

# Role of mitogen activated protein kinase cascade pathway and anti-stress response in liver transplantation

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**OBJECTIVE:** To explore the changes of mitogen activated protein kinase (MAPK) cascade pathway and anti-stress response of hepatocytes after liver transplantation.

**METHODS:** Ten normal liver specimens and 18 punctured donor liver specimens were divided into 3 groups: A (control:10), B (no rejection:10) and C (acute rejection:8). MAPK, Ras, Jun and heat shock protein 70 (HSP70) were tested immunohistochemically while Ras and HSP70 were tested by in situ hybridization. All sections were subjected to image analysis.

**RESULTS:** Protein expressions of MAPK, Ras and Jun were increased by an ascending order of groups A, B and C. The protein expression of HSP70 was the highest in group B but lower in group C.

Expressions of Ras and HSP70 mRNA were consistent with those of protein.

**CONCLUSIONS:** The changes of the MAPK cascade pathway and anti-stress response of hepatocytes after liver transplantation are one of regulation mechanisms for protecting the hepatocytes from damage after liver transplantation. This mechanism is active to support individual survival.

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**Key words:** liver transplantation; MAPK cascade pathway; anti-stress response; Ras; Jun; HSP70

## Introduction

Mitogen activated protein kinase (MAPK) cascade pathway is an important route which can conduct extracellular stimulatory signals into the nucleus and mediate cellular information transition. The cascade pathway mainly includes several pathways of Ras-extracellular signal-related protein kinase (Ras-ERK), c-jun amino kinase-stress activated protein kinase (JNK-SAPK), p38<sup>MAPK</sup> and ERK5. Many stimulators such as ischemia, stress,

and cytokines can activate MAPK through the MAPK cascade pathway. After being activated, MAPK can activate translocator of the nucleus to regulate expression of proto-oncogene and anti-stress protein gene, promote relevant protein expression, and response the stimulating signals from the extracellular.<sup>[1]</sup> This study was to explore the relationship between the MAPK cascade pathway and anti-stress reaction after liver transplantation.

## Methods

### Procurement of specimens

Eighteen specimens in experimental group were procured by puncture from recipients after liver transplantation performed from 1995 to present. Ten specimens in control group were obtained from normal liver during other relevant operations. The patients were in supine position when being punc-

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tured with the point at the cross of the anterior auxiliary line and the 10th or 11th intercostals. Two cm long allograft specimens were harvested and subsequently put into 10% neutral formaldehyde solution for immunohistochemistry. Fixation solution for in situ hybridization was 4% formaldehydum polymerisatum containing 1 mg/L diethyl-pyrocabonate (DEPC). The specimens were pretreated by poly-lysine and cut into sections about 8  $\mu$ m thick.

### Experimental groups

The specimens were divided pathologically into 3 groups of A (control:10), B (no rejection:10) and C (acute rejection:8). Histological manifestation was abnormal in group A. No or few inflammatory cells (lymphocytes, lymphoblasts, neutrophils and acidophils) in the triad were found in group B. In group C, however, there were infiltration of inflammatory cells, hepatocyte degeneration (spot necrosis, hydropic degeneration, fatty degeneration) or small necrotic area and existing mild or severe phlebitis around the triad. MAPK, Ras, Jun and HSP70 were tested immunohistochemically apart from Ras and HSP70 in situ hybridization in each case.

### Protein detection of MAPK, Ras and P53 by immunohistochemistry

Streptomycin-avidin-biotin-complex (SABC) was used. Rat anti-human monoclonal antibodies (MAPK McAb, Ras McAb, Jun McAb and HSP70 McAb) were bought from Gene Biotechnology Limited Company, China. SABC and DAB test kits were bought from Bost Biotechnology Limited Company, Wuhan, China. Immunohistochemically, sections of the specimens were dewaxed to water and added 3% H<sub>2</sub>O<sub>2</sub> to inactivate endogenous enzyme for 5 min. They were put in boiling 0.01 mol/L citrate buffer (pH 6.0) 5 min twice, washed twice with 0.1 mol/L PBS when buffer cooling, added with antigen restoratory liquid and washed after 20 min at room temperature, further added with goat serum blocking liquid and kept 20 min at room temperature. Superfluous liquid was discarded without washing and the sections were added with the first antibody to incubation at 37 °C for 1 hour

(MAPK McAb, Ras McAb, Jun McAb, HSP70 McAb), then washed with PBS. Again the sections were added with biotin-goat-anti-rat IgG to incubation at 37 °C for 20 min washed with PBS, added with SABC to incubation at 37 °C for 20 min, washed, and added mixture reagent (one drop reagent from A, B, C reagents to 1 ml distilled water from DAB test kit). They were colored at room temperature while controlling reaction time with a microscope. Finally, they were washed with distilled water, dehydrated, cleared, and sealed.

