

Assessing the efficacy of mammalian target of rapamycin inhibitors by phosphorylation of p70S6K in CD4-positive cells of liver transplant patients

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Abstract

The activity of p70S6 kinase located downstream of the mammalian target of rapamycin (mTOR) pathway is sensitive to mTOR inhibitors. However, the methods of assessing p70S6 kinase activity are still unclear. This study aimed to investigate p70S6 kinase activity in CD4-positive cells of liver transplant patients.

Liver transplant patients treated with mTOR inhibitors were recruited from Beijing Chaoyang Hospital between October 2014 and October 2016. The influence of mycophenolic acid (MPA) derivatives and prednisone on p70S6 kinase phosphorylation in CD4-positive cells was examined in liver transplant patients and healthy controls (HCs). The phosphorylation of p70S6K in CD4 + CD25^{hi} regulatory T cells (Treg cells) and CD4 + CD25- T effector cells was analyzed by phospho-flow cytometry.

The phospho-flow technique detected a significant loss of p70S6 kinase phosphorylation in CD4-positive cells of patients treated with mTOR inhibitors compared with HCs. MPA derivatives and prednisone did not affect p70S6 kinase phosphorylation significantly. No significant difference in p70S6 kinase phosphorylation was observed when the whole blood was stored within 3 hours at room temperature. The phosphorylation of p70S6K was significantly lower in CD4 + CD25^{hi} Treg cells than in CD4 + CD25-T effector cells in HCs. After liver transplant patients were treated with mTOR inhibitors, p70S6K phosphorylation was more reduced in CD4 + CD25-T effector cells than in CD4 + CD25^{hi} Treg cells.

The presence of phosphorylation of p70S6 kinase in CD4-positive cells was reduced in liver transplant patients who were treated by mTOR inhibitors.

Abbreviations: BD = Beckman Dickons, FKBP12 = FK506-binding protein of 12, HCs = healthy controls, MFI index = Mean Fluorescent Intensity index, MPA = mycophenolic acid, mTOR = mammalian target of rapamycin, mTORC1 = mTOR complex 1, PBMC = peripheral blood mononuclear cell, PBS = phosphate buffered saline, PI3K = phosphoinositide 3-kinase, Tregs = regulatory T cells.

Keywords: mTOR inhibitor, P70S6 kinase, phospho-flow cytometry

1. Introduction

The mammalian target of rapamycin (mTOR) is a central modulator in intracellular signaling pathways to regulate mammalian cell activities, such as cell proliferation, growth, survival, metabolism, and so on.^[1,2] The mTOR inhibitors

mainly include rapamycin (sirolimus) and everolimus, which were approved to prevent rejection in organ transplantation by the Food and Drug Administration in 2010. Both everolimus and sirolimus have a similar mechanism of action to inhibit mTOR. The mTOR inhibitors bind to FKBP12, which interacts with the FKBP12-binding domain of mTOR, thus leading to cell-cycle arrest in the G1 phase.^[3] P70S6 kinase located downstream of mTOR pathway and the phosphorylation of p70S6 kinase represent an important target for the sensitive detection of the pharmacodynamic effects of mTOR inhibitors on T-cell activation.^[2,4,5]

The trough level used in clinical work does not reflect any aspect of the individual's immune system.^[6] First, pharmacokinetics cannot measure the biological effects of drugs on immune cells and account for inter-subject variability in sensitivity toward the drug. Second, significant inter- and intra-individual differences in the trough level have been shown between transplant patients.^[7] Third, the final treatment effectiveness is reduced and disturbed because several different types of immunosuppressive agents are used simultaneously. Pharmacodynamic drug monitoring on immune cells represents an effort to receive more reliable information on the biological effects of immunosuppressive agents. It has been developed for mycophenolate mofetil and cyclosporine A.^[8-10] For instance, the determination of inosine-5'-monophosphate dehydrogenase activity was recently

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suggested to guide mycophenolate mofetil therapy after renal transplantation. This methodology could improve the survival and function of transplant patients.

