subjects. No patient met the exclusion criteria for the study. The primary analysis included 57 patients after 18 patients were excluded because of treatment discontinuation within 140 days for reasons other than ILD. Table 1 lists the characteristics of the 57 patients. No patient characteristics differed between the ILD and non-ILD groups within 140 days after the initiation of mTOR inhibitor therapy. Patient characteristics according to the ILD and non-ILD groups throughout the entire course of mTOR inhibitor therapy are presented in Table 2.

Association of the rs4796793 Genotype With the Incidence of ILD

Of the 57 patients, 10 (17.5%), 29 (50.9%), and 18 patients (31.6%) carried the CC, GC, and GG genotypes of rs4796793, respectively. Moreover, 23 patients (40.3%) developed ILD within 140 days after the initiation of mTOR inhibitor therapy. The results of the primary analysis are presented in Table 3. The ILD incidence rate was significantly higher in patients with the GG genotype compared with those carrying the CC + GC genotypes [77.8% (14/18) vs. 23.1% (9/39), odds ratio (OR) = 11.67, 95% confidential interval (CI) = 3.06–44.46, $p < 0.001$].

The ILD incidence according to overall treatment duration and the mTOR inhibitor used is presented in Table 4. Thirty-two patients (56.1%) developed ILD throughout the entire course of mTOR inhibitor therapy. The ILD

Table 1. Patient Characteristics According to the Incidence of Interstitial Lung Disease Within 140 Days of Mammalian Target of Rapamycin Inhibitor Treatment Initiation

	All Patients	ILD	Non-ILD	*p*
Patients (*n*)	57	23	34	
Institution A	33	17	16	-
Institution B	15	3	12	-
Institution C	5	0	5	-
Institution D	4	3	1	-
Institution E	0	0	0	-
Characteristics (*n*)				
Male	44	18	26	1.00*
Smoking	28	11	17	1.00*
Lung surgery	6	4	2	0.21*
Lung metastasis	43	18	25	0.76*
Characteristics [median (range)]				
Age	64 (35–82)	64 (52–79)	63 (35–82)	0.47†
Body weight (kg)	60.7 (38.5–87.1)	61.4 (46.2–87.1)	59.6 (38.5–81.4)	0.88†
Body mass index (kg/m^2)	22.2 (17.5–30.4)	22.3 (17.5–30.4)	22.2 (17.5–30.2)	0.90†
Number of metastatic sites	2 (0–6)	2 (1–4)	2 (0–6)	0.64†
Number of prior chemotherapies	2 (0–4)	2 (0–3)	2 (0–4)	0.82†
mTOR inhibitor				
Temsirolimus therapy (n)	28	14	14	0.18*
Temsirolimus initial dose [mg/kg, median (range)]	0.40 (0.29–0.62)	0.40 (0.29–0.54)	0.40 (0.32–0.62)	0.87†
Everolimus therapy, *n*	29	9	20	0.18*
Everolimus initial dose [mg/kg, median (range)]	0.16 (0.065–0.26)	0.17 (0.081–0.22)	0.16 (0.065–0.26)	0.80†
MSKCC risk classification (*n*)				
Favorable	8	6	2	0.77‡
Intermediate	38	12	26	
Poor	7	5	2	
Unknown	4	0	4	

ILD, interstitial lung disease; mTOR, mammalian target of rapamycin; MSKCC, Memorial Sloan Kettering Cancer Center. *Fisher's exact test, †Mann–Whitney *U*-test, ‡Cochran–Armitage trend test.

Table 2. Patient Characteristics According to the Incidence of Interstitial Lung Disease for the Entire Treatment Duration

