Original Article

P70S6 Kinase Phosphorylation: A New Site to Assess Pharmacodynamy of Sirolimus

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Abstract

Background: The phosphorylation of p70S6 kinase (p70S6K) represents an important target for sensitive detection on pharmacodynamic effects of sirolimus, but the methods of assessing p70S6K phosphorylation are still unclear. The aim of this study was to investigate p70S6K phosphorylation located down-stream of the mammalian target of rapamycin (mTOR) pathway in peripheral blood mononuclear cells (PBMCs) of liver transplant patients through different methods.

Methods: Seventy-five liver transplant recipients from Beijing Chaoyang Hospital of the Capital Medical University were analyzed in this study. Patients were divided into three groups, patient treated with sirolimus ($n = 22$), patient treated with tacrolimus ($n = 30$), patient treated with cyclosporine ($n = 23$). The p70S6K phosphorylation of PBMCs in patients and healthy control (HC, $n = 12$) were analyzed by phospho-flow cytometry and Western blotting. A correlation analysis of data from phospho-flow cytometry and Western blotting was performed. Intra-assay variability of p70S6K phosphorylation in HC and different patients were measured.

Results: Intra-assay variability of p70S6K phosphorylation in phospho-flow cytometry was from 4.1% to 8.4% and in Western blotting was from 8.2% to 18%. The p70S6K phosphorylation in patients receiving a sirolimus (19.5 ± 7.7) was significantly lower than in HC (50.1 ± 11.3, $P < 0.001$), tacrolimus (37.7 ± 15.7, $P < 0.001$) or cyclosporine treated patients (41.7 ± 11.7, $P < 0.001$). The p70S6K phosphorylation in HC (50.1 ± 11.3) was significantly higher than in tacrolimus (37.7 ± 15.7, $P < 0.01$) or cyclosporine-treated patients (41.7 ± 11.7, $P < 0.01$). There was correlation between data from phospho-flow cytometry and data from Western blotting ($r = 0.88$, $P < 0.001$).

Conclusions: The degree of mTOR inhibition by assessing p70S6K phosphorylation was established by phospho-flow cytometry and Western blotting. Assessment of p70S6K phosphorylation may play an adjunct role to on pharmacodynamically guide and individualize sirolimus based on immunosuppression.

Key words: P70S6 Kinase; Phospho-flow Cytometry; Sirolimus; Western Blotting

Introduction

Although sirolimus has a narrow therapeutic window, their application in solid organ transplantation is increasing (since) they have potent anti-inflammatory properties, which would improve graft function.[1,2] Sirolimus is able to inhibit the growth factor-induced T-cell proliferation by forming a complex with FK-binding protein 12 (FKBP12) which binds to mammalian target of rapamycin (mTOR) kinase (a specific cell-cycle regulatory protein) to suppress mTOR action.[3] Therefore, it is able to halt cell-cycle progression from G1 to S phase. The p70S6 kinase (p70S6K) located at the downstream of mTOR pathway.[4,5] The phosphorylation of the p70S6K represents an important target for a sensitive detection of the pharmacodynamic effects of sirolimus on T-cell activation.

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Almost all of the immunosuppressive agents have significant inter-individual and intra-individual therapeutic range.[6] Under- or overdosing would lead to acute allograft rejection or occurrence of adverse effects. Adjustment of the sirolimus dosage currently relies on sirolimus trough levels.[1,7] However, monitoring the trough level, which is used in clinical work, cannot always predict the effects of immunosuppressive drugs on immune cells. That is because it does not reflect any aspect of an individual's immune system.[8,9] Until now, a specific assay that determines the pharmacodynamic effects of mTOR inhibition on specific immune cell subsets has not been introduced into clinical routine. Pharmacodynamic drug monitoring on immune cells represents an effort to receive more reliable information on the biological effect of mTOR inhibition in an individual transplant recipient.[10]

The aim of this study was to investigate p70S6K phosphorylation in peripheral blood mononuclear

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cells (PBMCs) of liver transplant patients who received different immunosuppressive drug regimens with or without sirolimus by phospho-flow cytometry and Western blotting. A correlation analysis of data from phospho-flow cytometry and Western blotting were performed.

