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ORIGINAL ARTICLE

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A model of prediction system for adverse cardiovascular reactions by calcineurin inhibitors among patients with renal transplants using gene-based single-nucleotide polymorphisms

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Abstract The application of pharmacogenomic information to diagnostic assays is expected to improve the prediction of drug efficacy and toxicity, leading to appropriate therapeutic regimens for individual patients. Cardiovascular events are common and severe adverse drug reactions (ADRs) among transplant patients treated with calcineurin inhibitors (CNIs). We conducted case-control association studies using 50,947 gene-based single-nucleotide polymorphisms (SNPs) to identify genetic variations that might be associated with cardiovascular risk factors in 72 renal transplant recipients with CNI therapy. The overall incidence of cardiovascular events was 13.9% (10/72) among patients receiving cyclosporine or tacrolimus; arrhythmias in six patients (8.3%), ischemic heart diseases in two patients (2.8%), and heart failure in two patients (2.8%). On the basis of results of the genome-wide association studies, we attempted to establish a scoring system to predict individual risks for cardiovascular toxicity of cyclosporine and tacrolimus. Estimation of the predictive performance was carried out by the use of internal leave-one-out cross-validation test. When we combined arrhythmia, ischemic heart disease and heart failure cases as subjects with a cardiotoxicity phenotype, nine of ten ADR patients and 50 of 62 non-ADR patients were correctly classified into the respective categories using the top eight SNPs. In addition, the proportion of individuals in the control population ($n=246$) with scores over the cut-off (11.0%) was close to the cardiovascular ADR frequency (8.3%) among renal transplant patients in the previous clinical study. Our results open the possibility that prediction of CNI-induced cardiovascular complications can lead to better prognosis and quality of life among kidney-transplant patients, and to improved immunosuppressive regimens.

Keywords SNP · Genome-wide · Cyclosporine · Tacrolimus · Cardiotoxicity · Association study · Prediction score

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Introduction

Outcomes of organ transplantation have improved over the last two decades due to the availability of calcineurin inhibitors (CNIs; e.g., cyclosporine and tacrolimus) as immunosuppressive agents for the prevention of acute and chronic rejections. Although these agents bind primarily to different molecular targets [cyclophilin or FK506-binding protein-12 (FKBP12), respectively], they

exert similar pharmacological effects on signal-transduction pathways in T lymphocytes. It is generally accepted that by blocking dephosphorylation and subsequent translocation of the nuclear factor of activated T-cells (NFAT), CNIs inhibit the production of interleukin-2 (IL-2) (Shaw et al. 1988; McCaffrey et al. 1993). Extensive clinical experience has demonstrated that although both drugs are indispensable for immunosuppression after organ transplantation, due to a narrow window for therapeutic dosage it is difficult to find the dose appropriate to each patient. Under-dosing can lead to rejection of the transplanted organ, while over-dosing increases the risks of severe drug-specific adverse events including renal, pancreatic, and cardiovascular toxicity.

Cardiovascular complications, such as heart failure, myocardial infarction, and sudden cardiac death, are the leading causes of death following kidney transplantation, and CNIs are thought to be responsible for inducing these adverse cardiovascular reactions. Hypertension, myocardial ischemia, and arrhythmias have been reported in Japanese patients given tacrolimus therapy after renal transplantation (Seino et al. 2003), and multi-center studies in the United States and Europe have also documented hypertension, arrhythmias, and angina in recipients of kidney transplants who were treated with either cyclosporine or tacrolimus (Pirsch et al. 1997; Mayer et al. 1997; Margreiter, 2002).

The dosage of these drugs is usually decided by monitoring their concentrations in blood; however, the pharmacokinetic profiles of CNIs are not always associated with clinical efficacy or toxicity (Kahan and Grevel 1988; Kahan et al. 1990; Caruso et al. 2001). The evidence suggests that adverse drug reactions (ADRs) caused by CNIs might reflect not only differences in pharmacokinetics from one individual to another, but also variations in genes that alter drug response. Thus, a genome-wide approach seemed to be indicated for elucidating the mechanism of CNI-induced ADR.

We describe here the results of genome-wide association studies using single-nucleotide polymorphisms (SNPs) from the JSNP database (Haga et al. 2002) to identify genetic variations that might confer susceptibility to cardiovascular events under immunosuppressive regimens. On the basis of genotypic information from eight SNP loci at which we observed significant association with ADR, we developed a prediction-scoring system that was able to clearly separate two groups of patients; i.e., with or without ADRs.

