Role of the c-Jun N-terminal kinase signaling pathway in the activation of trypsinogen in rat pancreatic acinar cells

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Abstract. Bile acid causes trypsinogen activation in pancreatic acinar cells through a complex process. Additional research is required to further elucidate which signaling pathways affect trypsinogen activation when activated. The changes in the whole-genome expression profile of AR42J cells under the effect of taurolithocholic acid 3-sulfate (TLC-S) were investigated. Furthermore, gene groups that may play a regulatory role were analyzed using the modular approach of biological networks. The aim of the present study was to improve our understanding of the changes in TLC-S-stimulated AR42J cells through a genetic functional modular analysis. Whole-genome expression profile chip arrays were applied to detect genes that were differentially expressed in pancreatic acinar AR42J cells treated with TLC-S for 20 min. Based on the Human Protein Reference Database, a protein-protein interaction network was obtained, which was then processed by CFinder software to derive 14 modules. Among these 14 modules, the Gene Ontology Biological Processes enrichment analysis identified two as modules of interest. Kyoto Encyclopedia of Genes and Genomes map analysis revealed that MAP2K4, MAPK8 and FLNA are part of the c-Jun N-terminal kinase (JNK) pathway. The JNK signaling pathway is involved in regulating trypsinogen activation in rat pancreatic AR42J cells. Next, a regulatory network of seven kinase inhibitors was constructed. SP600125 is an ATP-competitive, efficient, selective and reversible inhibitor of JNK. The results were verified by four sets of experiments and demonstrated that trypsinogen activation is mediated by the JNK signaling pathway in the pathogenesis of acute pancreatitis (AP). The present study provided a useful reference for better understanding the pathogenesis of AP and identifying new targets to regulate trypsinogen activation, in addition to providing valuable information for the treatment of AP.

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Abbreviations: AP, acute pancreatitis; TLC-S, taurolithocholic acid 3-sulfate; RMA, robust multichip average; PPI, protein-protein interaction; HPRD, human protein reference database; DAVID, database for annotation visualization and integrated discovery; GO-BP, gene ontology biological processes; KEGG, Kyoto Encyclopedia of Genes and Genomes

Key words: acute pancreatitis, AR42J cell, bile acid, gene chip, modular analysis, c-Jun N-terminal kinase signaling pathway

Introduction

Acute pancreatitis (AP) is a type of sudden inflammation manifested by self-digestion, edema, hemorrhage, and even necrosis of pancreatic tissue due to trypsin activation in the pancreas caused by a variety of factors. AP is characterized by trypsinogen activation, leukocyte infiltration and tissue necrosis (1). However, the exact mechanism underlying the signaling pathways that regulate these biological processes in pancreatic cells has not been fully elucidated. At present, research is mostly focused on signal transduction mechanisms in acinar cells during AP. It is generally considered that the involved pathways include the Ca^{2+} signaling pathway (2), the mitogen-activated protein kinase (MAPK) pathway (3), the nuclear factor (NF)-κB pathway (4), the phosphoinositide 3-kinase (PI3K)/AKT pathway (5) and the NADPH pathway (6). According to their different roles in cells, these pathways may be divided into an abnormal trypsin secretion and activation signaling pathway, an inflammation signaling pathway, an oxidative damage signaling pathway, and an apoptosis and necrosis signaling pathway. The mechanisms of these signaling pathways are complex, and one type of cytokine may activate multiple signal transduction pathways. Furthermore, one signal transduction pathway may be activated by various cytokines. These pathways are regulated by various factors that form a complex activity network in cells.

In our previous experiment, the intracellular activation of trypsinogen was observed in rat AR42J cells pre-treated with 200 μM/l taurolithocholic acid 3-sulfate (TLC-S) for 20 min using (CBZ-Ile-Pro-Arg)2-rhodamine110 (BZiPAR) (7), confirming that TLC-S directly acts on pancreatic acinar cells to enhance the intracellular activation of trypsinogen.

Bile acid leads to trypsinogen activation in pancreatic acinar cells through a complex process. Additional research is required to further elucidate which signaling pathways affect trypsinogen activation when activated. Therefore, the changes in the whole-genome expression profile of AR42J cells under the effect of TLC-S were investigated. Furthermore, gene groups that may play a regulatory role were analyzed using the modular approach of biological networks. The aim of the present study was to provide a useful reference for identifying new targets that regulate trypsinogen activation mediated by the c-Jun N-terminal kinase (JNK) signaling pathway.

