World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i6.796

*World J Gastroenterol* 2011 February 14; 17(6): 796-803
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


BRIEF ARTICLE

# RRAS: A key regulator and an important prognostic biomarker in biliary atresia

Rui Zhao, Hao Li, Chun Shen, Shan Zheng

Rui Zhao, Hao Li, Chun Shen, Shan Zheng, Department of Pediatric Surgery, Children's Hospital of Fudan University, Shanghai 201102, China
Author contributions: Zhao R wrote the manuscript and performed the majority of experiments; Li H and Shen C coordinated and provided the collection of all the human materials; Zheng S designed the study and provided the financial support for this work.
Supported by National Natural Science Foundation of China, No. 30973139
Correspondence to: Shan Zheng, MD, Department of Pediatric Surgery, Children's Hospital of Fudan University, 399 Wanyuan Road, Shanghai 201102, China. szheng@shmu.edu.cn
Telephone: +86-21-64931007 Fax: +86-21-64931901
Received: September 14, 2010 Revised: November 3, 2010
Accepted: November 10, 2010
Published online: February 14, 2011

## Abstract

**AIM:** To characterize the differentially expressed gene profiles in livers from biliary atresia (BA) patients including, ascertain genes, functional categories and pathways that play a central role in the pathogenesis of BA, and identify the novel prognostic markers for BA.

**METHODS:** Liver tissue samples from control patients, neonatal cholestasis patients, and BA patients at the age of < 60 d, 60-90 d, and > 90 d were pooled for DNA microarray analysis. Bioinformatics analysis was performed using, series test cluster of gene ontology, and Pathway-Finder software. Reverse-transcription polymerase chain reaction was performed to confirm changes in selected genes. Relation between RRAS gene expression and prognosis of 40 BA patients was analyzed in a 2-year follow-up study.

**RESULTS:** The 4 identified significant gene expression profiles could confidently separate BA liver tissue from normal and other diseased liver tissues. The included genes were mainly involved in inflammation response and reconstruction of cellular matrix. The significant pathways associated with BA were primarily involved in autoimmune response, activation of T lymphocytes and its related cytokines. The *RRAS*, *POMC*, *SLC26A6* and *STX3* genes were important regulatory modules in pathogenesis of BA. The expression of RRAS was negatively correlated with the elimination rate of jaundice and positively correlated with the occurrence rate of cholangitis.

**CONCLUSION:** Autoimmune response mediated by T lymphocytes may play a vital role in the pathogenesis of BA. The *RRAS* gene is an important regulatory module in the pathogenesis of BA, which may serve as a novel prognostic marker for BA.



**Key words:** Biliary atresia; DNA microarray; Bioinformatics; RRAS; Prognostic biomarker

**Peer reviewer:** Beata Jolanta Jablońska, MD, PhD, Department of Digestive Tract Surgery, University Hospital of Medical University of Silesia, Medyków 14 St. 40-752 Katowice, Poland

Zhao R, Li H, Shen C, Zheng S. RRAS: A key regulator and an important prognostic biomarker in biliary atresia. *World J Gastroenterol* 2011; 17(6): 796-803 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i6/796.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i6.796

## INTRODUCTION

Biliary atresia (BA) is a devastating disease of infants, invariably leading to cirrhosis, end-stage liver disease, and death if untreated[1]. A recent review reported that BA may involve a primary perinatal hepatobiliary viral infection and a secondary autoimmune-mediated bile duct inju-

ry[2]. However, the cause and pathogenesis of BA remain largely unknown.

Microarray technology, emerged as an indispensable research tool for gene expression profiling, has been used to study the mechanism underlying BA, and allows the simultaneous analysis of thousands of transcripts within a single experiment[3]. Some studies have been performed to investigate the gene expression profiling of livers from BA patients[4-6]. However, to our knowledge, none of them was designed to identify genes that play a key role in the pathogenesis and prognosis of BA. In the current study, DNA microarrays for whole genome gene expression and bioinformatics analysis were used to characterize the differentially expressed gene patterns of normal livers and livers from BA patients at different ages, as well as to ascertain the genes and pathways that play a central role in the pathogenesis of BA. Furthermore, reverse-transcription polymerase chain reaction (RT-PCR) was performed to confirm the changes in selected genes. The relation between selected gene expression and prognosis of BA patients was also analyzed.

## MATERIALS AND METHODS

### Patients and specimens

Biopsy specimens were obtained from livers of 9 patients with BA, 3 patients with neonatal cholestasis and 3 control patients suffering from liver trauma (as normal control) at Children's Hospital of Fudan University from November 2007 to December 2008. Nine patients with BA were further divided into < 60 d group ($n$ = 3), 60-90 d group ($n$ = 3) and > 90 d group ($n$ = 3). Liver samples from 3 groups of BA patients, neonatal cholestasis and control groups were immediately dissolved in a RNAlater RNA stabilization reagent (Qiagen, Germany) and then stored at -80℃. Liver samples from each group were pooled and total RNA was isolated from them for DNA microarray experiments. Clinical data about these patients are summarized in Table 1. Liver samples were collected from the other 14 patients with neonatal cholestasis and 40 patients with BA for RT-PCR experiments. All subjects gave their informed consent to participate in the study which was approved by the Research Ethics Committee of Fudan University.