Methods

Patients

Seventy-five liver transplantation patients were admitted to the out- and in-patient care of the Hepatobiliary Surgery Department of Beijing Chaoyang Hospital of the Capital Medical University. The study protocol was approved by the institutional review board and conducted from May 2012 to December 2013. Patient inclusion criteria: (1) Patients gave written informed consent to participate. (2) All patients were treated with immunosuppressant after liver transplantation. Patient exclusion criteria: (1) Patients with immunological diseases, hepatitis, HIV positive, hematologic malignancies. (2) Patients with acute infection chronic infection. Seventy-five liver transplantation patients were divided into three groups, 22 patients treated with sirolimus, 30 patients treated with tacrolimus, 23 patients treated with cyclosporine, respectively. Twelve healthy control (HC) were served as a control group in this study. The age and gender distribution among liver transplantation patients and HC was not significantly different from each other ($P = 0.38$, $P = 0.61$; respectively). HC did not have an acute infection or immunological diseases in medical history.

Cell cultures

Peripheral blood mononuclear cells were separated from 20-ml heparinized blood samples by standard Ficoll-Paque density gradient centrifugation. This experiment was divided into two parts. For Part 1, the p70S6K phosphorylation in PBMCs of liver transplant patients receiving different immunosuppressive drug regimens by phospho-flow cytometry and Western blotting. Data were analyzed using FlowJo Software (Version 8.7.3, Tree Star, USA) in flow cytometry. The level of phosphorylation p70S6K was quantified by the mean fluorescence intensity index (MFI index). The reactive bands were detected by chemiluminescence and signal intensity was densitometrically quantitated in Western blotting (Image J, version 2.1.4.7, National Institutes of Health, USA). The level of phosphorylation p70S6K was quantified by relative gray density values which were calculated by dividing gray density of target band by gray density of loading control. For Part 2, PBMCs were stimulated with phorbol-12-myristate-13-acetate (PMA, Sigma, Germany), ionomycin (Sigma) and rapamycin (Sigma) (0 ng/ml, 0.1 ng/ml, 1 ng/ml, 5 ng/ml and 10 ng/ml) and analyzed by flow cytometry and Western blotting after 40 h of incubation in 5% CO_2 at 37°C.

Statistics analysis

All statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Data were given as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) for more than two groups. Data were compared using the two-tailed Student's *t*-test for normally distributed population and Mann–Whitney *U*-test for not normally distributed population. Correlation analysis was performed with Pearson's test after proving normal distribution of data. $P < 0.05$ was considered statistically significant.

Results

Baseline characteristics, laboratory data of liver transplantation patients and healthy volunteers

Seventy-five liver transplantation patients (32 females and 43 males, mean age 56 ± 11 years, range 13–72) and 12 HC (5 females, 7 males, mean age 52 ± 13 years old, range 18–75) were served as control group in this study. The age and gender distribution between liver transplantation

Table 1: Baseline characteristics and laboratory data of patients

Items	Liver transplantation			HC	*P*
	Sirolimus	Tacrolimus	Cyclosporine		
Total number	22	30	23	12	
Women/men	9/13	16/14	10/13	5/7	0.38
Age (years)	53 ± 13	55 ± 15	58 ± 10	52 ± 13	0.61
Time after transplantation (min)	28 ± 15	35 ± 27	85 ± 41		0.001
Trough level (ng/ml)	4.6 ± 1.4	5.1 ± 2.6	135.2 ± 29.0		0.001
Leukocytes (/μl)	7.1 ± 2.6	8.2 ± 3.1	6.0 ± 2.7		0.06
Platelets (10^3/μl)	1.9 ± 0.7	2.1 ± 0.5	2.5 ± 0.9		0.03
Creatinine (mg/dl)	1.9 ± 1.1	1.8 ± 0.6	1.6 ± 0.4		0.47
Total cholesterol (mg/dl)	208 ± 63	197 ± 47	251 ± 69		0.04
Cholesterin (mg/dl)	228 ± 48	196 ± 39	231 ± 52		0.01
HDL (mg/dl)	54 ± 17	58 ± 23	51 ± 21		0.32
LDL (mg/dl)	129 ± 27	101 ± 23	128 ± 47		0.006
Triglyceride (mg/dl)	271 ± 109	148 ± 69	172 ± 72		0.001

There were no significant differences of gender ratio and age in transplantation patients as compared to HC ($P > 0.05$). HDL: High-density lipoprotein; LDL: Low-density lipoprotein; HC: Healthy control.

patients and HC was not significantly different from each other ($P = 0.38$, $P = 0.61$, respectively) [Table 1].