Materials and methods

Detection of gene expression using a DNA microarray. The present study was performed in accordance with the Helsinki II Declaration, and approval for this study was obtained from the Committee for Medical Research Ethics of the First Affiliated Hospital of Harbin Medical University, which are the authority of research ethics in China. Pancreatic acinar AR42J cells were purchased from the China Center for Type Culture Collection (Wuhan, China) and were cultured in Ham's F12K culture medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37˚C and 5% CO_2. The cells were divided into two groups, namely the control and treatment groups. The cells in the control group did not receive treatment, while the cells in the treatment group were stimulated by TLC-S (200 μM/l) for 20 min. The dose of TLC-S and the treatment duration were as previously reported by Voronina *et al* (8) and Gerasimenko *et al* (9).

The cells were collected and total RNA was extracted according to the instructions for the TRIzol reagent (Invitrogen Life Technologies; Thermo Fisher Scientific, Carlsbad, CA, USA). Gene expression analyses were performed with the Rat 12x135K Gene Expression Array (NimbleGen Systems, Inc., Madison, WI, USA). Preparation of cDNA from 5 μg of total RNA, hybridizations, washes and detection were performed in accordance with the NimbleGen Gene Expression Analysis protocol (NimbleGen Systems, Inc.), and the slides were scanned using the Axon GenePix 4000B microarray scanner (Molecular Devices, LLC, Sunnyvale, CA, USA).

Scanned images in TIFF format were then imported into NimbleScan software (version 2.5) for grid alignment and expression data analysis. Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. The Probe level (*_norm_RMA.pair) files and Gene level (*_RMA.calls) files were generated following normalization. All gene level files were imported into Agilent GeneSpring GX software (version 11.5.1) for further analysis and were published in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63959). Differentially expressed genes were identified through fold change filtering.

Establishment and functional analysis of the modules of differentially expressed genes. The protein-protein interaction (PPI) network, which included 36,874 edges and 9,453 nodes (10), was acquired from the Human Protein Reference Database (HPRD). HPRD is a rich resource of diverse features of human proteins, which have been experimentally proven (11). The differentially expressed genes were mapped into the PPI network, and a file was obtained on the basis of genetic interaction pairs consisting of differentially expressed genes. The file was then imported to Cytoscape (version 2.6.3); the interaction type was 'default interaction'. Subsequently, 'layout', 'cytoscape layout', and 'spring embedded' was selected. Finally, a regulatory sub-network was constructed (12).

CFinder software (http://cfinder.org/) was used to identify functional modules in the sub-networks. Based on the clique percolation method, CFinder (13) is a software tool used to locate and visualize network modules and, thus, it may be applied to detect network clusters of a specified size.

The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to identify gene ontology biological processes (GO-BP) terms from the detected modules. The candidate gene groups were submitted to the DAVID database (http://david.abcc.ncifcrf.gov/); subsequently, using the complete genome of *Rattus norvegicus* as the background genes and 'functional annotation tool' as the analysis tool, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment results were obtained (the P-value cut-off was 0.05).

The KEGG database was used to identify pathway maps. The candidate genes in one module were imported into the DAVID pathway database (http://www.kegg.jp/kegg/pathway.html) as key words, and 'map' was selected; subsequently, a thumbnail image of the corresponding pathway was screened.

Cellular verification experiments. AR42J cells were grown to a density of 80-90%. A total of four groups of cells growing at logarithmic phase were treated as follows: Group A (control), AR42J cells cultured in normal medium; group B (TLC-S treatment), AR42J cells treated with TLC-S (200 μM) for 30 min; group C (SP600125 treatment), AR42J cells treated with SP600125 (40 μM) for 30 min; and group D (SP600125 + TLC-S treatment), AR42J cells pre-treated with SP600125 (40 μM) for 30 min prior to incubation with TLC-S (200 μM) for 30 min. Cells were harvested at the indicated time points for subsequent experiments.

Western blot analysis. Total proteins were extracted from control, TLC-S-treated, SP600125-treated and SP600125 + TLC-S-treated cultures at the abovementioned time points, and subjected to western blot analysis. Total proteins were separated by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions, and transferred onto PVDF membranes (Millipore, Billerica, MA, USA) by electroblotting. After blocking with non-fat dry milk in Tris-buffered saline and 500 μl Tween-20 (TBST) for 1 h at room temperature, the membrane was incubated at 4˚C overnight in a milk solution containing the following polyclonal rabbit antibodies: anti-p-JNK (1:1,000; cat. no. WLP1664; Wanleibio Co., Ltd., Shanghai, China), anti-p-JUN (1:500; cat. no. bs-3210R; Bioss, Inc., Beijing, China), and anti-β-actin (cat. no. WL0002; Wanleibio Co., Ltd.). After washing in TBST, the immunoreactive proteins were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG-HRP secondary antibodies (1:5,000; Beyotime, Haimen, China), followed by enhanced chemiluminescence (Wanleibio Co., Ltd.). The protein concentrations were determined using the microassay procedure of the Gel-Pro-Analyzer. Protein expression was determined by western blot analysis.