### mRNA detection of HSP70 and Ras by in situ hybridization

Pre-hybridization solution and Ras and HSP70 oligonucleotide probe hybridization solution were bought from Gene Biotechnology Limited Company, China, and so were SABC and ABC test kits. All reagents and containers were treated by DEPC. The sections were dewaxed to water, immersed into 3% H<sub>2</sub>O<sub>2</sub> at room temperature for 10 min, and washed twice by distilled water. Fresh diluted protease K 1  $\mu$ g/ml was added and digested for 15 min at 37 °C to expose mRNA nucleic acid segments. 20% glycerine 20 ml was added to the dry bottom of the test kit to keep its humidity. Twenty  $\mu$ l pre-hybridization solution was added to each section, which was kept at 37 °C for 4 hours. Finally the supernumerary liquid was absorbed without washing. Hybridization solution was added to each section, which was covered by protective membrane. After homeothermia at 40 °C overnight, cover glass was removed from each section. They were washed for 5 min twice with 2  $\times$  SSC at 37 °C, 15 min once with 0.5  $\times$  SSC, and 15 min once with 0.2  $\times$  SSC. The sections were kept in 3% BSA at 37 °C for 30 min and in seal solution at 37 °C for 30 min. Then biotin labeled anti-digoxin antibody derived from rat was added at 37 °C for 60 min to the sections, followed by washing with 0.5 mol/L PBS. SABC was added at 37 °C for 20 min to the sections, followed by washing with PBS. Further, biotin-peroxidase was added at 37 °C for 20 min to the sections, and washed by PBS. The sections were colored by DAB, retained, dehydrated and sealed.

**Table 1.** Protein expression of MAPK, Ras, Jun and HSP-70 (PI)

Group	n	MAPK	Ras	Jun	HSP70
A	10	0.88 ± 0.26	1.47 ± 0.17	0.71 ± 0.22	0.97 ± 0.14
B	10	1.15 ± 0.07	3.47 ± 0.55	0.93 ± 0.37	3.06 ± 0.23
C	8	9.33 ± 0.37	5.88 ± 0.62	1.58 ± 0.27	1.25 ± 0.13

**Table 2.** mRNA of Ras and HSP70

Group	n	Ras	HSP70
A	10	1.01 ± 0.02	0.81 ± 0.28
B	10	1.23 ± 0.03	2.09 ± 0.11
C	8	2.36 ± 0.42	0.71 ± 0.02

### Image analysis of immunohistochemistry and in situ hybridization

Four high visions were taken in each section to analyze gray degree with HPIAS-1000, and calculate the positive index of immunohistochemistry and in situ hybridization by the following equation; positive index (PI) = (positive signal area × mean gray value of positive signal)/measured area.

### Statistical analysis

Student's *t* test was used. Analysis of variance was performed with a statistical analysis system (SAS).

### Results

The positive indexes of MAPK, Ras, Jun and HSP70 by immunohistochemistry in each experimental group are shown in Table 1. There was no significant difference among the specimens within each group, but significant difference among groups ( $P < 0.05$ ). The protein expressions of MAPK, Ras and Jun were increased in an ascending order of group A, B and C. The protein expression of HSP70 was higher in group B than in group C.

The positive indexes of mRNA of Ras and HSP70 in situ hybridization in each experimental group are shown in Table 2. There was no significant difference in each group but between the groups ( $P < 0.05$ ).

The expressions of Ras and HSP70 mRNA were identical to those of protein.

### Discussion

MAPK, a family of protein kinases containing serine/threonine residue, is distributed widely in cellular plasma with a molecular weight of about 40–46 kDa. As a genetic product of ERK, it can lead to phosphorylation of threonine residue in myelin basic protein (MBP) in vitro. MAPK is a convergent signals pathway conducting from extracellular to intracellular because it can be activated by the coupling receptor pathway of cellular growth factor and G protein and by protein kinase C activated by phorbol ester.<sup>[2]</sup> The MAPK cascade pathway conducts the signals caused by growth factor or hormone for cell proliferation and differentiation, and mediates stress signals from extracellular environment to resist stress.