Assessing intra-assay variability of phosphorylation p70S6 kinase by phospho-flow cytometry

To investigate validity and repeatability of the assay, intra-assay precision was determined in healthy subjects ($n = 3$) [Table 1] and liver transplant patients receiving sirolimus, tacrolimus, or cyclosporine ($n = 3$ each). Isolated PBMCs were stained and assessed by phospho-flow cytometry in triplicates for each patient. The mean coefficient of variance (CV) values for intra-assay variability in different groups was from 4.1% to 8.4% [Table 2].

Assessing intra-assay variability of phosphorylation p70S6 kinase by Western blotting

To compare and confirm the data generated in the phospho-flow cytometry assay, the phospho-flow technique was compared with Western blotting analysis of phosphorylation of p70S6K in healthy subject ($n = 1$) and liver transplant patients receiving sirolimus, tacrolimus or cyclosporine ($n = 1$ each) [Figure 1a]. The bar graphs showed the respective analysis of triplicate Western blotting densitometric measurements expressed in pixel values (HC: 0.79 ± 0.10; cyclosporine: 0.63 ± 0.07; tacrolimus: 0.55 ± 0.06; sirolimus: 0.18 ± 0.03). The mean CV value for intra-assay variability was 8.2% for healthy individuals, 18% for patients receiving tacrolimus, 15% for cyclosporine and 16% for sirolimus, respectively. A representative Western blotting for phosphorylation of p70S6K was assessed in PBMCs of a healthy subject and a patient receiving cyclosporine, tacrolimus or sirolimus, respectively [Figure 1b].

Table 2: Assessing intra-assay variability by phospho-flow cytometry

Groups	Subject	MFI index		CV (%)	
		Mean	SD	Per subject	Per group *
Sirolimus	1	25.3	2.4	9.5	8.4 ± 1.4
	2	21.3	1.9	8.9	
	3	24.9	1.7	6.8	
Tacrolimus	1	43.4	2.7	6.2	6.3 ± 1.5
	2	51.4	2.5	4.9	
	3	39.8	3.1	7.8	
Cyclosporine	1	53.3	2.1	3.9	4.4 ± 1.9
	2	48.6	1.3	2.7	
	3	55.2	3.6	6.5	
HC	1	79.3	2.8	3.5	4.1 ± 1.1
	2	72.4	2.6	3.6	
	3	79.9	4.2	5.3	

MFI index: Mean fluorescence intensity index; SD: Standard deviation; CV: Coefficient of variance; HC: Health control; *Data were shown as mean ± SD.

The p70S6 kinase phosphorylation of peripheral blood mononuclear cells in patients treated with different immunosuppressive agents and healthy control

P70S6 kinase phosphorylation of PBMCs was assessed in patients treated with different immunosuppressive agent and HC [Figure 2]. The phospho-flow technique detected a significant loss of p70S6K phosphorylation at Thr389 in PBMCs of patients who treated with sirolimus ($n = 26$, mean MFI index: 19.5 ± 7.7) compared to HC ($n = 16$, mean MFI index: 50.1 ± 11.3, $P < 0.001$) and liver transplant recipients receiving an immunosuppressive therapy based on tacrolimus ($n = 35$, mean MFI index: 37.7 ± 15.7, $P < 0.001$) or cyclosporine ($n = 23$, mean MFI index: 41.7 ± 11.7, $P < 0.001$). The p70S6K phosphorylation in HC (50.1 ± 11.3) was higher than that in tacrlimus (37.7 ± 15.7, $P < 0.01$) and cyclosporine (41.7 ± 11.7, $P < 0.01$), but no significant difference was shown between tacrolimus (37.7 ± 15.7) and cyclosporine group (41.7 ± 11.7, $P = 0.29$).

