Safety and immunologic benefits of conversion to sirolimus in kidney transplant recipients with long-term exposure to calcineurin inhibitors

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Background/Aims: Sirolimus (SRL) is a promising immunosuppressant replacing calcineurin inhibitors (CNIs). This study was performed to evaluate the safety and immunologic benefits of conversion to SRL in stable kidney transplant (KT) recipients exposed to CNIs for long periods.

Methods: Fourteen CNI-treated KT recipients with stable renal function for more than 10 years were included. Either 2 or 3 mg per day of SRL was administered while CNIs were reduced by half starting on day 1, and then stopped 2 weeks after SRL introduction. The safety of SRL conversion was assessed considering the graft function, acute rejection, and graft loss. Immunologic alterations were measured via serial changes of T cell and B cell subsets after SRL conversion. Adverse effects of SRL conversion were also evaluated.

Results: Conversion to SRL was successful in nine patients (64.2%). Conversion to SRL preserved graft function as compared to the baseline value (p = 0.115). No acute rejection or allograft loss was observed during the follow-up period. Immune monitoring of T and B cells revealed a regulatory T cells increase after SRL conversion (p = 0.028). Most adverse events developed within 6 weeks after SRL conversion, and oral mucositis was the main cause of SRL withdrawal.

Conclusions: Conversion to SRL can be safe and has immunologic benefits in KT recipients with long-term CNI exposure. Close monitoring of mucocutaneous adverse events is, however, required in the early period after SRL conversion.

Keywords: Sirolimus; Calcineurin inhibitors; Kidney transplantation

INTRODUCTION

Calcineurin inhibitors (CNIs) are indispensable immunosuppressive agents in kidney transplantation (KT). However, CNI-associated adverse events such as nephrotoxicity, increase of diabetes mellitus, and secondary malignancy limit the use of these drugs [1-3]. To overcome such limitations, CNI minimization with non-nephrotoxic immunosuppressant is recommended in clinical practice.

Sirolimus (SRL), a type of mammalian target of rapamycin inhibitor, was introduced in the late 1990s and regarded as a promising immunosuppressant for replacing CNIs in KT patients. Compared to CNI, SRL causes less nephrotoxicity and has anticancer effects [4]. In addition, SRL provides an immunologic advantage by increasing regulatory T cells (T_{reg}) [5,6] and may prevent the development of chronic allograft nephropathy

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by suppressing the proliferation of smooth muscle cells and fibroblasts [7].

All these findings suggest that SRL may be beneficial for long-term CNI-exposed KT patients. However, most studies on CNI-to-SRL conversion have investigated the early post-transplant period [8-11], and there is little information on SRL conversion in patients with long follow-up periods. Therefore, we evaluated the safety and immunologic advantages of SRL conversion in long-term CNI-exposed KT recipients with stable graft function.

METHODS

Patients

Inclusion criteria included patients who had been followed-up for more than 10 years after KT; no history of rejection; estimated glomerular filtration rate (GFR) more than 50 mL/min/1.73 m^2 via modification of diet in renal disease (MDRD) with renal function change within 10% of baseline value in the past year; no immunosuppressive agent change during the past year; and proteinuria less than 0.3 protein (g)/creatinine (g) in spot urine. This study was approved by the Institutional Review Board (KC11OIS0917) of the Seoul St. Mary's Hospital, and all the patients provided written informed consent. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The study is registered at www.ClinicalTrials.gov (Identifier: NCT01592253).

SRL conversion protocol

We started patients on 2 or 3 mg/day of SRL and reduced CNI dose to 50% of the baseline value at the same time. After 2 weeks, we stopped CNIs entirely and titrated the SRL dose to maintain the blood levels between 3 and 8 ng/mL. We discontinued other immunosuppressive agents such as enteric-coated mycophenolate sodium, azathioprine, or mizoribine till 3 months after conversion, but maintained low-dose steroids [12].