### RNA extraction, processing and microarray analysis

Total RNA was extracted from liver tissue samples using the Trizol reagent (Invitrogen) according to its manufacturer's protocol, and then further purified using a NucleoSpin RNA clean-up kit (Macherey-Nagel, Germany). Quantification analysis of RNA was performed on a spectrophotometer and quality of RNA was analyzed by denaturing formaldehyde gel electrophoresis. Five micrograms of total RNA from each group was amplified and labeled with biotin using an Illumina total Prep RNA amp kit (Ambion, Austin,TX, USA) and hybridized to Illumina's Sentrix Human-6 (Version 3) Expression BeadChips containing 48000 transcripts (Illumina, San Diego, CA, USA). Three duplicated chips were also used in each group to test the variations in duplications from the same pooling. The hybridized Illumina chips were scanned on a BeadArray reader (Illumina, San Diego, CA, USA) and microarray analysis was performed using the BeadStudio software (Illumina, San Diego, CA, USA). Raw data were normalized using the cubic spline method and the resulting genes were filtered. Finally, only genes with a differential expression score (Diffscore) greater than 20 or less than -20 were included.

### Bioinformatics analysis of differentially expressed genes

Differentially expressed genes were analyzed by a series test of cluster (STC) to search a set of model expression profiles that were distinct in 5 groups as previously described[7,8]. These profiles were assigned to significant gene ontology categories using series test cluster of gene ontology (STC-GO)[9], and analyzed with the Pathway-Finder software to obtain the significance of pathway categories[10,11]. Moreover, dynamic gene networks were constructed to find the key genes that may play a central role in the pathogenesis of BA[12,13]. The principle and algorithmic details are available in supplementary data.

### Validation of microarray data by RT-PCR

RT-PCR was performed on liver tissue samples from 14 patients with neonatal cholestasis and 40 patients with BA to confirm changes in selected genes, including RRAS, POMC, SLC26A6 and STX3. Total RNA was extracted from liver tissue samples and purified as previously described[14]. Five micrograms of total RNA was reverse transcribed using MMLV reverse transcriptase (Merck, Germany) and random primers in a 20 μL reaction volume at 42℃ for 1 h. Oligonucleotide primers for the RRAS, POMC, SLC26A6, STX3 and β-actin are shown in Table 2. The PCR conditions were as follows: an initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 58℃ for 30 s, extension at 72℃ for 30 s, and a final extension at 72℃ for 7 min. The PCR products were separated by electrophoresis and UV illuminated on a 2% agarose gel containing ethidium bromide (0.5 μg/mL). The gel image was stored using the UVP gel documentation system 5000 (Ultra-Violet Products Ltd., Cambridge, United Kingdom). Expression levels of the selected genes relative to β-actin were measured with densitometric scanning using the multi-analysis/PC system (Bio-Rad, Hercules, California, USA).

### Prognostic biomarker and follow-up research

Fibrosis of liver biopsy specimens from 40 patients with BA was histologically classified into different groups. Elimination rate of jaundice (TB < 20 μmol/L) within 6 mo after operation, 2-year survival rates of cholangitis patients and of 40 BA patients were calculated. The diagnostic criteria for cholangitis included fever, increasing jaundice, acholic stools, with other causes of infection excluded. Follow-up data were obtained from our outpatient and inpatient referrals, as well as from interview by

**Table 1 Microarray analysis showing clinical characteristics of biliary atresia patients**

| Case No. | Gender | Age | TB/DB | ALT/AST | AKP/GGT | Albumin | Disease | Group |
|---|---|---|---|---|---|---|---|---|
| 1 | Female | 50 d | 171/130 | 132/254 | 604/609 | 35.8 | BA | 1 |
| 2 | Male | 49 d | 291/231 | 148/155 | 581/408 | 39.4 | BA | 1 |
| 3 | Male | 57 d | 148/117 | 189/166 | 493/535 | 35.2 | BA | 1 |
| 4 | Female | 73 d | 145/118 | 111/153 | 460/1460 | 36.3 | BA | 2 |
| 5 | Male | 84 d | 160/127 | 100/149 | 713/1278 | 37.3 | BA | 2 |
| 6 | Female | 66 d | 129/108 | 121/165 | 632/1235 | 39.2 | BA | 2 |
| 7 | Female | 103 d | 151/112 | 85/62 | 451/360 | 39.4 | BA | 3 |
| 8 | Female | 97 d | 118/89 | 74/78 | 522/501 | 34.3 | BA | 3 |
| 9 | Male | 110 d | 155/121 | 105/97 | 377/912 | 35.4 | BA | 3 |
| 10 | Female | 77 d | 102/89 | 201/137 | 234/317 | 39.4 | Cholestasis | 4 |
| 11 | Male | 64 d | 137/108 | 404/267 | 584/1044 | 37.2 | Cholestasis | 4 |
| 12 | Female | 55 d | 144/112 | 389/266 | 612/1339 | 41.0 | Cholestasis | 4 |
| 13 | Male | 4 yr | 16/9 | 33/35 | 200/50 | 39.4 | Liver trauma | 5 |
| 14 | Male | 6 yr | 10/4 | 20/25 | 192/44 | 40.1 | Liver trauma | 5 |
| 15 | Male | 4 yr | 12/4.7 | 29/37 | 101/38 | 37.1 | Liver trauma | 5 |

TB: Total bilirubin; DB: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AKP: Alkaline phosphatase; GGT: γ-glutamyl transferase; BA: Biliary atresia.