	ILD	Non-ILD	*p*
Patients (*n*)			
Institution A	20	13	-
Institution B	7	8	-
Institution C	2	3	-
Institution D	3	1	-
Institution E	0	0	-
Characteristics (*n*)			
Total	32	25	
Male	23	21	0.35*
Smoking	15	13	0.79*
Lung surgery	4	2	0.69*
Lung metastasis	23	20	0.55*
Characteristics [median (range)]			
Age	64 (52–82)	63 (35–81)	0.25†
Body weight (kg)	60.5 (38.5–87.1)	60.7 (40.6–81.4)	0.46†
Body mass index (kg/m^2)	22.3 (17.5–30.4)	22.2 (17.5–30.2)	0.97†
Number of metastatic sites	2 (1–6)	2 (0–4)	0.48†
Number of prior chemotherapies	2 (0–3)	2 (0–4)	0.76†
mTOR inhibitor			
Temsirolimus therapy (*n*)	16	12	>0.99*
Temsirolimus initial dose [mg/kg, median (range)]	0.40 (0.29–0.58)	0.40 (0.32–0.62)	0.70†
Everolimus therapy, *n*	16	13	>0.99*
Everolimus initial dose [mg/kg, median (range)]	0.17 (0.065–0.26)	0.15 (0.082–0.20)	0.50†
MSKCC risk classification (*n*)			
Favorable	6	2	0.82‡
Intermediate	19	19	
Poor	5	2	
Unknown	2	2	

ILD, interstitial lung disease; mTOR, mammalian target of rapamycin; MSKCC, Memorial Sloan Kettering Cancer Center. *Fisher's exact test, †Mann–Whitney *U*-test, ‡Cochran–Armitage trend test.

incidence rate was significantly higher in patients with the GG genotype compared with those carrying the CC + GC genotypes [100% (18/18) vs. 35.9% (14/39), OR = not applicable, $p < 0.001$]. The distribution of patients with each rs4796793 genotype significantly differed between the ILD and non-ILD groups over the entire treatment period for everolimus and temsirolimus.

The results of sensitivity analysis are shown in Table 5. The association between the incidence of ILD within 140 days and rs4796793 genotype was not significant in group A; however, there were significant associations with other groups. On the one hand, all groups had a significant association between the incidence of ILD over the entire period and rs4796793 genotype.

Comparison of Individual Everolimus Apparent Clearances in Patients According to the Incidence of ILD

The individual everolimus apparent clearances as determined by the empirical Bayesian method in a subset of the patients[24] are shown according to the incidence of ILD for the entire treatment period (Fig. 1). There

Table 3. The Association of the Risk of Interstitial Lung Disease Within 140 Days of Mammalian Target of Rapamycin Inhibitor Treatment Initiation With the rs4796793 Genotype of Signal Transducer and Activator of Transcription 3 (*STAT3*)

SNP/Genotype	ILD	Non-ILD	OR (95% CI)	*p*
rs4796793				<0.001
CC + GC	9 (23.1%)	30 (76.9%)	1	
GG	14 (77.8%)	4 (22.2%)	11.67 (3.06–44.46)	

CI, confidential interval; ILD, interstitial lung disease; OR, odds ratio.

Table 4. The Association of the Risk of Interstitial Lung Disease During the Entire Course of Mammalian Target of Rapamycin Inhibitor Treatment With the rs4796793 Genotype of *STAT3*

	Incidence in the Entire Period				Incidence in the Entire Period (Temsirolimus)				Incidence in the Entire Period (Everolimus)			
Genotype	ILD	Non-ILD	OR (95% CI)	p*	ILD	Non-ILD	OR (95% CI)	p*	ILD	Non-ILD	OR (95% CI)	p*
CC	3	7		<0.001	1	2		<0.001	2	5		0.007
GC	11	18	–		5	10	–		6	8	–	
GG	18	0			10	0			8	0		
CC + GC	14	25	1	<0.001	4	14	1	<0.001	8	13	1	0.003
GG	18	0	–		10	0	–		8	0	–	
CC	3	7	1	0.09	1	2	1	0.56	2	5	1	0.19
GC + GG	29	18	3.76 (0.86–16.43)		15	10	3.00 (0.24–37.67)		14	8	4.38 (0.68–27.98)	

CI, confidential interval; ILD, interstitial lung disease; OR, odds ratio.
*p value less than 0.017 was considered statistically significance for multiple comparison.

was no difference of everolimus apparent clearances between the ILD and non-ILD groups.

Time-to-Event Analyses in Each rs4796793 Genotype Group

Time-to-event analyses according to the rs4796793 genotype were performed in all 75 patients (Fig. 2). The cumulative incidence of ILD in patients with the GG genotype was significantly higher compared with that in patients carrying the CC + GC genotypes ($p < 0.001$). The progression-free survival and time-to-treatment failure did not significantly differ between the genotype groups ($p = 0.69$ and $p = 0.96$, respectively). The discontinuation rate attributable to progressive disease did not differ between the genotype groups ($p > 0.99$), whereas the discontinuation rate attributable to ILD was higher for the GG genotype compared with the CC + GC genotype groups ($p < 0.001$). Objective response and disease control were evaluated in 64 patients with available data, and the rates did not differ by genotype (Table 6).