Safety of SRL conversion

We evaluated the safety of SRL conversion using allograft function (estimated GFR via MDRD) at 2, 6, 12, and 24 weeks after conversion. Acute rejection was sus-

pected if serum creatinine increased to more than 30% of the baseline value after SRL conversion. Rejection was confirmed via biopsy. Graft loss was defined as declining graft function that required dialysis, retransplantation, or death with functioning graft. We measured trough level of CNIs at baseline and 2 weeks after conversion. We checked SRL levels and performed laboratory tests including complete blood count, blood chemistry, urinalysis, and urine chemistry at 2, 6, 12, and 24 weeks after conversion.

Changes of immune profile after SRL conversion

We sampled peripheral blood before and 6 months after conversion to SRL. Peripheral blood mononuclear cells (PBMCs) were isolated, and cell cultures were performed as described previously [13]. Flow cytometric study of PBMCs was performed to analyze human intracellular cytokine production. The PBMCs were stimulated with phorbol myristate acetate and ionomycin in the presence of GolgiStop (BD Biosciences, San Diego, CA, USA) for 4 hours. For surface staining, cells were stained with combinations of the following monoclonal antibodies: CD4-PE/Cy7 (BioLegend, San Diego, CA, USA); CD45RA-FITC (BD Pharmingen, San Diego, CA, USA); CD8-APC (Pharmingen), and CD25-APC (Pharmingen). Staining for chemokine receptor CCR7 was performed using monoclonal antibodies to it. Cells were washed, fixed, permeabilized, and stained to detect intracellular cytokines with monoclonal antibodies specific to interferon γ (IFN-γ, eBioscience); interleukin 4 (IL-4, eBioscience); IL-17 (FITC, eBio64DEC17, IgG1, κ; eBioscience); Foxp3 (FITC, PCH101, IgG2a, κ; eBioscience); IFN-γ (PE, B27, IgG1, κ; Pharmingen), and IL-10-APC (JES3-19F1, IgG2a, κ; Pharmingen). Appropriate isotype controls were used for gate setting for measuring cytokine expression. Cells were analyzed on a FACS Calibur flow cytometry system (BD Biosciences).

Adverse events of SRL conversion

During each outpatient clinic visit, we examined adverse events that developed after SRL conversion. These included mucositis, folliculitis, dermatitis, or any signs of infection. We monitored the following: development of proteinuria as spot urine protein-to-creatinine ratio (PCR), diabetes mellitus via fasting glucose level, dyslipidemia via fasting lipid level or lipid lowering agents,

leukopenia, anemia, thrombocytopenia, increasing aspartate or alanine aminotransferase, and requirement of antihypertensive medication. We defined the patient's situation as tolerable when the patient had successfully received SRL for 6 months without any serious adverse events.

Statistical analysis

Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). The comparison of numerical variables at baseline, 2, 6, 12, and 24 weeks from conversion was performed using the Friedman test. The comparison of numerical variables before and 24 weeks after conversion was performed using the Wilcoxon signed-rank test. The results are presented as mean ± standard deviation or standard error, counts and percentages, or median with ranges depending on the data type. The p values < 0.05 were considered significant.

RESULTS

Patients

A total of 45 KT patients met the inclusion criteria in Seoul St. Mary's hospital. Among them, 14 patients (31%) agreed to participate in this study, and they were ultimately enrolled. The mean duration from KT to initiation of the study was 14.7 ± 2.9 years (median, 13.5; range, 12 to 19.5). The mean MDRD-GFR was 72.2 ± 20.2 mL/min/1.73 m². Only one patient was highly sensitized (her flow cytometric crossmatch was positive for B cells), so she had taken plasmapheresis three times before KT. Other baseline patient characteristics are presented in Table 1. Of the 14 patients, eight (57%) were receiving cyclosporine (CsA) with a median dosage of 150 mg (range, 75 to 175); the mean blood trough level was 95.8 ± 58.4 ng/mL. The remaining patients (n = 6, 43%) were receiving tacrolimus (TAC) with a median dosage of 2 mg (range, 2 to 4); the mean blood trough level was 4.2 ± 2.1 ng/mL. The patients' immunosuppressive regimens are also shown in Table 1.