The aim of this study was to investigate p70S6K phosphorylation in CD4-positive cells of liver transplant patients treated with mTOR inhibitors and HCs by phospho-flow cytometry. The extent of p70S6K phosphorylation under different storing conditions was examined.

2. Methods

2.1. Patients

Thirty liver transplant patients were investigated in Beijing Chaoyang Hospital of the Capital Medical University, China. The study protocol was approved by the Ethics Committee of our institution (14-ke-01/2014.07.121) and the study was conducted from October 2014 to October 2016. All the patients gave written informed consent. The inclusion criteria were as follows:

- (1) Patients gave written informed consent to participate.
- (2) All patients were treated with mTOR inhibitors after liver transplantation.

The exclusion criteria were as follows:

- (1) patients with immunological diseases, hepatitis, human immunodeficiency virus (HIV) positivity, or hematologic malignancies; and
- (2) Patients with acute or chronic infection.

Ten healthy controls (HCs) served as a control group in this study. The age and gender distribution among liver transplant patients and HCs was not significantly different ($P = .42$, $P = .58$, respectively).

2.2. Lymphocyte staining and flow cytometry data analysis

The fixation and analysis were performed immediately after collecting the heparinized blood samples. Freshly isolated peripheral blood mononuclear cells (PBMCs) were harvested, washed with 2 mL of phosphate-buffered saline (PBS), and resuspended in 500 μ L of PBS (for 1×10^6 cells). Then, the PBMCs were fixed using 500 μ L of pre-warmed Beckman Dickons (BD) Phosflow Fix Buffer 1 at 37°C for 10 minutes and washed with PBS. The surface staining antibody was added in appropriate volumes to 90 μ L of PBMC suspension and stained for 30 minutes in the dark at room temperature. BD Phosflow Per/Wash Buffer 1 was diluted (1:10) in distilled water prior to use. The samples were washed twice with 3 mL of BD Per/Wash Buffer 1 and then incubated for 15 minutes with 3 mL of BD Per/Wash Buffer 1 on ice. After the PBMCs were washed with 3 mL of BD Phosflow Per/Wash Buffer 1 twice, the Phospho-p70S6 Kinase (Thr389) (1A5) was added to 90 μ L of PBMC suspension and stained for 30 minutes in the dark at room temperature. The stained Phospho-p70S6 Kinase (Thr389) (1A5) was washed with 3 mL of BD phosflow per/wash Buffer 1, and the PBMCs were incubated with 10 μ L of Anti-Mouse IgG2a-PE for 30 minutes in the dark at room temperature. After washing with 3 mL of BD phosflow per/wash Buffer 1, the PBMCs were resuspended in 300 μ L of PBS and finally analyzed by flow cytometry. The acquisition was performed on a FACSCalibur flow cytometer (BD, USA) using the CellQuest software (BD, USA). Data were analyzed using FlowJo Software (BD, USA), Version 8.7.3. The level of phosphorylation at Thr389 of p70S6

kinase was quantified using the PE mean fluorescence intensity (MFI) index. The MFI index was calculated using the following formula: MFI index = [(MFI of anti-Fas Ab-stained cells – MFI of control IgG1-stained cells)/MFI of control IgG1-stained cells].

2.3. Statistical analysis

All statistical analysis was performed by SPSS version 17.0 (SPSS Inc., IL). Data were expressed as mean \pm standard deviation (SD) and compared using the 2-tailed Student *t* test for normally distributed population and Mann–Whitney *U* test for not normally distributed population. The significance of the difference between independent group enumeration data was determined using the Fisher exact test. A *P* value less than .05 was considered statistically significant.

3. Results

3.1. Baseline characteristics of liver transplant patients and HCs

This study included 30 liver transplant patients (17 female and 13 male, mean age 54 ± 12 years, range 18–76). PBMCs were collected at various time points following transplantation. The patient characteristics are shown in Table 1. Clinical chemistry was performed on the same specimen used for FACS acquisition. Further, 10 HCs (6 female and 4 male, mean age 53 ± 11 years, range 16–82) served as the control group in this study. The age and gender distribution between liver transplant patients and HCs was not significantly different ($P = .47$, $P = .72$, respectively) (Table 1). The medical history of HCs did not reveal acute infection or immunological diseases. No renal diseases were observed during the time of measurement.