Assessing p70S6 kinase phosphorylation by phospho-flow cytometry is comparable to the Western blotting method

Correlation between MFI index and Western blotting values was shown in various immunosuppressive agent groups. MFI index was used to indicate the flow cytometry results while normalized mean pixel value was used to indicate the Western blotting data. The analysis of the obtained results revealed a high correlation coefficient ($n = 75$, $r = 0.88$, $P < 0.001$) [Figure 3].

Incubation of peripheral blood mononuclear cells with rapamycin, phorbol-12-myristate-13-acetate, and ionomycin

To confirm the validity of the phospho-flow assay *in vitro* experiments, the phosphorylation status of p70S6 in mitogen-stimulated PBMCs was incubated in presence or

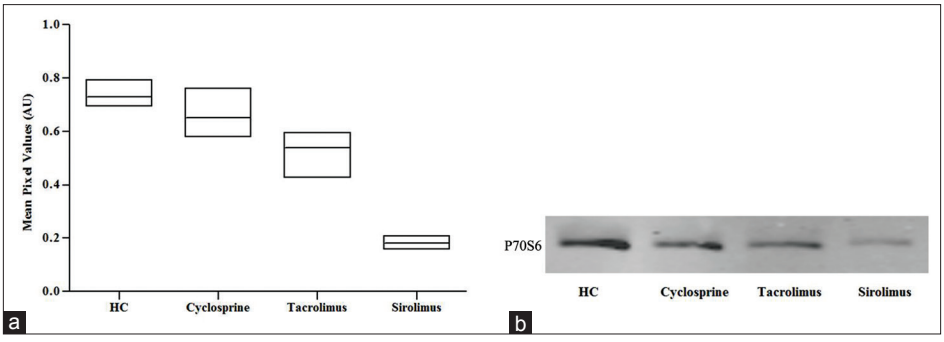

Figure 1: Assessing intra-assay variability by Western blotting (a and b).

absence of rapamycin plus PMA and ionomycin for 40 h. Phospho-flow analysis revealed that addition of rapamycin reduced the p70S6K phosphorylation in PBMCs of healthy volunteers (n = 3) in a dose-dependent manner [Figure 4a] and this was confirmed in a representative Western blotting [Figure 4b].

Discussion

Transplantation rejection is an adaptive immune response caused by cell-mediated and humoral immunity. Activation of T-cell, interleukin-2 (IL-2) production and secretion play important roles in transplantation. The sirolimus bind to FKBP12, which interacts with the FKBP12 rapamycin binding domain of mTOR, leads to a decrease in messenger RNA translation and protein synthesis. This results in cell-cycle arrested in G1 phase, and it is important to suppress immunological response.

The phosphorylation of a protein correlates with its biological functions. For kinases, phosphorylation enhances their enzymatic activity and conduct signals to downstream regulations. Previous studies found there were three phosphorylation sites of p70S6K (S404, Thr389 and Thr229) to be sirolimus sensitive and showed that dephosphorylation of Thr389 most closely correlated with loss of kinase activity.[11,12] Moreover, site-directed mutational analysis has revealed that Thr389 represents the principal site of sirolimus mediated inactivation of p70S6K and most closely correlates with loss of kinase activity.[13] Inhibition of mitogen-induced p70S6K activation *in vivo* with either by treatment with the neutralizing antibodies or immunosuppressant sirolimus severely compromises the processing ability through the G1 phase in the cell cycle.[14] The aim of this study was to investigate Thr389 phosphorylation of p70S6K in PBMCs of liver transplant patients who received different immunosuppressive drug and HC by phospho-flow cytometry and Western blotting.