Materials and methods

Subjects

DNAs from a total of 72 patients who were treated with either cyclosporine or tacrolimus after renal transplantation were obtained at the University of Tokyo for a case-control association study. Controls consisted of 246 members of the general population recruited through the University of Tokyo. All subjects were Japanese and provided written informed consent to participate in the study in accordance with the process approved by the Ethical Committees at the SNP Research Center, The Institutes of Physical and Chemical Research (RIKEN), Yokohama, Japan, and The Institute of Medical Science at the University of Tokyo, Tokyo, Japan.

Dosage and use of these drugs were pursuant to the standards indicated for kidney transplantation by the Ministry of Health, Labor and Welfare, Japan, and the doses were adjusted for each patient to maintain blood concentrations within the target range. In the present study, the median loading doses given by oral administration were 2.8 and 0.082 mg/kg/day for cyclosporine and tacrolimus, respectively.

SNPs and genotyping

We selected 52,608 SNPs from the JSNP database according to tag-SNP information (Tsunoda et al. 2004) for genome-wide association studies. The SNPs were genotyped using the multiplex PCR-based Invader assay (Third Wave Technologies, Madison, Wis.). DNA extraction, design of PCR primers, multiplex PCR experiments, and Invader assay were performed as previously described (Iida et al. 2001; Ohnishi et al. 2001).

Statistical analyses and prediction-score system

We carried out statistical analysis for association by comparing two patient groups, with or without cardiovascular ADR, by means of Fisher's exact test using dominant-inheritance and recessive-inheritance models. SNPs were rank-ordered according to the lowest *P* value in both models. When a "risk allele" contributed to cardiovascular events in a dominant-inheritance manner, we scored an individual as 2, 1 or 0 according to homozygosity for the risk allele, heterozygosity for the risk allele, or homozygosity for the low-risk allele, respectively. If a risk allele contributed to cardiotoxicity in a recessive-inheritance manner, we assigned scores of 2 to individuals with homozygosity for the risk allele and zero to individuals with other genotypes. Then we calculated a prediction score for each patient by adding his or her scores from each of the SNP loci. The discriminating SNPs for the prediction-scoring system were chosen according to a "leave-one-out" procedure.

Results

Patient characteristics

Table 1 summarizes clinical information for the 72 CNI-treated patients in the study. Cardiovascular events were reported in ten patients (13.9%). Among ten patients

Table 1 Patient characteristics and cardiovascular events

Recipient age	46.7 ± 9.0 years (mean ± SD, range: 27–65 years)
Gender	
Male	48
Female	24
Calcineurin inhibitor	
Cyclosporine	52
Tacrolimus	20
Cardiovascular events	
No events	62 (86.1%)
Arrhythmias	6 (8.3%)
Ischemic heart diseases	2 (2.8%)
Heart failure	2 (2.8%)

with the cardiovascular ADR, we noted arrhythmias in six cases (8.3%) and premature contraction in three, as well as atrial fibrillation, sinus tachycardia, and QT prolongation in one patient each. The frequency of each of ischemic heart diseases and heart failure was observed in two each of these patients. The 12-h trough blood concentrations at the onset of cardiac symptoms were measured in six patients treated with cyclosporine and in two with tacrolimus. Mean trough concentrations were within the therapeutic range at the time of onset of adverse reactions in most of the patients with cardiovascular complications, and were not statistically different from those in patients without ADR (Fig. 1).

Genome-wide association studies

Initially, we performed a case-control association study with six arrhythmia patients and 62 non-ADR patients by genotyping recipients of kidney transplants using a high-throughput multiplex PCR-Invader assay method and 52,608 gene-based SNPs. These SNPs were selected from the JSNP database based on the haplotype block structure estimated previously (Tsunoda et al. 2004). The success rate for this genotyping was 96.8% (50,947 SNPs), and the distribution of *P* values is shown in Table 2. We observed associations between two patient groups at ten SNP loci for which *P* values were < 0.001 by a dominant-inheritance model, and at seven SNP loci with a recessive-inheritance model. Then, we combined arrhythmia, ischemic heart disease and heart failure cases as subjects with a cardiotoxicity phenotype and performed a case-control association study. We observed associations with *P* values of < 0.001 at 14 SNPs according to the dominant-inheritance model between the two groups; eight SNPs revealed *P* values of < 0.001 by the recessive-inheritance model (Table 2).