**Table 2 Sequences of primers in selected genes used in reverse-transcription polymerase chain reaction**

| Gene name | Primer sequences |
|---|---|
| RRAS | F: TTGGTCGGGAACAAGGCAGAT |
| | R: CTCGTCCACGTTGAGACGCAGT |
| POMC | F: GAGAGCAGCCAGTGTCAGG |
| | R: GAAGTGGCCCATGACGTACT |
| SLC26A6 | F: CGGTATCCTGTGCGTGACT |
| | R: GGAAGTGCCAAACAGGAAGT |
| STX3 | F: GGCAAAAAGACAACCGATGA |
| | R: TGTCGTGAAGCTCCTTGATG |
| β-actin | F: GGGAAATCGTGCGTGCATT |
| | R: CAGGCAGCTCGTAGCTCTT |

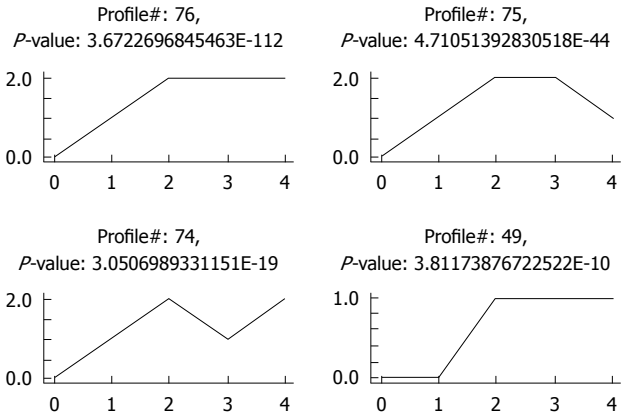


**Figure 1 Four most significant expression profiles in liver samples from biliary atresia patients.** 0: Normal control group; 1: Neonatal cholestasis group; 2: Group of biliary atresia (BA) patients > 90 d; 3: Group of BA patients at the age of 60-90 d; 4: Group of BA patients < 60 d. Y axis represents the expression change expressed as log2 [v(i)/v(0)].

telephone or questionnaires. These patients were further classified based on the follow-up data, including presence of jaundice 6 mo after operation, occurrence of cholangitis within 2 years after operation, and 2-year survival rate.

### *Statistical analysis*

Data were expressed as mean ± SD. Variations in duplications were detected by Fisher's exact test, $\chi^2$ test, *t*-test and Cochran-Mantel-Haenszel test using the STATA 8.0 software (Stata Co., College Station, TX, USA). Pair-wise test was used to confirm the limited variations in duplications from the same pooling. $P < 0.05$ was considered statistically significant.

## RESULTS

### *Differentially expressed gene profiles in liver tissue samples from BA patients*

Total RNA was extracted from liver tissues of the normal group, neonatal cholestasis group, and 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d). Denaturing formaldehyde gel electrophoresis showed no degradation (data not shown). Illumina's Sentrix Human-6 (Version 3) Expression BeadChip was used for each of pooled liver tissue samples from the 5 groups. Pair-wise test confirmed a limited variation in duplications from the same pooling (data not shown). A total of 795 differentially expressed genes were identified from different groups with a Diffscore greater than 20 or less than -20. Further STC analysis yielded 80 expression profiles. Of these expression profiles, 20 were statistically significant ($P < 0.05$) and 4 had the lowest *P* value (Figure 1). These 4 expression profiles could confidently separate livers in groups of BA patients from those in normal and neonatal cholestasis groups. Specifically, all the differentially expressed genes in 5 groups were included in profile 76, which showed no significant difference in the 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d). The 611 genes represented in profile 75 were mainly involved in inflammation mediated by activation of T lymphocytes, and reconstruction of extracellular matrix. As shown in Figure 1, the global expression level of

**Table 3 Significant pathways involved in pathogenesis of biliary atresia**

| Pathway name | *P* value | Profile No. |
|---|---|---|
| Cell adhesion molecules | 0.000118 | Profile49 |
| Regulation of actin cytoskeleton | 0.000739 | Profile49 |
| T Leukocyte transendothelial migration | 0.001738 | Profile49 |
| Asthma | 0.002023 | Profile49 |
| Allograft rejection | 0.003243 | Profile49 |
| Systemic lupus erythematosus | 2.44E-05 | Profile74 |
| Lysosome | 0.0002109 | Profile74 |
| NF-kappa B signaling pathway | 0.0036605 | Profile74 |
| MAPK signaling pathway | 0.0052322 | Profile74 |
| Allograft rejection | 0.0058515 | Profile74 |
| Graft-versus-host disease | 0.0071277 | Profile74 |
| Type I diabetes mellitus | 0.00781 | Profile74 |
| Chemokine signaling pathway | 1.81E-08 | Profile75 |
| Matrix_Metalloproteinases | 6.52E-07 | Profile75 |
| Cytokine-cytokine receptor interaction | 3.59E-05 | Profile75 |
| T cell receptor signaling pathway | 4.34E-05 | Profile75 |
| Antigen processing and presentation | 0.000337 | Profile75 |
| Leukocyte transendothelial migration | 0.0010211 | Profile75 |
| Lysosome | 2.09E-07 | Profile76 |
| Toll-like receptor signaling pathway | 0.000284 | Profile76 |
| T cell receptor signaling pathway | 0.000388 | Profile76 |
| Chemokine signaling pathway | 0.000755 | Profile76 |
| Asthma | 0.000841 | Profile76 |
| Matrix_Metalloproteinases | 0.001402 | Profile76 |
| Allograft rejection | 0.001697 | Profile76 |

the genes in profile 75 was much lower in livers from the group of BA patients at the age of < 60 d than from the groups of BA patients at the age of 60-90 d and > 90 d. The 372 genes represented in profile 74 were associated with an apoptotic pathway and inflammatory response mediated by nuclear factor-κB (NF-κB). The global expression level of profile 74 genes was much lower in the group of BA patients at the age of 60-90 d than in the groups of BA patients at the age of < 60 d and > 90 d. Moreover, the 285 genes represented in profile 49 were mainly involved in inflammatory response mediated by the major histocompatibility complex (MHC) class II antigen. The global expression level of profile 49 genes was much higher in the 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d) than in the normal and neonatal cholestasis groups. Little variance in gene expression was observed neither in the 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d) nor in the normal and neonatal cholestasis groups (See supplementary data for a complete list of these 4 expression profiles).