Differences in Activity and Expression of STAT3 and Everolimus-Induced Expression Changes of EMT Indicators in Lung-Derived Cell Lines

The cell profiles for each lung-derived cell line are presented in Figure 3A. A549 and LK-2 cells carry the CC and GC genotypes, respectively, whereas the other cell lines carry the GG genotype. As shown in Figure 3B, STAT3 expression was not associated with genotype in each cell line. STAT3 activity, defined as the ratio of p-STAT3/total STAT3 expression, was higher in EBC-1 and SK-MES-1 cells compared with that in A549 cells, whereas a trend for genotype-related STAT3 activity was not evident in the six cell lines. Everolimus-induced expression changes of EMT indicators in A549, EBC-1, ABC-1, COR-L23, and SK-MES-1 cells are presented in Figure 3C. Everolimus significantly increased the expression of the mesenchymal indicators, N-cadherin and vimentin, in EBC-1 cells. It also significantly increased the expression of fibronectin in ABC-1, SK-MES-1, and COR-L23 cells, and decreased the expression of E-cadherin in SK-MES-1 and COR-L23 cells. Everolimus did not induce significant expression changes in the EMT indicators in A549 cells using the same drug concentrations. In ABC-1 and COR-L23 cells, the expression of N-cadherin and vimentin was not detected by Western blot analysis.

DISCUSSION

This study revealed a significant association between *STAT3* polymorphism and the incidence of mTOR inhibitor-induced ILD. This is the first report to identify a genetic risk factor for mTOR inhibitor-induced ILD. The mTOR inhibitor-induced ILD incidence rate within 140

Table 5. Sensitivity Analysis to Evaluate the Interinstitution Bias for the Association Between the Incidence of Interstitial Lung Disease and the rs4796793 Genotype of *STAT3*

	Incidence Within 140 Days				Incidence in the Entire Period			
Group/Genotype	ILD	Non-ILD	OR (95% CI)	*P*	ILD	Non-ILD	OR (95% CI)	*P*
Group A (*n* = 24)				0.139				<0.001
CC + GC	3	15	1		6	12	1	
GG	3	3	4.60 (0.41–57.08)		6	0	–	
Group B (*n* = 42)				<0.001				<0.001
CC + GC	6	20	1		9	17	1	
GG	14	2	21.11 (3.47–242.28)		16	0	–	
Group C (*n* = 52)				<0.001				<0.001
CC + GC	9	26	1		13	22	1	
GG	14	3	12.68 (2.71–84.85)		17	0	–	
Group D (*n* = 53)				0.001				<0.001
CC + GC	9	29	1		14	24	1	
GG	11	4	8.42 (1.92–45.82)		15	0	–	

Group A: institution B + C + D, Group B: institution A + C + D, Group C: institution A + B + D, Group D: institution A + B + C. CI, confidential interval; ILD, interstitial lung disease; OR, odds ratio.

days of treatment initiation was similar to the findings of a previous study of patients with RCC[5,8]. However, the rate of mTOR inhibitor-induced ILD incidence was higher over the complete observation period because this study included patients with longer treatment periods compared with previous reports[5,8]. Our study did not demonstrate an association with any patient demographics, including the reported risk factors of mTOR inhibitor- and EGFR-TKI-induced ILD[5,12], other than *STAT3* polymorphism, with ILD incidence.