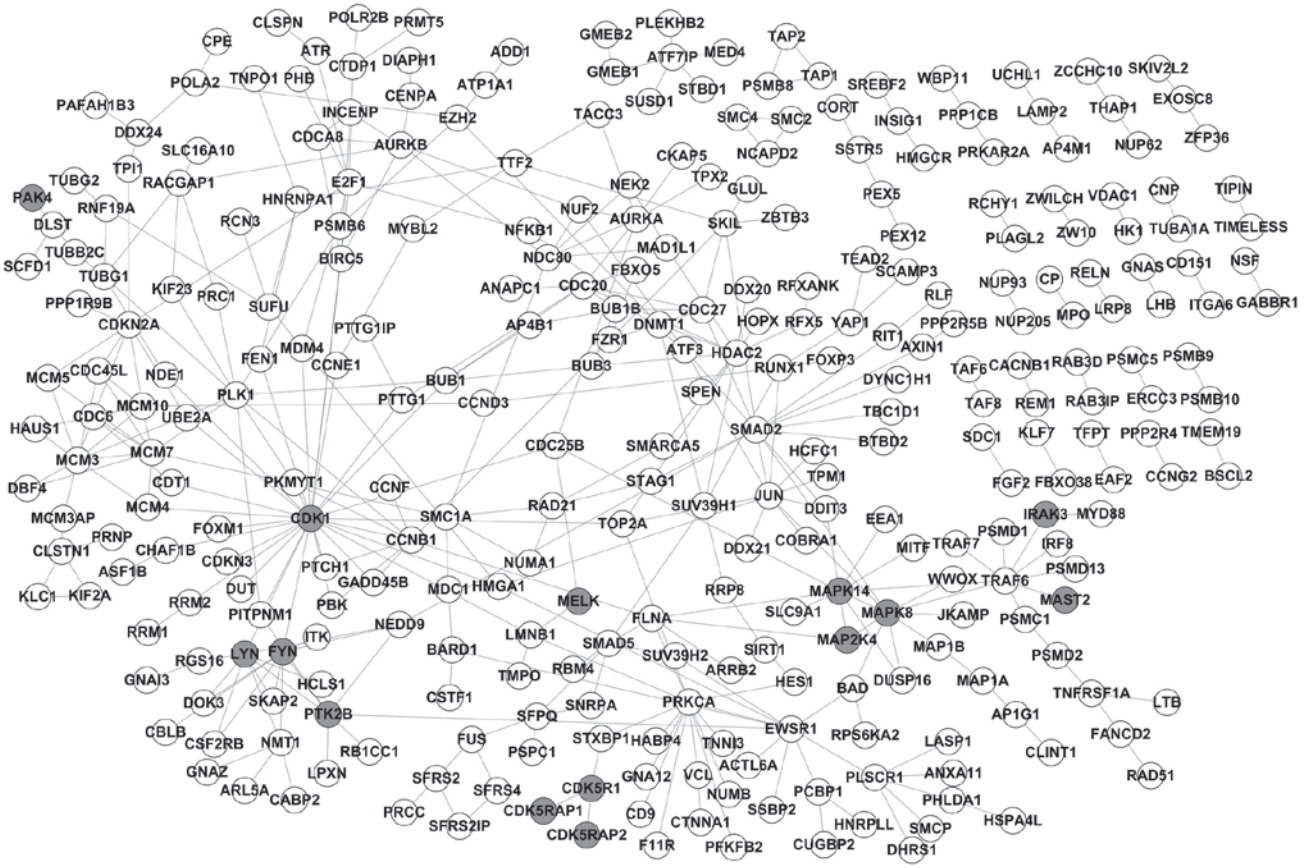

Figure 1. Interaction sub-network of differentially expressed genes of AR42J cells treated with taurolithocholic acid 3-sulfate (TLC-S) was constructed based on a protein-protein interaction network acquired from the Human Protein Reference Database. White nodes refer to differentially expressed genes, whereas gray nodes refer to differentially expressed genes that encoded protein kinases.