### *Involvement of significant pathways in BA focused on autoimmune response associated with inflammatory response of T lymphocytes*

Based on the Kyoto Encyclopedia of Genes and Genomes Database and the most significant 4 gene expression profiles, Fisher's exact test and $\chi^2$ test were performed to identify the significant pathways involved in BA as previously described[15]. The significant pathways ($P < 0.01$) highly associated with BA were mainly focused on (1) autoimmune response associated with asthma, systemic lupus erythematosus, allograft rejection graft-versus-host disease, type I diabetes mellitus, antigen processing and presentation; (2) activation of T lymphocytes and inflammatory response including transendothelial migration of T leukocytes, cell adhesion molecules, NF-κB and MAP kinase (MAPK) signaling pathways, chemokine signaling pathway, cytokine-cytokine receptor interaction, transendothelial migration of leukocytes, Toll-like receptor signaling pathway; and (3) reconstruction of extracellular matrix, including matrix-metalloproteinases. The significant pathways are shown in Table 3.

**Table 4 Genes with the highest degree and k-core in dynamic gene networks**

| Gene symbol | Definition | Degree | k-core |
|---|---|---|---|
| RRAS | Homo sapiens related RAS viral (r-ras) oncogene homolog (RRAS), mRNA | 12 | 6 |
| POMC | Homo sapiens POMC, transcript variant 1, mRNA | 12 | 6 |
| SLC26A6 | Homo sapiens SLC26A6, transcript variant 3, mRNA | 12 | 6 |
| STX3 | Homo sapiens STX3, mRNA | 10 | 6 |

**Table 5 Reverse-transcription polymerase chain reaction showing relative expression levels of RRAS, POMC, SLC26A6 and STX3 (mean ± SD)**

| Group | POMC | SLC26A6 | RRAS | STX3 |
|---|---|---|---|---|
| Biliary atresia ($n$ = 40) | 0.58 ± 0.090 | 0.43 ± 0.054 | 0.89 ± 0.103 | 0.61 ± 0.074 |
| Neonatal cholestasis ($n$ = 14) | 0.41 ± 0.081 | 0.30 ± 0.029 | 0.47 ± 0.074 | 0.51 ± 0.045 |
| *P*-value | 0.031 | 0.023 | 0.004 | 0.017 |

### *Construction of dynamic gene networks*

Dynamic gene networks[16] were constructed to find the key regulators that may play a central role in the pathogenesis of BA (Figure 2). Circles indicate genes in the 4 expression profiles, solid lines indicate direct interactions, size of circles indicates their interactions with other molecules, coloring is classified according to the k core and red indicates high k core. The genes with the highest degree and k-core from Figure 2, are listed in Table 4, including the related RAS viral (r-ras) oncogene homolog (RRAS), POMC, SLC26A6 and STX3 genes, indicating that they play a crucial role in the pathogenesis of BA.

### *Expression of RRAS, POMC, SLC26A6 and STX3 genes in liver tissue samples from patients with BA confirmed by RT-PCR*

The mRNA expression levels of RRAS, POMC, SLC26A6 and STX3 in liver tissue samples from 40 patients with BA and 14 patients with neonatal cholestasis, measured in order to validate the results derived from microarray data, were significantly higher in liver tissue samples from patients with BA than from those of neonatal patients