Development of a prediction-scoring system

On the basis of results of the genome-wide association studies, we attempted to establish a scoring system to predict individual risks for cardiovascular toxicity of cyclosporine and tacrolimus. To determine the optimal number of the SNPs for prediction of ADR risk, an internal leave-one-out cross validation was employed. One of the patients was withheld, and a rank-ordered SNP list and prediction score were generated from the remaining subjects. The withheld patient was tested whether the patient was classified as ADR or non-ADR groups according to a cut-off score at the middle of the medians for ADR and non-ADR groups. The process was repeated for each of all subjects. The rates of correct classification into ADR and non-ADR groups, sensitivity and specificity, were calculated. When we combined arrhythmia, ischemic heart disease and heart failure cases, the number of the SNPs used for the scoring influenced the sensitivity and specificity in the prediction (Fig. 2). Using the eight SNPs that showed the most significant associations, nine of ten ADR patients and 50 of 62 non-ADR patients were correctly classified into the respective categories. Thus, the maximum values for sensitivity and specificity were 90.0 and 80.6%, respectively (Fig. 2). For comparison between arrhythmia and non-ADR groups, we obtained the highest values for sensitivity and specificity, 83.3 and 50.0%, respectively, when the top eight SNPs on each rank-ordered list were used for the scoring in the cross-validation (data not shown). In fact, the prediction-scoring system using the data from all subjects with the top eight SNPs for a combination of three cardiovascular ADRs clearly separated the two patient groups (Fig. 3). To evaluate the system further, we investigated DNA from 246 members of the general Japanese population. As shown in Fig. 3, the scores of these controls ranged from zero to ten with a median of four. When we set a cut-off score at 6.5 using the average for median

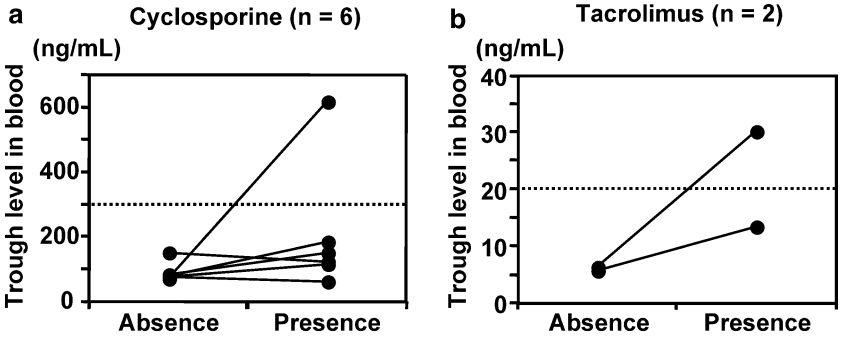

Fig. 1 Twelve-hour trough concentrations of cyclosporine (**a**) and tacrolimus (**b**) in whole blood from renal-transplant recipients at the point of the absence or presence of ADR. *Dotted lines* represent the maximum whole-blood therapeutic range generally accepted for treatment of renal-transplant patients

Table 2 Distribution of *P* values in genotyping of patients with or without cardiovascular events

P value	*Arrhythmias*		*Cardiotoxicity total*	
	Dominant model	*Recessive model*	*Dominant model*	*Recessive model*
≥0.01	50,772	50,847	50,734	50,835
$<1\times10^{-2}$	165	93	199	104
$<1\times10^{-3}$	10	7	11	7
$<1\times10^{-4}$	0	0	3	1
Total	50,947	50,947	50,947	50,947

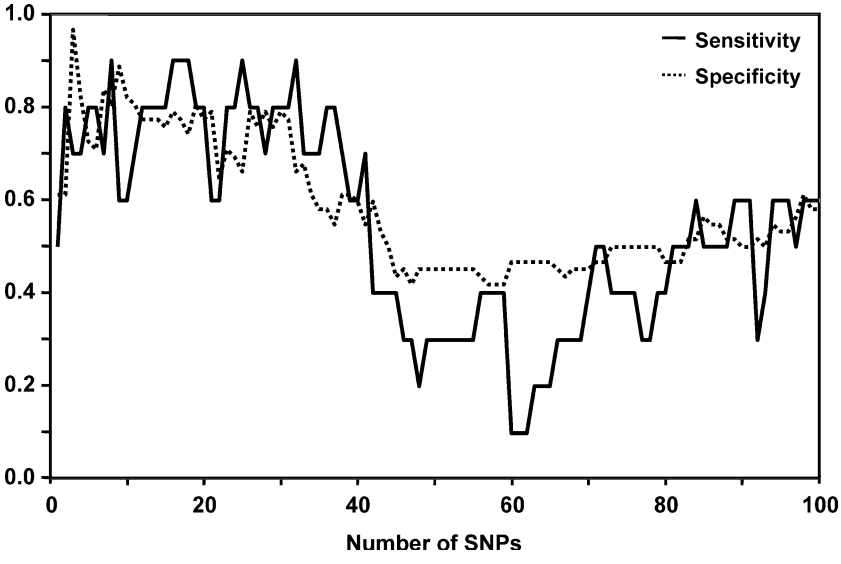

Fig. 2 Optimization of the number of SNPs for prediction-scoring system using the data from combination analysis of arrhythmia, ischemic heart disease and heart failure cases. *Solid and dotted lines* represent the sensitivity and specificity calculated by the prediction scores in leave-one-out test for cross-validation using the top 100 SNPs on the rank-ordered lists