Trypsinogen activation assay. Following the abovementioned treatment, $2x10^6$ cells were disrupted by sonication on ice (40 kHz, 3-5 pulses, 3 sec each time; the process was repeated after 10 min for a total of three times). The cell homogenate was diluted with 500 μl buffer (10 mM HEPES, pH 7.5; 15% ethanol). Diluted cell homogenate (100 μl) was then added to 24-well plates, followed by the addition of 400 μl buffer. Finally, the fluorescent substrate BZiPAR (Molecular Probes, Inc., Eugene, OR, USA) was added to a final concentration of 2 μM. The reaction was incubated in the dark for 10-15 min prior to fluorescence intensity measurement using a multifunctional microplate reader (excitation, 496 nm; emission, 521 nm; ELX-800; BioTek, Winooski, VT, USA).

Statistical analysis. Data are expressed as mean ± standard deviation and analyzed with the SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA). Analysis of variance was used to determine statistically significant differences among the three groups, and means of every two groups were detected with the Student-Newman-Keuls test (q-test). $P<0.05$ was considered to indicate statistically significant differences.

Results

Construction of interaction sub-networks of differentially expressed genes. A total of 11,292 genes were detected, and 1,124 upregulated and 498 downregulated genes were identified in the TLC-S treatment group. There were 36,875 rat PPI pairs according to the HPRD. By mapping the differentially expressed genes onto PPI pairs, 375 PPI pairs of differentially expressed genes were obtained, and a sub-network diagram was constructed as illustrated in Fig. 1 using Cytoscape software.

Results derived from modular detection using CFinder software. CFinder software was applied for the modular detection of the abovementioned obtained PPI pairs of differentially expressed genes. Under the condition of k=3, 14 modules were identified (Fig. 2).

Functional analysis of each module. A GO-BP functional enrichment analysis was performed on the 14 modules by the DAVID software. The functional analysis of each gene group revealed that, in modules 2, 3 and 6, the differentially expressed genes were mainly enriched in 'Cell cycle' (rno04110) functions; in modules 4 and 7, the function of the differentially expressed genes was mainly the 'MAPK signaling pathway' (rno04010). Particularly in module 4, differentially expressed genes, such as MAP2K4, MAPK14 and MAPK8, encode kinases (Fig. 3).

A total of six differentially expressed genes in module 4 were imported into the KEGG map. It was observed that MAP2K4, MAPK8 and FLNA are part of the JNK pathway in the 'MAPK signaling pathway' (map04010) (Fig. 4). MAP2K4 is a MAPK kinase (MAPKK) and phosphorylates the downstream MAPK MAPK8 (JNK1).

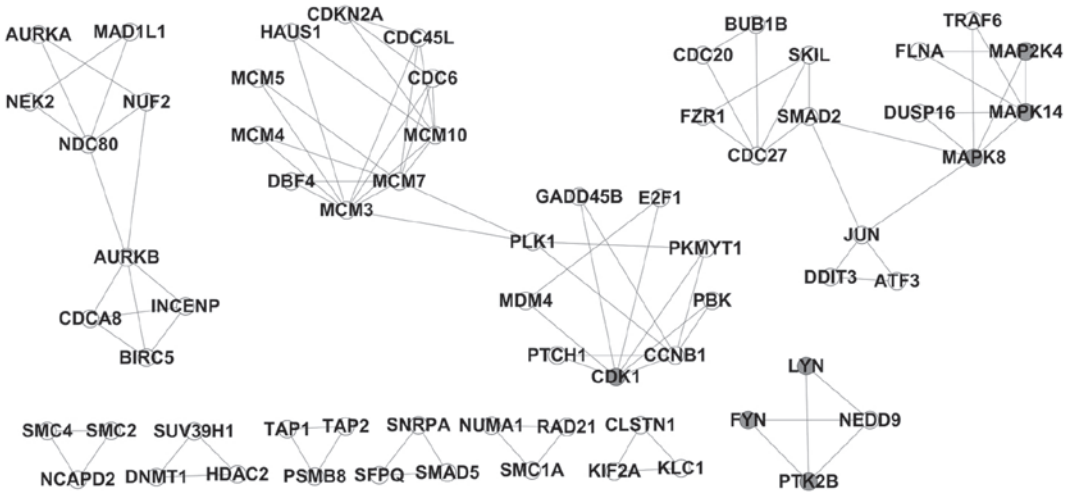

Figure 2. Following module analysis of the interaction sub-network, 14 modules were identified applying CFinder software under the condition of k=3. White nodes refer to differentially expressed genes, whereas gray nodes refer to differentially expressed genes that encoded protein kinases.