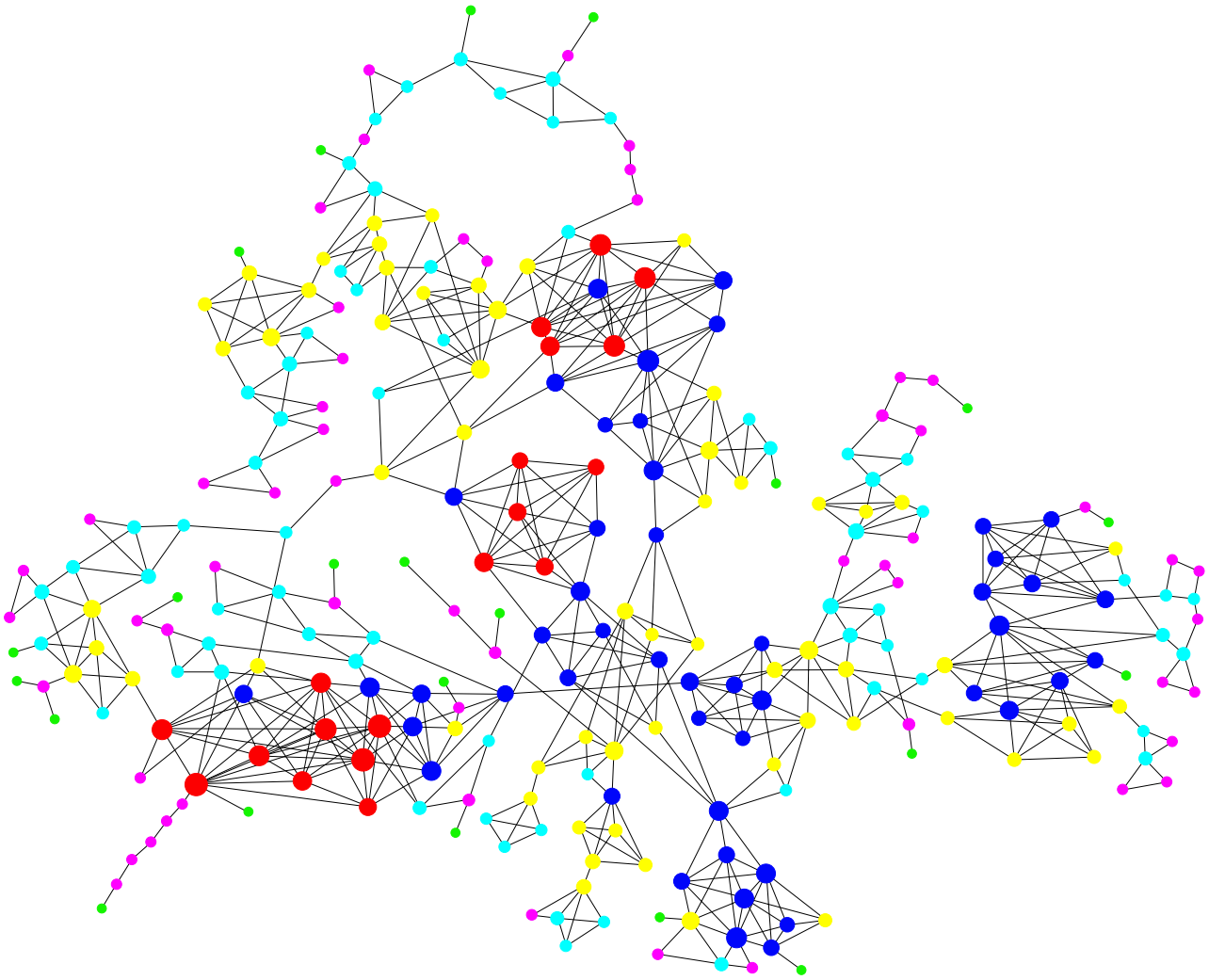

**Figure 2 Dynamic gene networks constructed showing the key regulators that may play a central role in the pathogenesis of biliary atresia.** Circles indicate genes in the 4 expression profiles, solid lines indicate direct interactions, and size of circles indicates their interactions with other molecules. Coloring is classified according to the k core, and red indicates high k core.

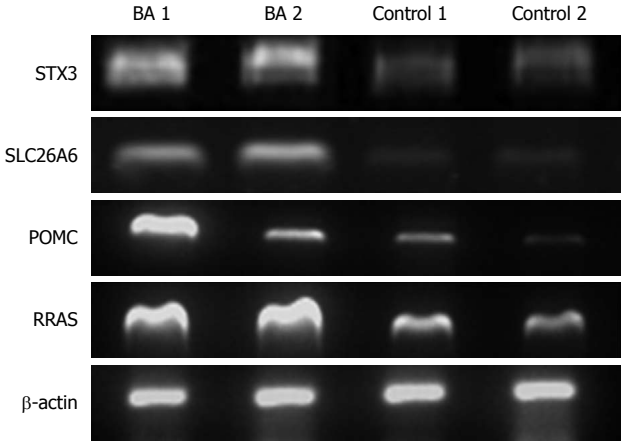


**Figure 3 Reverse-transcription polymerase chain reaction showing expression levels of RRAS, POMC, SLC26A6, and STX3 genes.** BA: Biliary atresia; Control: Neonatal cholestasis.

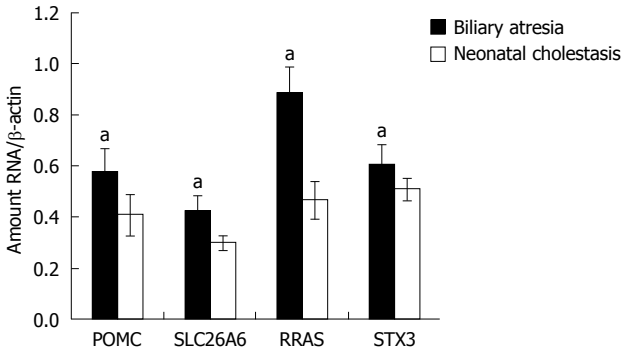


**Figure 4 Reverse-transcription polymerase chain reaction showing significantly higher relative expression levels of RRAS, POMC, SLC26A6 and STX3 genes in biliary atresia patients ($n$ = 40) than in neonatal cholestasis patients ($n$ = 14).** [a]$P < 0.05$ *vs* liver tissue samples from neonatal cholestasis patients.

with cholestasis ($P < 0.05$, Table 5, Figures 3 and 4). The mRNA expression level of the RRAS gene increased 1.9-fold in BA patients ($P < 0.05$).

### *Correlation between expression of RRAS in liver tissue samples and prognosis of BA patients*

To address whether the RRAS expression is associated with the prognosis of BA patients, we performed a 2-year

**Table 6 Fibrosis scores for different groups of biliary atresia patients at different ages**

| Group | Fibrosis score (*n*) | | | | | Total |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | |
| < 60 d | 1 | 3 | 8 | 2 | 0 | 14 |
| 60-90 d | 0 | 2 | 3 | 6 | 4 | 15 |
| > 90 d | 0 | 0 | 1 | 4 | 6 | 11 |
| Total | 1 | 5 | 12 | 12 | 10 | 40 |