3.2. P70S6 kinase phosphorylation in CD4-positive cells of patients treated with mTOR inhibitors and HCs

P70S6 kinase phosphorylation in CD4-positive cells was assessed in patients treated with mTOR inhibitors and HCs (Fig. 1). The phospho-flow technique detected a significant loss of p70S6K phosphorylation in CD4-positive cells of patients treated with mTOR inhibitors ($n = 30$), Mean Fluorescent Intensity Index (MFI index): 21.3 ± 6.9 compared with HCs ($n = 10$) MFI: 52.4 ± 10.5 , $P < .05$.

Table 1

No significant differences in gender ratio and age were observed in transplant patients compared with HCs ($P > .05$).

	Sirolimus	HC	<i>P</i>
Total number	30	10	
Women/men	17/13	6/4	.47
Age (year)	54 ± 12	53 ± 11	.72
Leukocytes (/ μ L)	7.1 ± 3.6	6.5 ± 2.1	.09
Creatinine (mg/dL)	2.1 ± 1.1	2.3 ± 1.5	.58
Total cholesterol (mg/dL)	196 ± 53	201 ± 69	.32
Cholesterolin (mg/dL)	243 ± 47	197 ± 43	.01
HDL (mg/dL)	52 ± 14	51 ± 11	.52
LDL (mg/dL)	132 ± 22	110 ± 29	.006
Triglyceride (mg/dL)	237 ± 89	143 ± 59	.001

HC = healthy control, HDL = high-density lipoprotein, LDL = low-density lipoprotein.

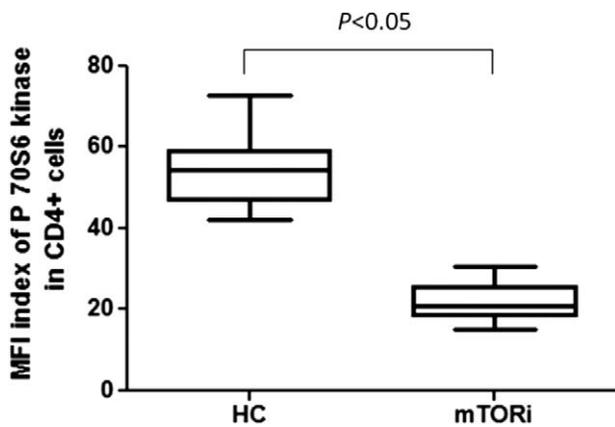


Figure 1. CD4-positive cells of HCs and liver transplant recipients treated with mTOR inhibitors were isolated and stained for p70S6 kinase using phospho-flow cytometry. The bar graph shows that p70S6 kinase phosphorylation in CD4-positive cells was significantly reduced in patients treated with mTORs compared with HCs ($P < .05$). mTOR=mammalian target of rapamycin, HC=healthy control.

3.3. Influence of storage duration on p70S6 kinase phosphorylation

The time-dependent intra-assay variability was determined in heparinized blood samples collected from 5 HCs to evaluate the influence of storing conditions. These blood samples were either processed by phospho-flow cytometry immediately (0 hour) or processed after storage at room temperature for 3, 6, and 24 hours (Fig. 2). The MFI index of the 5 HCs was 68.1 ± 18.3 , 65.0 ± 17.9 , 44.39 ± 13.8 , and 25.4 ± 10.3 at 0 hour, 3 hours, 6 hours, and 24 hours after withdrawal, respectively. The MFI index analysis demonstrated similar results at 0 and 3 hours after blood sample collection (68.1 ± 18.3 vs 65.0 ± 17.9 , $P > .05$, Fig. 2).