To investigate validity and repeatability of the assay, intra-assay precision was determined in healthy subject and liver transplant patients receiving sirolimus, tacrolimus, or cyclosporine. Isolated PBMCs were stained and assessed by phospho-flow cytometry and Western blotting in triplicates. The range of mean CV value of intra-assay variability assessed by phospho-flow cytometry was from 4.1% to 8.4%. Meanwhile,

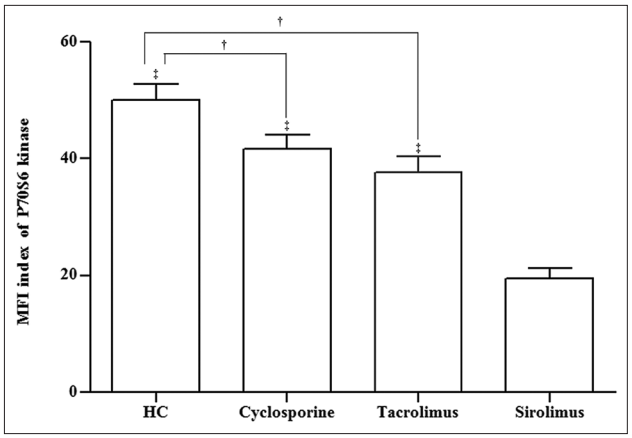

Figure 2: The p70S6 kinase phosphorylation of peripheral blood mononuclear cells in patients treated with different immunosuppressive agents and health control. MFI index: Mean fluorescence intensity index ($^{\ddagger}P < 0.001$ vs. sirolimus group, $^{\dagger}P < 0.05$).

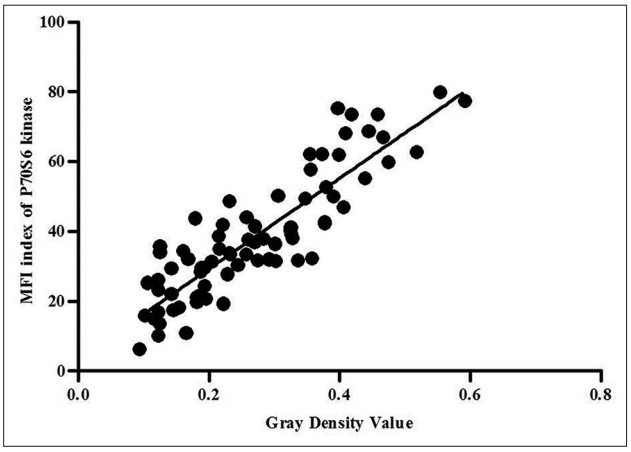

Figure 3: Correlation between MFI index and Western blotting value. MFI index: Mean fluorescence intensity index.

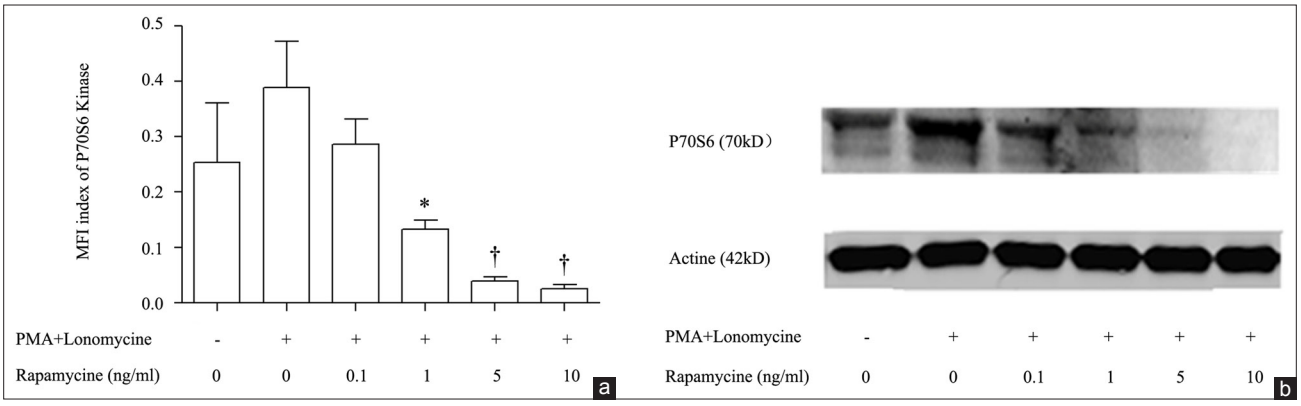

Figure 4: (a) Phospho-flow cytometry detected dose-dependent decrease of p70S6 kinase (p70S6K) phosphorylation in peripheral blood mononuclear cells (PBMCs) *in vitro*. MFI index: Mean fluorescence intensity index ($^{*}P < 0.05$, $^{\dagger}P < 0.01$ vs. phorbol-12-myristate-13-acetate + ionomycin treated PBMCs); (b) A representative experiment about p70S6K was analyzed in parallel by Western blotting.