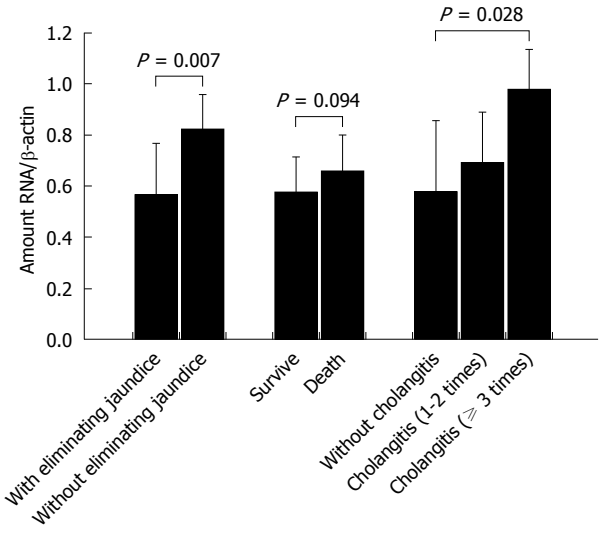


**Figure 5 Correlation between expression of RRAS in liver tissue samples and prognosis of biliary atresia patients within 2 years.**

follow-up study in 40 patients with BA. Six months after operation, The RRAS expression level was significantly higher in patients with their jaundice not eliminated than in those with their jaundice eliminated ($P$ = 0.007). Furthermore, the expression level of RRAS was significantly higher in patients with cholangitis reoccurred 3 or more times than in those with no cholangitis recurred ($P$ = 0.028). However, no significant difference in RRAS expression was found between the surviving and dead patients ($P$ = 0.094, Figure 5). In addition, the fibrosis was more serious in group of BA patients at the age of > 60 d than in group of BA patients at the age of < 60 d (Table 6), which was consistent with that observed in profile 75.

## DISCUSSION

The pathogenesis of BA has not yet been delineated. It has been shown that factors such as genetic susceptibility, congenital heteroplasia, and infectious and abnormal immune response lead to BA[17-23], and its clinical course and surgical outcome are correlated with the age of such patients[24-26]. Although there are some studies involving DNA microarrays in BA[4-6], very few studies are available on gene expression profiling of BA at its different stages of clinical course. That is why the clinicopathologic characteristics of BA vary with the age of such patients.

The differential gene expression patterns of RRAS in liver tissue samples from BA patients at different ages, as well as normal liver tissue samples and liver tissue samples from neonatal cholestasis patients were characterized in this study using the expression DNA microarray technology and bioinformatics. The 4 significant expression profiles identified using STC could confidently separate BA liver tissues from normal and diseased liver tissues. STC-GO analysis revealed that the genes represented in the 4 profiles were mainly involved in inflammatory response and reconstruction of extracellular matrix. Notably, as validated by fibrosis classification, profile 75 showed that the expression level of genes involved in fibrosis and inflammation was much lower in BA patients at the age of < 60 d than in those at the age of > 60 d, which may explain why BA patients at the age of < 60 d often have a good prognosis after a Kasai's operation[27]. Additionally, this phenomenon may also result from fibrosis due to the continuous hepatic inflammatory response-induced activation of stellate cells[28]. Moreover, a set of genes were involved in apoptosis represented in profile 74, which is in agreement with the reported findings[29]. In this profile, the BA patients at the age of > 90 d and < 60 d showed obvious inflammatory response and apoptosis mediated by NF-κB, which might be associated with the inflammatory response of local bile ducts in BA patients at the age of < 60 d and severe inflammatory response induced by fibrosis in BA patients at the age of > 90 d.

Mack *et al*[30,31] showed that $CD4^+$ Th1-mediated bile duct inflammation is responsible for the development of BA. Profile 74 in the present study contains a set of genes associated with MHC class Ⅱ antigen-mediated Th1 inflammatory response, which is in agreement with the findings of Mack *et al*[31] and Osada *et al*[32]. It is well known that antigens associated with MHC class Ⅱ can bind to T cell receptors of $CD4^+$ Th1 cells, and thereby produce functional T lymphocytes. Furthermore, the significant pathways highly associated with BA were mainly focused on the autoimmune response, activation of T lymphocytes and its related cytokines, suggesting that autoimmune response mediated by T lymphocytes may play a vital role in the pathogenesis of BA, which is consistent with the widely accepted hypothesis of BA[2,22,23,32].

In this study, the RRAS, POMC, SLC26A6 and STX3 genes were found to be important regulatory modules in BA. The RRAS gene is a component of the MAPK signaling pathway with GTP kinase activity[33,34]. The MAPK pathway is associated with BA[35]. Based on the results of this study, it is reasonable to speculate that the RRAS gene plays an important role in the pathogenesis of BA. The human POMC gene is located on chromosome 2p23.3 encoding a preprohormone. The adrenocorticotropin hormone and α melanocyte-stimulating hormone are cleavage products of POMC, which are associated with immune regulation and participate in the pathogenesis of experimental autoimmune encephalomyelitis[36,37]. The SLC26A6 is an anion exchanger involved in the secretion of bile acid. The STX3 has been implicated in the development and differentiation of dendritic cells[38,39]. Nonetheless,

the precise role of these genes in the pathogenesis of BA needs to be further elucidated.

Given the key role of RRAS gene in the pathogenesis of BA, we evaluated the relation between the expression of RRAS and prognosis of BA patients through a 2-year follow-up study. The RRAS expression was negatively correlated with the elimination rate of jaundice and positively correlated with the occurrence rate of cholangitis, indicating that up-regulation of RRAS expression may inhibit the recovery of BA from jaundice and cholangitis *via* activation of the MAPK pathway, continuous inflammatory response, inflammatory cell infiltration, as well as activation of stellate cells[40]. However, no significant difference was found in the 2-year survival rate of patients with different expression levels of RRAS. Validation may require a long-term follow-up and a larger number of subjects.

In summary, autoimmune response mediated by T lymphocytes may play a vital role in the pathogenesis of BA. The RRAS gene and its related MAPK pathway are important regulatory modules in the pathogenesis of BA, which may serve as a novel prognostic marker for BA.

## ACKNOWLEDGMENTS

The authors thank Dr. Duan Ma for his technical assistance and Song Zai for his collection of clinical samples.