3.4. Influence of (mycophenolic acid) MPA and prednisone on p70S6 kinase phosphorylation

Many immunosuppressive agents were used in clinical treatment for sufficient effects of immunosuppression. MPA and prednisone

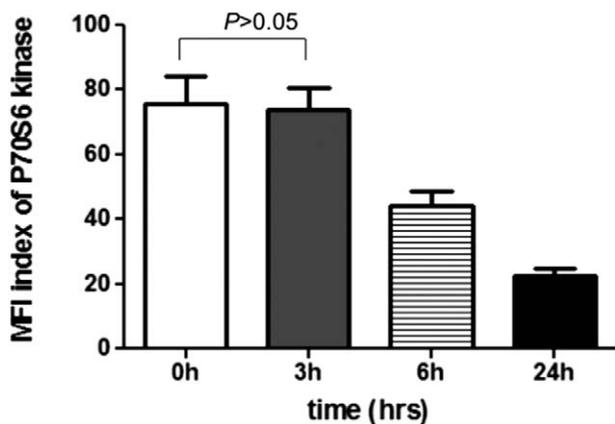


Figure 2. MFI index of p70S6 kinase in healthy controls after storing for different durations. No significant difference was observed between the MFI index of p70S6 kinase after 0 and 3 hours at room temperature ($P > .05$, $n = 5$). MFI index=Mean Fluorescent Intensity index.

Table 2

Influence of MPA and prednisone on p70S6 kinase phosphorylation.

	mTOR inhibitor	n	MFI index (mean \pm SD)	P (+ vs -)
MPA	+	13	29.1 ± 7.5	.49
	-	17	31.2 ± 6.3	
Prednisone	+	18	52.2 ± 11.3	.37
	-	12	51.2 ± 9.3	
Combination	+	10	53.2 ± 6.3	.61
	-	8	$57.1.2 \pm 6.8$	

MFI index = Mean Fluorescent Intensity Index, MPA = mycophenolic acid, mTOR = mammalian target of rapamycin.

were the important ones of these. The p70S6 kinase phosphorylation of CD4-positive cells was performed in patients treated with MPA or prednisone after liver transplantation. MPA and prednisone did not affect p70S6 kinase phosphorylation significantly in the mTOR inhibitor group (Table 2).

3.5. P70S6 kinase phosphorylation of CD4 + CD25^{hi} Treg cells and CD4 + CD25^{lo} T effector cells after treatment with mTOR inhibitors

The phosphorylation of p70S6K was significantly lower in CD4 + CD25^{hi} Treg cells than in CD4 + CD25^{lo} T effector cells ($n = 8$) in HCs. The phosphorylation of p70S6K selectively reduced in CD4 + CD25^{lo} T effector cells, leaving CD4 + CD25^{hi} Treg cells unimpaired in patients treated with mTOR inhibitors ($P < .05$, Fig. 3).

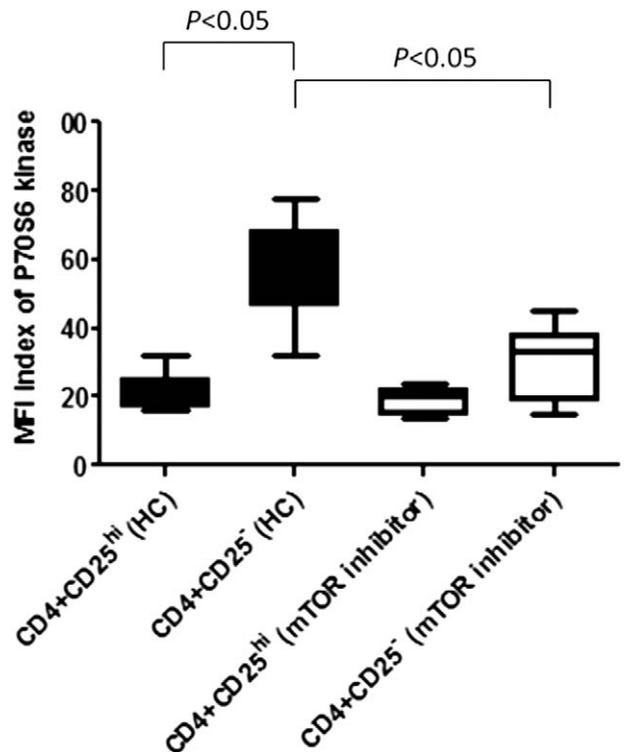


Figure 3. The bar graph demonstrating differential phosphorylation status of p70S6K in CD4+CD25^{hi} Treg and CD4+CD25^{lo} T effector cells. The mTOR inhibitor mainly inhibits the phosphorylation of p70S6K in CD4 + CD25^{lo} T effector cells ($n = 8$).