Safety of SRL conversion

The 6-month conversion success rate was 64.2% (n = 9), while 35.8% of patients (n = 5) ceased receiving SRL.

Table 1. Baseline characteristics of patients

Variable	Value
Male sex	2 (14.3)
Age, yr	54.3 ± 10.5
Retransplantation	2 (14.3)
Time from transplant to conversion, yr	14.7 ± 2.9
Type of donor	
Deceased	6 (42.9)
Living	8 (57.1)
eGFR by MDRD, mL/min/1.73 m²	72.2 ± 20.2
Urine protein-to-creatinine ratio	0.08 ± 0.08
Mismatched no. of HLA antigen	2.6 ± 2.1
Causes of end stage renal disease	
Chronic glomerulonephritis	8 (57.1)
Hypertensive nephropathy	1 (7.1)
Unknown	5 (35.7)
Comorbid disease	
New onset diabetes after transplantation	1 (7.1)
Hypertension	7 (50)
Immunosuppressant regimen	
Cyclosporin	1 (7.1)
Cyclosporin + steroid	3 (21.4)
Cyclosporin + enteric-coated mycophenolic acid	3 (21.4)
Cyclosporin + azathioprine + steroid	1 (7.1)
Tacrolimus + steroid	1 (7.1)
Tacrolimus + enteric-coated mycophenolic acid	2 (14.3)
Tacrolimus + mizoribine	2 (14.3)
Tacrolimus + azathioprine	1 (7.1)

Values are presented as number (%) or mean ± SD.
eGFR, estimated glomerular filtration rate; MDRD, modification of diet in renal disease; HLA, human leukocyte antigen.

Renal allograft function at baseline and 2, 6, 12, and 24 weeks after SRL conversion was not significantly different (71.2 ± 17.6, 71.0 ± 17.1, 72.0 ± 16.7, 71.2 ± 17.6, and 67.7 ± 14.6 mL/min/1.73 m² via MDRD-GFR respectively), indicating good graft function preservation after SRL conversion (p = 0.115) (Fig. 1). There was no acute rejection or graft loss after SRL conversion. Fig. 2 shows trough levels of TAC and CsA before and 2 weeks after SRL conversion and SRL trough levels at 2, 6, 12, and 24 weeks after SRL conversion. The 50% dosage reduction of CsA
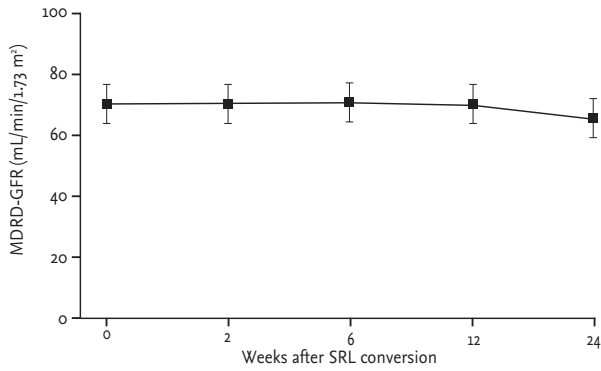
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Figure 1. Renal allograft function during 24 weeks after sirolimus conversion (n = 9, p = 0.115). Note no significant change of graft function after sirolimus (SRL) conversion. Black dots indicate means; error bars indicate standard errors. MDRD, modification of diet in renal disease; GFR, glomerular filtration rate.

or TAC caused the trough level to decrease by 48.7% and 27.2%, respectively (Fig. 2A and 2B). Levels of SRL were variable in the early period of conversion but gradually stabilized and reached the target level (3 to 8 ng/mL) by 12 weeks post-conversion (mean trough level, 7.4 ng/mL). The levels were not different statistically (p = 0.919), but showed decreasing pattern throughout the study periods (Fig. 2C).