## COMMENTS

### Background

Biliary atresia (BA) is an inflammatory obliterative cholangiopathy with unknown etiology, leading to progressive fibrosis and cirrhosis. Although there are some studies involving DNA microarrays on BA, very few studies are available on gene expression profiling of BA at different stages of its clinical course, which is why the clinicopathologic characteristics of BA vary with the age.

### Research frontiers

Microarray technology and bioinformatics, emerged indispensable research tools for gene expression profiling, have been used to study the pathogenesis of BA and allow the simultaneous analysis of thousands of transcripts within a single experiment. In this study, genes that play a key role in the pathogenesis and prognosis of BA were identified.

### Innovations and breakthroughs

In the current study, DNA microarrays for whole genome gene expression and bioinformatics analysis were used to characterize the differentially expressed gene patterns in normal livers and livers from BA patients at different ages, as well as to ascertain genes and pathways playing a central role in the pathogenesis of BA. The results demonstrate that *RRAS* gene and its related MAP kinase (MAPK) pathway are important regulatory modules in the pathogenesis of BA, which may serve as a novel prognostic marker for BA.

### Applications

By identifying genes and pathways playing a central role in the pathogenesis of BA, this study may represent a future strategy for therapeutic intervention in treatment of BA.

### Terminology

*RRAS* gene is a component of the MAPK signaling pathway with GTP kinase activity. The MAPK pathway is associated with BA. Consequently, it is reasonable to speculate that the RRAS gene plays an important role in the pathogenesis of BA.

### Peer review

This paper is interesting and valuable for other researchers. BA is a pediatric liver disease, which can lead to liver-related death and is the most common indication for liver transplantation in children. Therefore, the early proper treatment of BA with Kasai procedure is important in this group of patients. Early diagnosis of BA and knowledge of its prognostic factors can improve the treatment outcome of BA. Different prognostic factors have been described in the literature, but no report is available on RRAS as a key regulator and an important prognostic biomarker for BA identified by DNA microarray and bioinformatics.

## REFERENCES


1 **Hartley JL**, Davenport M, Kelly DA. Biliary atresia. *Lancet* 2009; **374**: 1704-1713
2 **Mack CL**. The pathogenesis of biliary atresia: evidence for a virus-induced autoimmune disease. *Semin Liver Dis* 2007; **27**: 233-242
3 **Young RA**. Biomedical discovery with DNA arrays. *Cell* 2000; **102**: 9-15
4 **Leonhardt J**, Stanulla M, von Wasielewski R, Skokowa J, Kübler J, Ure BM, Petersen C. Gene expression profile of the infective murine model for biliary atresia. *Pediatr Surg Int* 2006; **22**: 84-89
5 **Chen L**, Goryachev A, Sun J, Kim P, Zhang H, Phillips MJ, Macgregor P, Lebel S, Edwards AM, Cao Q, Furuya KN. Altered expression of genes involved in hepatic morphogenesis and fibrogenesis are identified by cDNA microarray analysis in biliary atresia. *Hepatology* 2003; **38**: 567-576
6 **Bezerra JA**, Tiao G, Ryckman FC, Alonso M, Sabla GE, Shneider B, Sokol RJ, Aronow BJ. Genetic induction of proinflammatory immunity in children with biliary atresia. *Lancet* 2002; **360**: 1653-1659
7 **Ramoni MF**, Sebastiani P, Kohane IS. Cluster analysis of gene expression dynamics. *Proc Natl Acad Sci USA* 2002; **99**: 9121-9126
8 **Miller LD**, Long PM, Wong L, Mukherjee S, McShane LM, Liu ET. Optimal gene expression analysis by microarrays. *Cancer Cell* 2002; **2**: 353-361
9 **Gene Ontology Consortium**. The Gene Ontology (GO) project in 2006. *Nucleic Acids Res* 2006; **34**: D322-D326
10 **Yi M**, Horton JD, Cohen JC, Hobbs HH, Stephens RM. WholePathwayScope: a comprehensive pathway-based analysis tool for high-throughput data. *BMC Bioinformatics* 2006; **7**: 30
11 **Draghici S**, Khatri P, Tarca AL, Amin K, Done A, Voichita C, Georgescu C, Romero R. A systems biology approach for pathway level analysis. *Genome Res* 2007; **17**: 1537-1545
12 **Schlitt T**, Palin K, Rung J, Dietmann S, Lappe M, Ukkonen E, Brazma A. From gene networks to gene function. *Genome Res* 2003; **13**: 2568-2576
13 **Barabási AL**, Oltvai ZN. Network biology: understanding the cell's functional organization. *Nat Rev Genet* 2004; **5**: 101-113
14 **Micke P**, Ohshima M, Tahmasebpoor S, Ren ZP, Ostman A, Pontén F, Botling J. Biobanking of fresh frozen tissue: RNA is stable in nonfixed surgical specimens. *Lab Invest* 2006; **86**: 202-211
15 **Kanehisa M**, Goto S, Kawashima S, Okuno Y, Hattori M. The KEGG resource for deciphering the genome. *Nucleic Acids Res* 2004; **32**: D277-D280
16 **Prieto C**, Risueño A, Fontanillo C, De las Rivas J. Human gene coexpression landscape: confident network derived from tissue transcriptomic profiles. *PLoS One* 2008; **3**: e3911
17 **Arikan C**, Berdeli A, Kilic M, Tumgor G, Yagci RV, Aydogdu S. Polymorphisms of the ICAM-1 gene are associated with biliary atresia. *Dig Dis Sci* 2008; **53**: 2000-2004
18 **Arikan C**, Berdeli A, Ozgenc F, Tumgor G, Yagci RV, Aydogdu S. Positive association of macrophage migration inhibitory factor gene-173G/C polymorphism with biliary atresia. *J Pediatr Gastroenterol Nutr* 2006; **42**: 77-82
19 **Awasthi A**, Das A, Srinivasan R, Joshi K. Morphological and immunohistochemical analysis of ductal plate malformation: correlation with fetal liver. *Histopathology* 2004; **45**: 260-267
20 **Allen SR**, Jafri M, Donnelly B, McNeal M, Witte D, Bezerra J, Ward R, Tiao GM. Effect of rotavirus strain on the murine