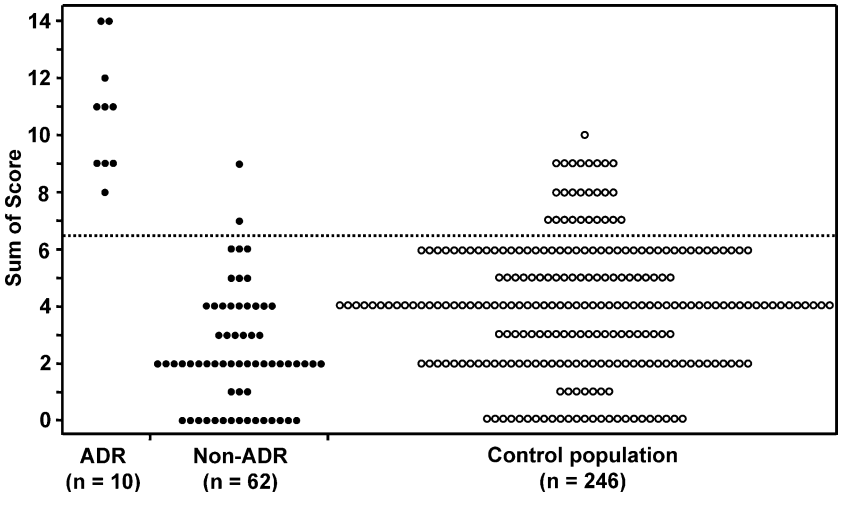

Fig. 3 Prediction scores using the top eight SNPs in the rank-ordered list with the data from combination analysis of arrhythmia, ischemic heart disease and heart failure cases. *Filled circles* indicate scores in 72 learning cases (ten patients with ADR, 62 without). *Open circles* represent scores for 246 individuals from the general Japanese population

values of ADR and non-ADR groups, the proportion of subjects in the control population with scores higher than the cut-off value was 11.0%.

Discussion

ADRs often deteriorate the quality of life and are sometimes fatal. In an attempt to predict genetic risks for these events, many investigators have looked for associations between ADRs and polymorphisms in drug-metabolizing enzymes; however, few useful examples have emerged to allow consistent prediction of ADR prior to drug administration. ADRs due to overdosing frequently correlate with blood concentrations of parent drugs or active metabolites, an indicator of systemic drug exposure. However, in the present study, the mean trough concentrations of CNIs in renal transplant

Table 3 Top eight SNPs associated with arrhythmias, ischemic heart diseases and heart failure

Gene	SNP ID	Group[a]	Genotype[b]			*P* value [odds ratio (95% confidence interval)]		
			11	*12*	*22*	Sum	Genotype *11* versus *12* + *22*	Genotype *11* + *12* versus *22*
STK38L	rs2242185	ADR	9	0	1	10	0.000029	0.44
		Non-ADR	12	35	15	62	[37.5 (4.32–325)]	[2.87 (0.34–24.6)]
GRIK1	rs2248218	ADR	9	0	1	10	0.000029	0.43
		Non-ADR	12	34	16	62	[37.5 (4.32–325)]	[3.13 (0.37–26.7)]
MYO18B	rs2018701	ADR	6	3	1	10	0.000034	0.035
		Non-ADR	2	29	31	62	[45.0 (6.77–299)]	[9.00 (1.07–75.4)]
GRIK1	rs2256478	ADR	9	1	0	10	0.000077	0.10
		Non-ADR	14	32	16	62	[30.9 (3.59–265)]	[Not available]
TNFRSF11A	rs2277731	ADR	4	2	4	10	0.00020	0.73
		Non-ADR	0	30	32	62	[Not available]	[1.60 (0.41–6.23)]
SLC12A8	rs2981503	ADR	0	4	6	10	1	0.00020
		Non-ADR	0	0	62	62	[Not available]	[Not available]
SGSH	rs2071148	ADR	10	0	0	10	0.00024	0.36
		Non-ADR	24	30	8	62	[Not available]	[Not available]
CUGBP2	rs2765981	ADR	1	8	1	10	1	0.00028
		Non-ADR	3	14	45	62	[2.19 (0.20–23.4)]	[23.8 (2.80–203)]