Immunologic benefits of SRL conversion

Figs. 3 and 4 show the immunologic changes in six of the nine successfully converted patients. Three of them had received CsA, while the remaining three had received TAC before SRL conversion.

The percentage of CD4+ T_{reg} increased at 24 weeks after SRL conversion (6.3% ± 3.2% vs. 8.6% ± 5.4%, p = 0.028)

(Fig. 3A). There were no differences in the percentages of Th1 (23.4% ± 9.8% vs. 24.1% ± 10.4%, p = 0.917), Th2 (3.5% ± 4.2% vs. 1.3% ± 1.2%, p = 0.6), or Th17 (1.3% ± 0.6% vs. 2.1% ± 1.1%, p = 0.249) (Fig. 3B-3D). The percentage of CD8+ $T_{naïve}$ (44.0% ± 13.8% vs. 39.8% ± 9.0%, p = 0.462), CD8+ central memory T cells (TCM) (27.1% ± 10.5% vs. 30.6% ± 12.7%, p = 0.753) or CD8+ effector memory T cells (T_{EM}) (13.0% ± 9.3% vs. 15.3% ± 4.2%, p = 0.6) also did not alter after SRL conversion (Fig. 4A-4C). The percentage of IFN-γ producing CD8+ $T_{naïve}$ (59.7% ± 26.1% vs. 50.2% ± 41.8%, p = 0.6) and T_{CM} (57.4% ± 15.1% vs. 38.1% ± 24.7%, p = 0.075) did not change (Fig. 4D and 4E), but the percentage of IFN-γ producing CD8+ T_{EM} reduced after conversion (58.5% ± 18.9% vs. 37.9% ± 25.4%, p = 0.046) (Fig. 4F). No significant difference was seen between B cell subsets. The percentage of memory B cells (22.6% ± 11.5% vs. 30.9% ± 11.1%, p = 0.345), mature B cells (39.1% ± 9.8% vs. 35.1% ± 11.3%, p = 0.293), and immature B cells (5.1% ± 3.6% vs. 9.7% ± 9.3%, p = 0.345) did not change 24 weeks after SRL conversion.

Adverse events of SRL conversion

Total cholesterol, low-density lipoprotein, and triglyceride levels increased after SRL conversion (178 ± 22 mg/dL vs. 223 ± 58 mg/dL, p < 0.001; 97 ± 28 mg/dL vs. 149 ± 45 mg/dL, p < 0.001; and 94 ± 38 mg/dL vs. 114 ± 57 mg/dL, p = 0.018; respectively). Urine PCR also increased after conversion (0.08 ± 0.08 vs. 0.39 ± 0.40, p = 0.026). Of the 14 patients, 11 (79.6%) experienced mucocutaneous adverse events. Oral mucositis (n = 7), dermatitis (n = 4), facial and leg edema (n = 3), folliculitis (n = 1), acne (n = 1), and diarrhea (n = 1) were all recorded. Most events occurred within 6 weeks after conversion and led to
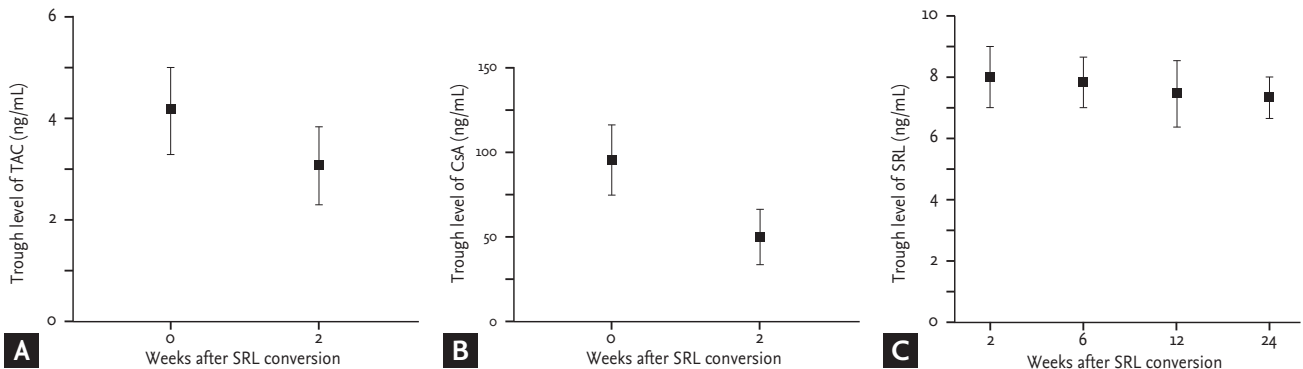