model of biliary atresia. *J Virol* 2007; **81**: 1671-1679
21 **Szavay PO**, Leonhardt J, Czech-Schmidt G, Petersen C. The role of reovirus type 3 infection in an established murine model for biliary atresia. *Eur J Pediatr Surg* 2002; **12**: 248-250
22 **Shivakumar P**, Sabla G, Mohanty S, McNeal M, Ward R, Stringer K, Caldwell C, Chougnet C, Bezerra JA. Effector role of neonatal hepatic CD8+ lymphocytes in epithelial injury and autoimmunity in experimental biliary atresia. *Gastroenterology* 2007; **133**: 268-277
23 **Mack CL**, Falta MT, Sullivan AK, Karrer F, Sokol RJ, Freed BM, Fontenot AP. Oligoclonal expansions of CD4+ and CD8+ T-cells in the target organ of patients with biliary atresia. *Gastroenterology* 2007; **133**: 278-287
24 **Serinet MO**, Wildhaber BE, Broué P, Lachaux A, Sarles J, Jacquemin E, Gauthier F, Chardot C. Impact of age at Kasai operation on its results in late childhood and adolescence: a rational basis for biliary atresia screening. *Pediatrics* 2009; **123**: 1280-1286
25 **Wadhwani SI**, Turmelle YP, Nagy R, Lowell J, Dillon P, Shepherd RW. Prolonged neonatal jaundice and the diagnosis of biliary atresia: a single-center analysis of trends in age at diagnosis and outcomes. *Pediatrics* 2008; **121**: e1438-e1440
26 **Davenport M**, Puricelli V, Farrant P, Hadzic N, Mieli-Vergani G, Portmann B, Howard ER. The outcome of the older (> or =100 days) infant with biliary atresia. *J Pediatr Surg* 2004; **39**: 575-581s
27 **Altman RP**, Buchmiller TL. The jaundiced infant: biliary atresia. In: Grosfeld JL, O'neill JA, Fonkalsrud EW, Coran AG, editors. Pediatric surgery. St. Louis: Mosby, 2006: 1603-1619
28 **Kershenobich Stalnikowitz D**, Weissbrod AB. Liver fibrosis and inflammation. A review. *Ann Hepatol* 2003; **2**: 159-163
29 **Erickson N**, Mohanty SK, Shivakumar P, Sabla G, Chakraborty R, Bezerra JA. Temporal-spatial activation of apoptosis and epithelial injury in murine experimental biliary atresia. *Hepatology* 2008; **47**: 1567-1577
30 **Mack CL**, Tucker RM, Sokol RJ, Karrer FM, Kotzin BL, Whitington PF, Miller SD. Biliary atresia is associated with CD4+ Th1 cell-mediated portal tract inflammation. *Pediatr Res* 2004; **56**: 79-87
31 **Mack CL**, Tucker RM, Sokol RJ, Kotzin BL. Armed CD4+ Th1 effector cells and activated macrophages participate in bile duct injury in murine biliary atresia. *Clin Immunol* 2005; **115**: 200-209
32 **Osada M**, Tolkacheva T, Li W, Chan TO, Tsichlis PN, Saez R, Kimmelman AC, Chan AM. Differential roles of Akt, Rac, and Ral in R-Ras-mediated cellular transformation, adhesion, and survival. *Mol Cell Biol* 1999; **19**: 6333-6344
33 **Muraji T**, Hosaka N, Irie N, Yoshida M, Imai Y, Tanaka K, Takada Y, Sakamoto S, Haga H, Ikehara S. Maternal microchimerism in underlying pathogenesis of biliary atresia: quantification and phenotypes of maternal cells in the liver. *Pediatrics* 2008; **121**: 517-521
34 **Kinbara K**, Goldfinger LE, Hansen M, Chou FL, Ginsberg MH. Ras GTPases: integrins' friends or foes? *Nat Rev Mol Cell Biol* 2003; **4**: 767-776
35 **Jafri M**, Donnelly B, McNeal M, Ward R, Tiao G. MAPK signaling contributes to rotaviral-induced cholangiocyte injury and viral replication. *Surgery* 2007; **142**: 192-201
36 **Taylor AW**. Modulation of regulatory T cell immunity by the neuropeptide alpha-melanocyte stimulating hormone. *Cell Mol Biol* (Noisy-le-grand) 2003; **49**: 143-149
37 **Taylor AW**, Kitaichi N. The diminishment of experimental autoimmune encephalomyelitis (EAE) by neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH) therapy. *Brain Behav Immun* 2008; **22**: 639-646
38 **Ohana E**, Yang D, Shcheynikov N, Muallem S. Diverse transport modes by the solute carrier 26 family of anion transporters. *J Physiol* 2009; **587**: 2179-2185
39 **Despars G**, O'Neill TJ, O'Neill HC. Use of gene profiling to describe a niche for dendritic cell development. *Immunol Cell Biol* 2007; **85**: 567-570
40 **Tsukada S**, Westwick JK, Ikejima K, Sato N, Rippe RA. SMAD and p38 MAPK signaling pathways independently regulate alpha1(I) collagen gene expression in unstimulated and transforming growth factor-beta-stimulated hepatic stellate cells. *J Biol Chem* 2005; **280**: 10055-10064

**S- Editor** Sun H **L- Editor** Wang XL **E- Editor** Zheng XM