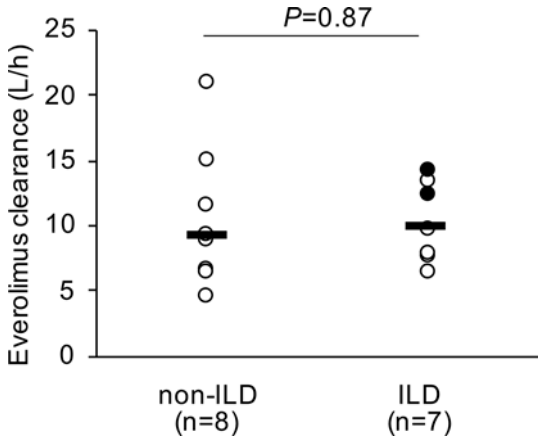

Figure 1. Comparison of individual everolimus apparent clearances in patients according to the incidence of interstitial lung disease for the entire treatment period. Individual clearance was estimated by the empirical Bayes method in a previous report[24]. Each open circle represents a patient carrying the CC or GC genotype of rs4796793 on *STAT3* (*n* = 13). Each closed circle represents a patient carrying the GG genotype (*n* = 2). The bar shows the median value for each group.

A study of renal transplant recipients suggested that the area under the blood concentration–time curve or trough concentrations of everolimus or sirolimus were not associated with the incidence of ILD[13,29]. Some of the patients in our study participated in the previous pharmacokinetic analysis[24]; thus, we examined the association between individual everolimus clearance and ILD incidence (Fig. 1). The results indicated that the individual clearance of everolimus was not associated with ILD incidence, which supports the previous findings[24].

The genotype distribution of the *STAT3* polymorphism rs4796793 in this study was not significantly different from that in a Japanese population ($p = 0.774$), namely, Hapmap-JPT (GG, 37%; GC, 48%; CC, 15%) obtained from the NCBI Database (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?subsnp_id=ss77261856). The rs4796793 polymorphism may affect the expression of STAT3, because it is located in the 5′-flanking region of *STAT3*[21]. Of the reports related to the phenotype of rs4796793, one found that the expression of *STAT*3 mRNA in B-lymphocyte cells was lower in cells carrying the C allele of rs4796793, although this polymorphism had no direct influence on *STAT3* promoter activity[21]. Another report indicated that *STAT3* expression in peripheral blood lymphocytes and melanoma cell lines did not differ in an rs4796793 genotype-dependent matter[30]. Knowledge of the biological phenotype associated with this polymorphism is controversial. Our study focused on a single polymorphism in *STAT3*. Numerous polymorphisms in *STAT3* have been identified, and some were demonstrated to have linkage disequilibrium[21]. Considering previous and present perceptions, the association of ILD and *STAT3* polymorphisms may not be specific to rs4796793; rather, this association could result from other polymorphisms