After being activated, MAPK can stay in plasma to activate other protein kinases or enter the nucleus to regulate gene expression of Fos, Myc and Jun by phosphorylation transcription factor, characterized by regulation of cell cycle, cell proliferation and differentiation, and anti-stress response. The transcription factor compound AP-1 composed by Jun and Fos is one of the substrates of the MAPK cascade pathway.

As a family of conservative proteins in evolution, molecular chaperones can alter or modify other proteins to affect or regulate their function. Studies<sup>[3–5]</sup> focused on heat shock protein (HSP) family. HSP70 is one kind of important and conservative molecules of the HSP family whose principal function is to promote precise folding of newborn polypeptide chain and prevent irreversible aggregation. It is essential to molecular rearrangement, protein depolymerization and trans-membrane transport of the new born polypeptide. Therefore, HSP70 is an important functional protein in cellular anti-stress response. Many gene-coding proteins relevant to cellular survival directly or indirectly can be induced by ischemia and immune injury. They include survival genes such as HSP and growth factor as well as anti-apoptosis genes.<sup>[6]</sup> It was found in a reversible cerebral ischemia model that the MAPK cascade pathway is activated to exert effects on DNA transcription and mRNA transla-

tion, which support cell survival and increase HSP mRNA expression in the ischemic area.<sup>[7]</sup>

HSP is a substrate of the MAPK cascade pathway and its expression is beneficial to repairment of tissue structure and function. After liver transplantation, allografts experience immunological attack and non-immunological injury, indicating that hepatocytes are in a stress condition. These affect the expression of molecular chaperones such as HSP70. High expression of HSP70 in allograft indicates a repair response to injury. It is also found that HSP70 has protective effect on allograft in a transgenic mice model.<sup>[8]</sup>

Protein expressions of MAPK, Ras and Jun are highly significant in hepatocytes after transplantation, especially after rejection. It is indicated that signal transduction is affected by immunologic and non-immunologic factors after liver transplantation. The MAPK cascade pathway is affected by signals that are transmitted to plasma by G protein in membrane. The protein expression of MAPK increases to have signals be transmitted to the nucleus for cellular anti-stress response through the pathways of Ras-ERK and JNK-SAPK. Ras plays a role in the MAPK cascade pathway while reinforces to conduct signals into the intracellular as a member of the micro-G protein family. The activity of the JNK-SAPK pathway can be increased by C-Jun which is phosphorylated by JNK protein. The increasing protein of MAPK, Ras and Jun indicates the transmission and magnification of signals to the intracellular in different aspects of the MAPK cascade pathway. When rejection occurs, the hepatocyte membrane is stimulated more intensely by extracellular factors, donor special antibodies, and activated CD4<sup>+</sup>/CD8<sup>+</sup> T cells so that the protein expression of MAPK, Ras and Jun is increased. Detection of protein and mRNA has shown that gene transcription and translation are increased synchronally. It is indicated that the whole cascade pathway proceeds effectively.

The expression of protein and mRNA of HSP70 in hepatocytes increases rapidly after liver transplantation but decreases after rejection although the signals of MAPK are intensified during this course. This finding suggests the characteristics of balance between magnification and inhibi-

tion in the course of signal transmission. If the signal intensity of the MAPK cascade pathway for stimulating is beyond a set limit, intracellular phosphorylation will be activated and inhibitory effects will be presented as inhibition of gene transcription and translation. If rejection can't be controlled effectively, hepatocytes may fail to respond to injury effectively, resulting in apoptosis or necrosis. The decreasing protein and mRNA expression of HSP70 after rejection shows the significance of early diagnosis and treatment of rejection at a molecular biological level.

The reaction of the MAPK cascade pathway and anti-stress response of hepatocytes is a protective response to keep cellular function normal despite it is strong or not. Hepatocytes participate in almost all biochemical metabolisms in organisms, so this self-protection mechanism is active to support individual survival. It is a precise regulatory mechanism formed during the course of evolution. Recognizing injury and stress of allograft after liver transplantation at a molecular level is helpful to seek new methods and drugs for relieving injury after rejection and protecting the allograft.

### Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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