[a] *ADR* patients with cardiovascular symptoms; *Non-ADR* patients without cardiovascular symptoms
[b] *1* risk allele; *2* less-risk allele

patients with cardiovascular ADRs were comparable to those in patients with normal cardiac function (Fig. 1), suggesting that some biological factor(s), not an excess of drug, might be responsible for the emergence of cardiovascular toxicity after administration of cyclosporine or tacrolimus. Therefore, we included two subjects with extremely higher whole-blood concentrations compared with the maximum therapeutic range in the case-control association studies.

We profiled the SNP-genotype patterns of renal transplant patients according to the number of risk alleles each carried, with a view to predicting risk for ADR by means of a scoring system based on data for the SNPs significantly associated with CNI-induced ADR in our patient samples. Because of the small number of subjects, estimation of the predictive performance was not done in an independent group, but by the use of internal leave-one-out cross-validation test. In the validation study, the maximum values for sensitivity and specificity were 90.0 and 80.6%, respectively, in the combination analysis of arrhythmia, ischemic heart disease and heart failure cases, while we obtained the highest values for sensitivity and specificity, 83.3 and 50.0%, respectively, in the comparison between arrhythmia and non-ADR groups. Thus, the prediction-scoring system using data from the combination analysis of three cardiovascular events was more promising compared with the comparison analysis between the arrhythmia and non-ADR groups, probably due to the small number of the arrhythmia subjects. Moreover, the proportion of individuals in the control population ($n = 246$) with scores over the cut-off value (11.0%) was close to the cardiovascular ADR frequency (8.3%) among transplant patients in the previous clinical study (Seino et al. 2003), indicating the feasibility of using our scoring system as a means of predictive genetic testing to distinguish between ADR and non-ADR patients before administration of CNI drugs. Considering the CNI-induced cardiovascular ADR frequency, the positive predictive value was calculated to be only 29.6%, while the negative predictive value was 98.9%. These results indicate that about 70% of patients should show false-positive results, but suggest that our system can be used to exclude high-risk patients with CNI-induced cardiotoxicity among renal transplant recipients.

Coronary vasospasm is generally suspected as the cause of the CNI-associated arrhythmias and myocardial ischemia. In fact, reversible vasospasm was observed in several CNI-treated patients in the present study (data not shown). Proposed mechanisms by which these drugs cause coronary vasospasm include inhibition of expression of the gene encoding endothelial nitric oxide synthetase (eNOS) and activation of the endothelin (*ET*) gene (Weis et al. 2000). In support of this hypothesis, several groups have demonstrated that administration of CNIs significantly increases concentrations of ET, a potent vasoconstrictive peptide, in plasma from organ-transplant recipients (Haug et al. 1995; Slowinski et al. 2002). In addition, it is well-known that the endothelial dysfunction is often observed in patients with heart failure. Several in vitro and clinical studies demonstrated that tumor necrosis factor-α (TNF-α) induced the endothelial dysfunction as a result of downregulation of eNOS expression and an increase in apoptosis (Agnoletti et al. 1999; Fichtscherer et al. 2001). Thus, unknown factor(s) associated with endothelial function might be responsible for the risk of these three cardiovascular ADRs, arrhythmias, ischemic heart disease and heart failure.

Table 3 summarizes the top eight SNPs used for our prediction-scoring system. No association of any of those genes with cardiac functions has been reported; however, variations in the functions and/or expression levels of these genes do appear to contribute to the cardiovascular

toxicity of CNI drugs. For example, one of them, *SLC12A8*, belongs to the cation-coupled chloride ion cotransporter family (*SLC12*) (Hebert et al. 2004), of which two other members (*SLC12A6* and *SLC12A7)* possess transport activity in potassium ion efflux (Mount et al. 1999) and might play important roles in the genesis of arrhythmias following myocardial ischemia (Yan et al. 1996). Although the substrates transported by *SLC12A8* are unknown, our data suggest that polymorphism in this gene may be an important determinant of risk for cardiovascular toxicity with CNI therapy.

In conclusion, through a genome-wide association study we identified genes that might be related to the adverse cardiovascular events often observed in renal transplantation recipients who are given CNI immunosuppressants. Although further validation and improvement of the scoring system described here will be required, using a very large number of patients, our results open the possibility that prediction of CNI-induced cardiovascular complications can lead to better prognosis and quality of life among kidney-transplant patients, and to improved immunosuppressive regimens.

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