Figure 2. Trough level of (A) tacrolimus (TAC), and (B) cyclosporin (CsA) before and 2 weeks after sirolimus (SRL) conversion, (C) trough level of sirolimus at 2, 6, 12, and 24 weeks after SRL conversion. Black dots indicate means; error bars indicate standard errors.
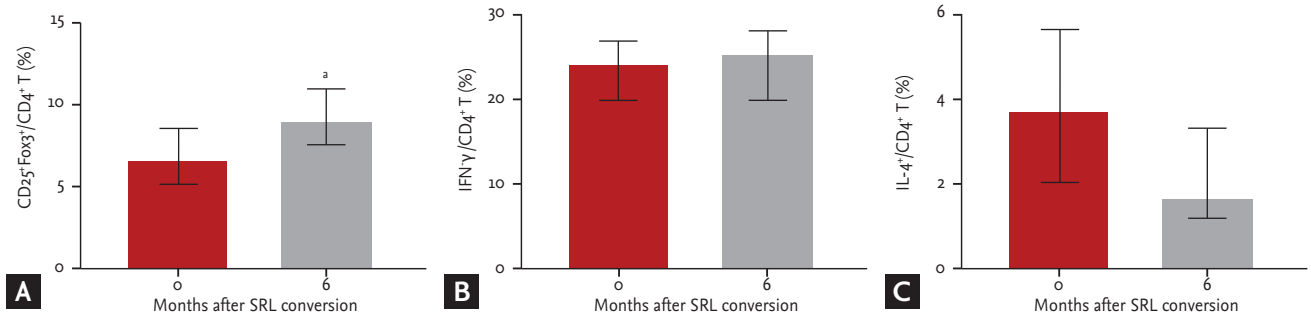
Figure 3. Effect of conversion from calcineurin inhibitors to sirolimus on CD4$^+$ T lymphocyte subpopulations within the peripheral blood mononuclear cell population isolated from kidney transplant recipients (n = 6). The percentage of (A) regulatory T cells (T$_{reg}$), (B) Th1, (C) Th2, and (D) Th17 cells before and after 6 months sirolimus (SRL) conversion were compared. Note significant increase of CD25$^+$Foxp3$^+$/CD4$^+$ T cells (T$_{reg}$) after SRL conversion (6.3% ± 3.2% vs. 8.6% ± 5.4%). IFN-γ, interferon γ; IL-4, interleukin 4. ^{a}p 0.028, Wilcoxon signed-rank test.
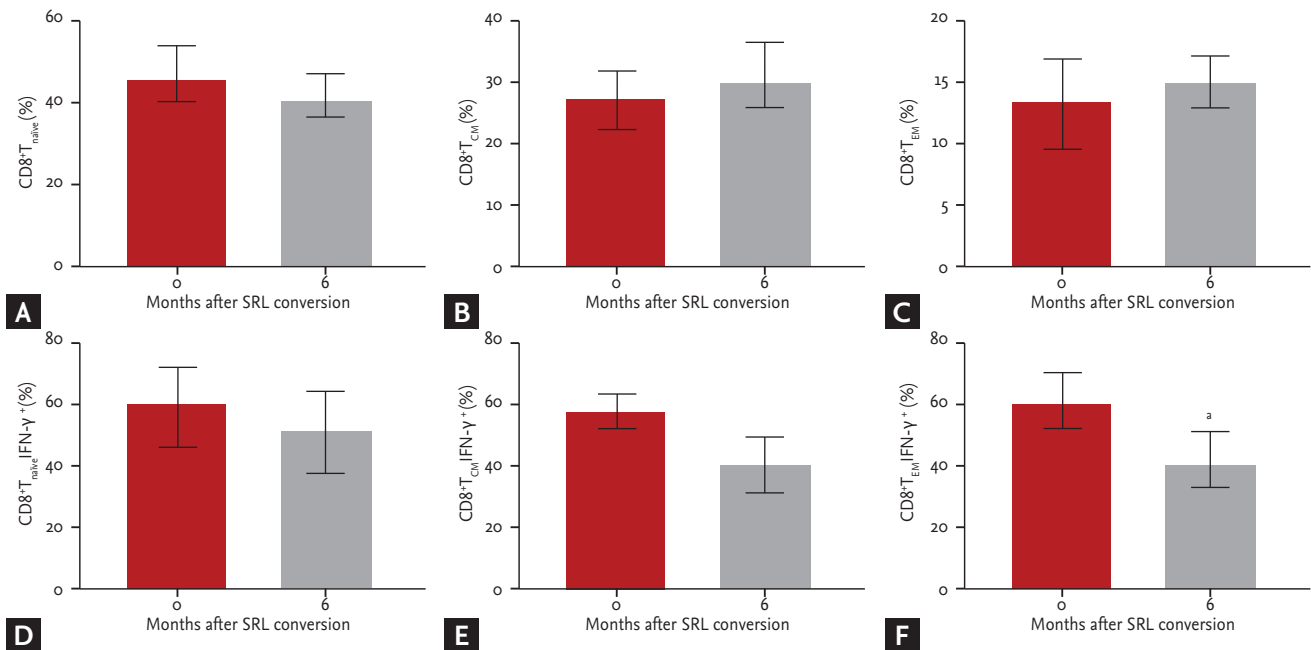