4. Discussion

The application of mTOR inhibitors in solid organ transplantation has increased because they have potent anti-inflammatory properties and prevent acute rejection. The therapeutic monitoring of mTOR inhibitor therapy is based on trough levels, which do not necessarily reflect the biological effects of the (Phosphoinositide 3-kinase) PI3K/Akt/mTOR pathway. The p70S6 kinase is located downstream of the mTOR pathway. The phosphorylation of p70S6K represents an important target for the sensitive detection of the pharmacodynamic effects of mTOR on T-cell activation. The aim of this study was to investigate p70S6 kinase phosphorylation in CD4-positive cells of liver transplant patients. In this study, the MFI index of CD4-positive cells was significantly lower in patients treated with mTOR inhibitors than in HCs.

The phosphorylation of a protein correlates with its biological functions. For kinases, phosphorylation typically enhances their enzymatic activity, conducting signals for downstream regulation.^[11] While activating PI3K kinase cascade, signaling begins on the cell surface and passes from PI3K kinase to mTOR, and finally to p70S6 kinase. The p70S6 kinase is stimulated by the activation of (mTOR complex 1) mTORC1. This leads to the increase in messenger RNA translation and results in T-cell proliferation and upregulation of further surface molecules, which is important for initiating or amplifying the immunological response.^[12–14] The mTOR inhibitors are able to form a complex with FK-binding protein 12 (FKBP12), which binds to mTOR to suppress its activity.^[15] Therefore, it is able to halt cell-cycle progression from the G1 phase to S phase by inhibiting mTOR so as to halt the phosphorylation of p70S6 kinase in T-cell proliferation.

The mTOR inhibitors can be used in combination with MPA to reduce drug toxicity and ensure effective immunosuppression. MPA is an anti-proliferative agent. It inhibits lymphocyte proliferation by limiting the availability of purine, which is critical for the biosynthesis of DNA and RNA. The target of MPA in lymphocytes is not involved in the mTOR pathway. As expected, the analysis of p70S6 kinase phosphorylation in patients with or without MPA and prednisone therapy showed no differences, thus excluding a confounding effect of MPA and prednisone.

The phosphorylation of proteins is often a reversible event and may change within a few hours.^[16] Therefore, the time of sample collection and duration of sample preparation were investigated in this study. It demonstrated that p70S6 kinase phosphorylation was indeed susceptible to storage time in lymphocytes. The blood samples were processed within 3 hours after collection to obtain reliable results. Therefore, the differences in p70S6 kinase phosphorylation in different patients did not result from the collecting time and storage condition. Drug absorption, distribution and interaction, metabolism, and elimination might be responsible for these differences. In addition, the collection of samples and isolation of CD4-positive cells within 3 hours are feasible in clinical practice.

In this study, CD4 + CD25^{hi} Treg cells showed lower levels of p70S6K activity compared with CD4 + CD25⁻ T effector cells in

HCs. The p70S6K activity of CD4 + CD25⁻ T effector cell subset was significantly lower in liver transplant patients treated with mTOR inhibitors than in HCs. However, the CD4CD25^{hi} Treg subset was virtually not affected. These results indicated that the p70S6K activity of the CD4 + CD25⁻ T effector cell subset could be primarily inhibited by mTOR inhibitors.

In conclusion, the presence of phosphorylation of p70S6 kinase in CD4-positive cells was reduced in liver transplant patients treated with mTOR inhibitors.

Author contributions

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