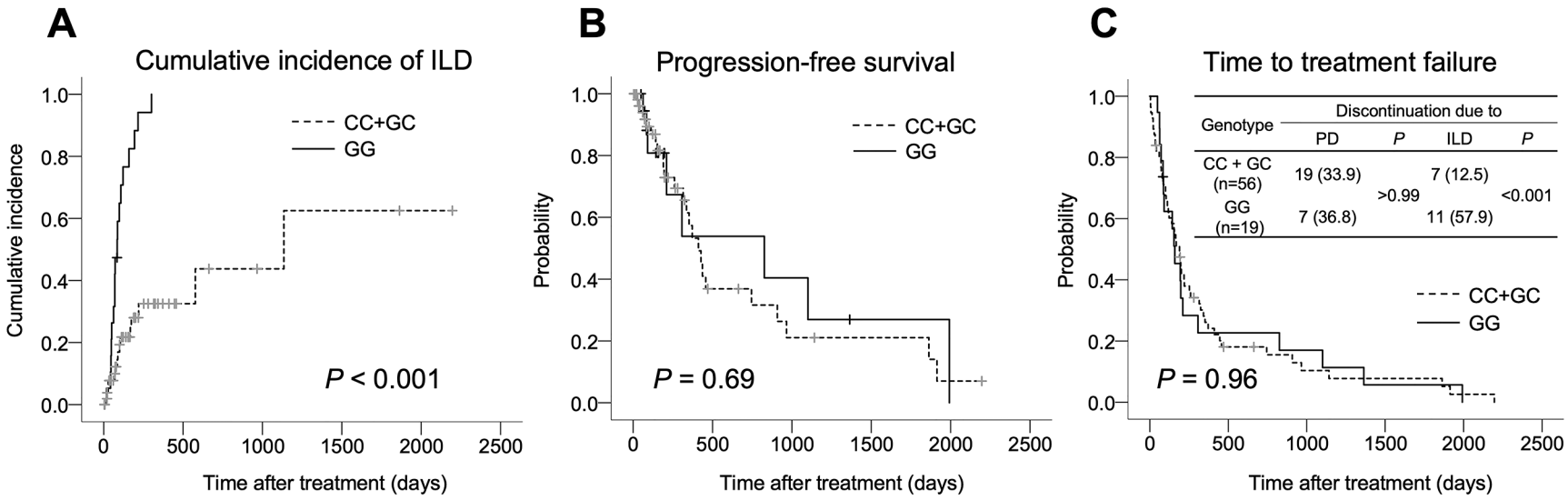

Figure 2. Time-to-event analysis according to the rs4796793 genotype of signal transducer and activator of transcription 3 (*STAT3*). Time to event analyses for all enrolled patients were performed using Kaplan–Meier curves. The solid line represents patients carrying the GG genotype of rs4796793 on *STAT3*, and the dotted line represents patients carrying the GC and CC genotypes. (A) The cumulative incidence of ILD for each genotype group. (B) Progression-free survival for each genotype group. (C) Time-to-treatment failure for each genotype group. The table in (C) presents the rate of treatment discontinuation attributable to PD and ILD according to the rs4796793 genotype. *p* values in the figures and table were calculated using the log-rank test and Fisher's exact test, respectively. ILD: interstitial lung disease, PD: progressive disease.

Table 6. The Association of the rs4796793 Genotype of *STAT3* With the Outcomes of Mammalian Target of Rapamycin Inhibitor Treatment

Genotype	Total	Objective Response	OR (95% CI)	Disease Control	OR (95% CI)
CC + GC	46	8 (17.4%)	1	41 (89.1%)	1
GG	18	3 (16.7%)	0.95 (0.22–4.07)	16 (88.9%)	0.98 (0.17–5.55)

CI, confidential interval; OR, odds ratio.

coexisting with rs4796793 or in conjunction with certain polymorphisms. Consequently, our in vitro study only focused on the biological phenotype related to EMT, but not the molecular phenotype of rs4796793.

Inhibition of mTOR signaling has been reported to have contradictory effects on EMT. mTOR inhibitors reverse transforming growth factor-β (TGF-β)-induced EMT[31–33]. Meanwhile, chronic inhibition of mTOR activity using mTOR inhibitors and silencing of the mTOR complex using RNA interference promote EMT mediated by TGF-β-independent pathways[34,35]. Our study revealed that everolimus treatment altered the expression of EMT markers in cell lines carrying the GG genotype of rs4796793, but not in A549 cells carrying the CC genotype, although long-term exposure to mTOR inhibitors induced EMT in a previous report[34]. However, our in vitro experiments were preliminary; therefore, conclusions regarding the functional effects of the rs4796793 polymorphism cannot be drawn. There may be other factors related to the induction of EMT in lung cells other than *STAT3* polymorphisms, because changes in EMT indicator expression differed in cell lines with the same rs4796793 genotype. Moreover, we did not perform experiments for EMT using cells with a heterogeneous genotype of rs4796793, such as LK-2 cells, because we considered it difficult to interpret the changes of multiple markers as the phenotype on the heterogenous genotype. Further experiments using normal lung cell lines are needed to study this phenomenon.

We propose two different mechanisms to explain the higher rate of mTOR inhibitor-induced ILD in GG genotype carriers. First, as mentioned above, EMT-related responses to mTOR inhibitor exposure in alveolar epithelial cells can differ between *STAT3* genetic variants, thereby explaining differences in ILD incidence. This hypothesis is partially supported by our in vitro findings. Second, differences in immune responses between rs4796793 genotypes may affect the incidence of ILD, as STAT3 is a master regulator of the immune response[36–38]. STAT3 depletion in various immune cells enhances the antitumor immune response by suppressing negative immune regulators, such as regulatory T cells, and activating immune effectors, such as $CD8^+$ T cells[38]. Therefore, the function or expression of STAT3 and/or the response to mTOR inhibitors in various immune cells differ between *STAT3* genotypes. The potency of the immune system may also be determined by the *STAT3* genotype. Some studies have suggested that mTOR inhibitors can enhance anticancer immunity, although it remains unclear whether mTOR inhibition suppresses or potentiates the immune response[39–41]. Hyperimmunity, which may be caused by the combination of *STAT3* polymorphism and mTOR inhibition, can cause immune-related adverse events including ILD and is associated with a good therapeutic outcome following immune checkpoint inhibition[42,43]. *STAT3* genetic variants may determine the potency of systemic immunity during exposure to mTOR inhibitors.