Figure 4. Effect of conversion from calcineurin inhibitors to sirolimus (SRL) on CD8$^+$ T lymphocyte subpopulations within the peripheral blood mononuclear cell population isolated from kidney transplant recipients (n = 6). The percentage of (A) CD8$^+$T$_{naïve}$, (B) CD8$^+$T$_{CM}$, (C) CD8$^+$T$_{EM}$, and those of interferon γ (IFN-γ) producing cells (D, E, F) before and after 6 months SRL conversion were compared. Note significant decrease of IFN-γ producing CD8$^+$TCM cells after SRL conversion (58.5% ± 18.9% vs. 37.9% ± 25.4%). T$_{CM}$, central memory T cells; T$_{EM}$, effector memory T cells. ^{a}p 0.046, Wilcoxon signed-rank test.

withdrawal. The main cause of discontinuation was intolerable oral mucositis (n = 3). Other causes were facial dermatitis (n = 1) and diarrhea (n = 1). Development of leukopenia, anemia, thrombocytopenia, abnormality of liver function tests, increase of fasting glucose, or infections was not observed.

DISCUSSION

The results of our study clearly demonstrated that SRL conversion is safe in long-term (mean, 14.7 years) CNI-exposed KT recipients with stable graft function. Conversion to SRL did not affect graft function, and

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there were no episodes of acute rejection or graft loss. Furthermore, conversion to SRL provided immunologic benefits by increasing T_{reg}. These findings suggest that conversion to SRL is recommendable in long-term CNI-exposed KT patients with stable graft function.