the mean CV values for of intra-assay variability assessed by Western blotting ranged from 8.2% to 18%. Compared with Western blotting, phospho-flow cytometry showed a smaller intra-assay variability and better stability and repeatability.

Hartmann *et al*.[15] showed patients treated with sirolimus had a reduced phosphorylation of p70S6K in PBMCs as compared to patients treated with tacrolimus, cyclosporine by Western blotting. In this study, MFI index of PBMCs in patients treated with sirolimus was significantly lower than those with cyclosporine, tacrolimus, and HC. MFI index of patients treated with cyclosporine and tacrolimus was significantly lower than HC [Figure 2]. Sirolimus blocks the mTORC1 and results in a decrease of p70S6K phosphorylation. Cyclosporine and tacrolimus are calcineurin inhibitors, the former binding to calcineurin, and the latter to FKBP12. In both cases the dephosphorylation of the transcription factor nuclear factor of activated T-cell is inhibited, resulting in a decrease of IL-2 production from T-cell.[16] IL-2 is a T-cell-derived-cytokine which is secreted as a soluble molecule by activated T-cell and activates dendritic cells in immune response and regulates growth and proliferation of T-cell, B-cell and natural killer cells. Interaction of IL-2 with its respective receptor leads to activation of various signaling pathways, including the PI3K-AKT pathway results in up-regulation of mTOR activity. Therefore, the activity of p70S6K in patients receiving cyclosporine or tacrolimus could be reduced by inhibition of IL-2 production and decreased in its paracrine stimulatory effects. Since tacrolimus is more potent calcineurin inhibitor, it may result in a slightly lower p70S6K phosphorylation compared to that of cyclosporine-treated patients [Figure 2].

In order to further validate the data from the phospho-flow cytometry assay, the activity of p70S6K of PBMCs was measured by Western blotting, and the mean gray value was calculated as well. Both methods were comparable and highly correlating with each other ($n = 75$, $r = 0.88$, $P < 0.001$) [Figure 3]. Analysis of the phosphorylation status of the p70S6K via phospho-flow technique provides reliable information on the extent of mTOR inhibition exerted by sirolimus, and the result is comparable to the Western blotting method.

Phorbol-12-myristate-13-acetate and ionomycin were stimulators in immunology assays, which were able to up-regulate p70S6K phosphorylation.[17] In this present study, PBMCs from HC were isolated and incubated with rapamycin in various concentrations. PMA and ionomycin in the presence of IL-2 for 40 h. The results suggested that the activity of p70S6K was up-regulated by PMA inomycin stimulation and suppressed by sirolimus in a dose-dependent manner, which was also verified by Western blotting.

The results shown above were consistent with the one of research of Leogrande *et al*.[18] which indicated that the decrease in p70S6K phosphorylation was caused by the PBMCs stimulated with insulin and incubated with rapamycin. The present study also indicated that sirolimus can effectively inhibit the activity of mTOR pathway *in vitro* and *in vivo*.

Sirolimus has significant inter-individual and intra-individual therapeutic range. Inhibition of mTOR pathway did not correlate with the respective sirolimus trough levels, a finding described by Hartmann *et al*. by Western blotting technique. These results suggest that the adjustment of sirolimus dosage in organ transplantation patients exclusively depended on trough level and needed further evaluation. Pharmacodynamic drug monitoring on immune cells represents an effort to receive more reliable information on the biological effect of mTOR inhibition in an individual transplant recipient.[19,20]

In conclusion, a robust phospho-flow cytometry assay that determines the degree of mTOR inhibition by assessing p70S6K phosphorylation was established. Quantification of p70S6K phosphorylation may play an adjunct role to pharmacodynamically guide an individualized sirolimus based on immunosuppression.

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