In the present study, the rs4796793 genotype was not associated with therapeutic outcomes, such as progression-free survival, time-to-treatment failure, objective response rate, and disease control rate. However, the rate of treatment discontinuation attributable to ILD was higher for the GG genotype compared with the other genotypes. A suggested decision tree for the recommended management of mTOR inhibitor-associated ILD according to severity was presented by multiple study groups[44,45]. Most of our patients with mild mTOR inhibitor-induced ILD, as well as patients with early ILD detection, achieved complete resolution following mTOR inhibitor dose reduction or administration of corticosteroid therapy[46,47]. Therefore, it is important to continue mTOR inhibitor therapy with close monitoring and careful dose adjustments[48]. This intensive disease management may prolong the duration of mTOR inhibitor treatment in patients with the GG genotype of rs4796793.

Our study featured a limitation. This was a small-scale retrospective observational study, and the number of enrolled patients did not reach the statistical sample size estimated in the study protocol. Moreover, the patients at one institution accounted for over half of all entered patients and exhibited a relatively high incidence of ILD. Therefore, the significant association between the incidence of ILD within 140 days and *STAT3* polymorphism was not found in the subgroup except for the patients from a large contributing institution. However, the association between the incidence of ILD over the entire period and *STAT3* polymorphism in this subgroup was retained. We considered that the sensitive analysis using subgroups demonstrated the robustness of the association of the

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Cell line	Cell Type	rs4796793 genotype
A549	Lung adenocarcinoma	CC
LK-2	Lung squamous cell carcinoma	GC
EBC-1	Lung squamous cell carcinoma	GG
ABC-1	Lung adenocarcinoma	
SK-MES-1	Lung squamous cell carcinoma	
COR-L23	Lung large cell carcinoma	