First, we evaluated whether conversion to SRL is actually safe in long-term CNI-exposed KT patients. Although graft function was stable and no acute rejection episodes were noted after SRL conversion, we did not observe further improvement of graft function after SRL, as observed in other studies of early SRL conversion [14,15]. This may be related to the minimal dose of CNIs received by patients in our study, which infers minimal toxicity before SRL conversion. The lack of acute rejection episodes in this study seems to be related to the conversion timing. Our patients were also immunologically stable before conversion; therefore, conversion to SRL may not have affected the overall immune status. Taken together, our study suggests that SRL conversion is safe without leading to deteriorating graft function, development of acute rejection, or graft loss.

Treatment with CNIs induces the production of proinflammatory cells, such as Th17, and reduces that of anti-inflammatory phenotypes, such as T_{reg}, by blocking the transcription of the gene encoding IL-2 [16,17]. We reported previously that SRL treatment decreased Th17 and increased T_{reg} *in vitro* and *in vivo* [18]. Therefore, we expected that SRL conversion would provide immunologic advantages by reciprocal regulation of the Th17/T_{reg} axis. In our study, SRL conversion increased the proportion of T_{reg} but did not change the proportion of Th17. Additionally, IFN-γ producing CD8⁺ TEM decreased, which was associated with late allograft dysfunction in our previous study [19]. Based on these immunologic findings, SRL may have higher favorable effects by regulating the Th17/T_{reg} axis and by decreasing IFN-γ producing CD8⁺ T_{EM} in KT recipients when compared to current CNI therapy. In B cell subpopulations, however, SRL conversion did not induce any change. This may be because of our patients' stable immune status before conversion.

The SRL conversion was successful in nine of 14 patients (64.2%). However, the five patients (35.8%) who withdrew did so primarily owing to mucocutaneous inflammation, mostly within 6 weeks after conversion. The conversion rate and adverse events were similar to previous studies [9,10,15,20], but serious adverse events such as pneumonitis did not occur in our study. The leading cause of SRL discontinuation was oral mucositis, experienced by half of the enrolled patients. It was thought that trough level of SRL was relatively high in early period (2 weeks vs. 6 months, 7.9 ng/mL vs. 7.3 ng/mL, respectively). A previous report suggested that 3 to 7 days of topical clobetasol application was an effective treatment for SRL-induced aphthous ulcers [21]. Close monitoring of mucocutaneous complications and treatment with topical steroid ointment might be helpful, but as these patients were unable to tolerate these adverse events, we decided to withdraw these patients during the early period of SRL conversion. In addition, SRL caused hyperlipidemia and proteinuria, but the degree of proteinuria in our study was not clinically significant (PCR, 0.39 ± 0.40).

Our study had some limitations. First, the number of enrolled patients was small (31% participation rate) in spite of a detailed explanation of the advantages of SRL. Eligible patients were required to have received CNIs for more than 10 years. Therefore, it was difficult for clinicians to introduce new immunosuppressant to stable KT recipients who were also reluctant to change it. Second, there was no control group for comparing the change of renal function and adverse events after conversion. Third, the follow-up duration (6 months) was too short to observe any potential anticancer effect of SRL. A larger number of patients and long-term follow-up are needed in further studies.

In summary, SRL conversion in long-term CNI-exposed KT recipients with stable graft function is a safe and immunologically beneficial switch in terms of graft function, acute rejection and changes of T cell subsets. Drawbacks included intolerably severe mucocutaneous adverse events, ultimately resulting in study withdrawal. Close monitoring and effective treatment of mucocutaneous adverse events is required in the early period after SRL conversion to ensure success.

KEY MESSAGE

1. Sirolimus (SRL) conversion in long-term stable kidney transplantation recipients is safe.
2. SRL conversion has immunologic advantage via increasing regulatory T cell (T_{reg}).

3. Mucocutaneous complications are the most common side effect of SRL occurred in early period of SRL conversion.

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