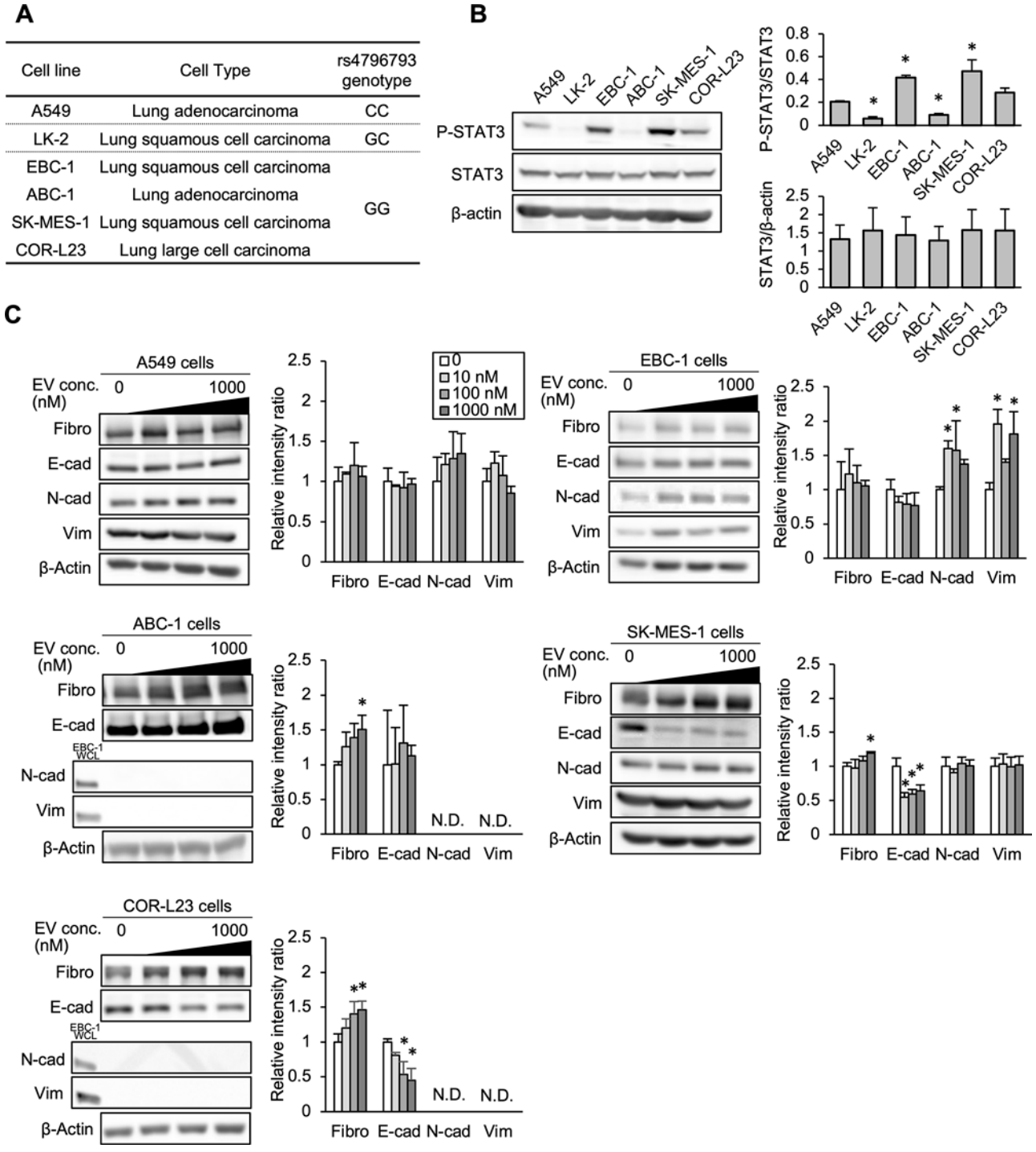

Figure 3. Expression and activity of STAT3 and everolimus-induced changes in expression of epithelial–mesenchymal transition (EMT)-related proteins in lung carcinoma cell lines. (A) Cell type and rs4796793 genotype in lung cancer cell lines. (B) The expression and activity of STAT3 were analyzed by Western blot analysis. The activity of STAT3 was defined as the intensity ratio of phosphorylated STAT3 to total STAT3. STAT3 expression was defined as the intensity ratio of total STAT3 to β-actin. Each bar represents the mean ± standard deviation (SD) ($n = 3$, $^*p < 0.05$ vs. A549 cells). p Values were calculated using Dunnett's test. (C) Everolimus-induced changes in the expression of EMT-related proteins analyzed by Western blotting. The cells were incubated in medium containing 0, 10, 100, or 1000 nM everolimus for 72 h. "EBC-1 WCL" in the ABC-1 and COR-L23 cell section was used to validate the experimental method. Each bar represents the mean ± SD ($n = 3$, $^*p < 0.05$ vs. 0 μM). p Values were calculated using Dunnett's test. E-cad: E-cadherin, EV: everolimus, Fibro: fibronectin, N-cad: N-cadherin, Vim: vimentin, WCL: whole-cell lysate.

incidence of ILD and *STAT3* polymorphism, although it was clear that the primary endpoint may reflect the trend of one institution having the most enrolled patients. In addition, the statistical power of the present analysis was extremely high (96.3%) at a significance level of 0.05 (two-tailed).

Our clinical study clearly demonstrated that patients with the GG genotype of *STAT3* polymorphism rs4796793 (−1697C/G) have a higher risk for mTOR inhibitor-induced ILD compared with those carrying other genotypes. Our in vitro study indicated the possibility that EMT induction by mTOR inhibitors in lung cells increases the risk of mTOR inhibitor-induced ILD in patients with the GG genotype of rs4796793. This genotype may be predictive of the risk for developing ILD before the initiation of mTOR inhibitor therapy. We hope that our findings are applied for the selection of treatment for RCC or intensive care for preventing ILD.

ACKNOWLEDGMENTS: *This work was supported in part by JSPS KAKENHI Grant Nos. 16K08401 and 19K07191 and by the Takeda Science Foundation. We thank all of the participating patients, Professor Kohji Takara at Himeji Dokkyo University for providing the human lung-derivative cell lines, and Dr. Masayuki Miyazaki for the arranging and cooperating with the conduct of this study at Nagoya University Hospital. The authors declare no conflicts of interest.*

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