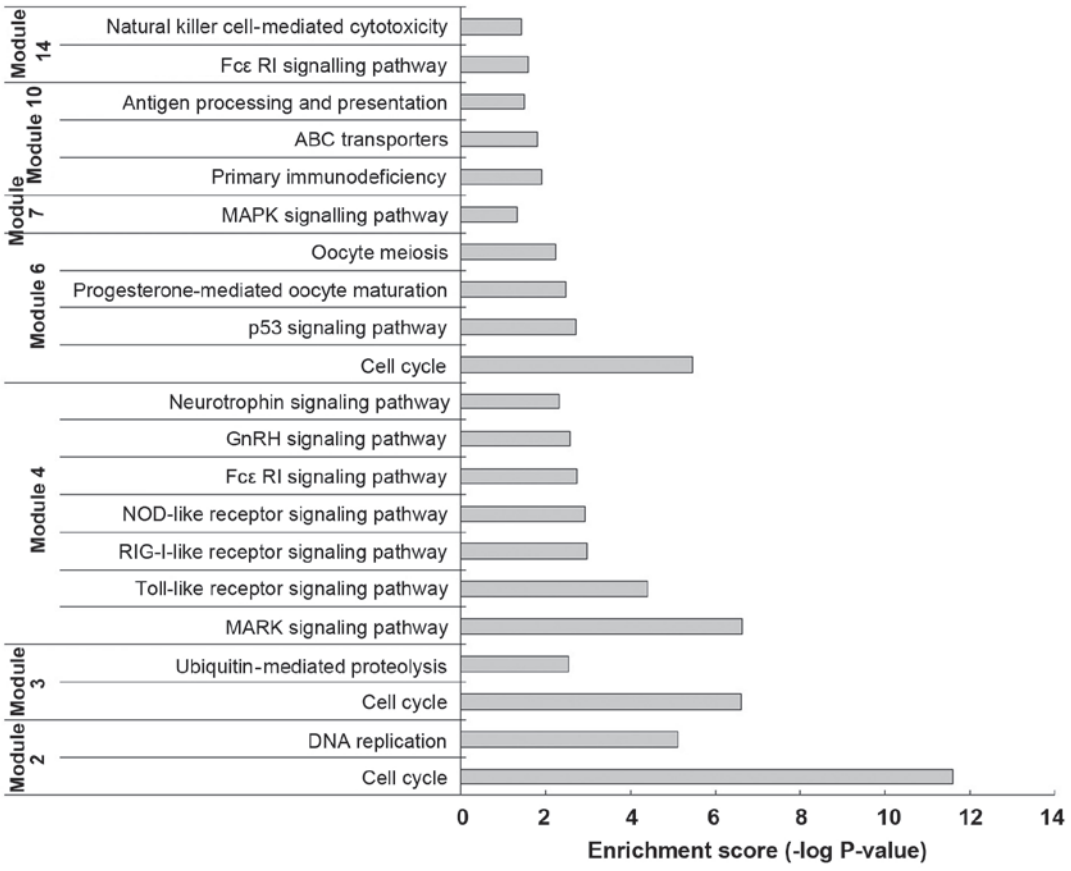

Figure 3. Gene ontology biological processes (GO-BP) analysis of each module. The enrichment score of each GO term was computed (enrichment score = –log P-value) and sorted in descending order using Database for Annotation, Visualization and Integrated Discovery software.

A regulatory network (Fig. 5A) of seven kinase inhibitors was constructed and arranged by the number of kinases regulated: LY294002 (n=6); SP600125 and PD98059 (n=5); and lapatinib, roscovitine, CI-1040, bosutinib, Torisel, selumetinib, imatinib, AZD7762, PIK-75, tozasertib and flavopiridol (n=2). A regulatory network (Fig. 5B) constructed with three kinases from module 4 contained LY294002 (PI3K inhibitor), SP600125 (JNK inhibitor) and PD98059 (MEK inhibitor). Thus, SP600125 (JNK inhibitor) was selected for the following experiments.

Western blot results. Western blots of the control and TLC-S groups revealed that, during trypsinogen activation, the p-JNK levels were markedly increased in AR42J cells following TLC-S treatment. In the TLC-S and SP600125 + TLC-S groups, the p-JNK levels following TLC-S stimulation were

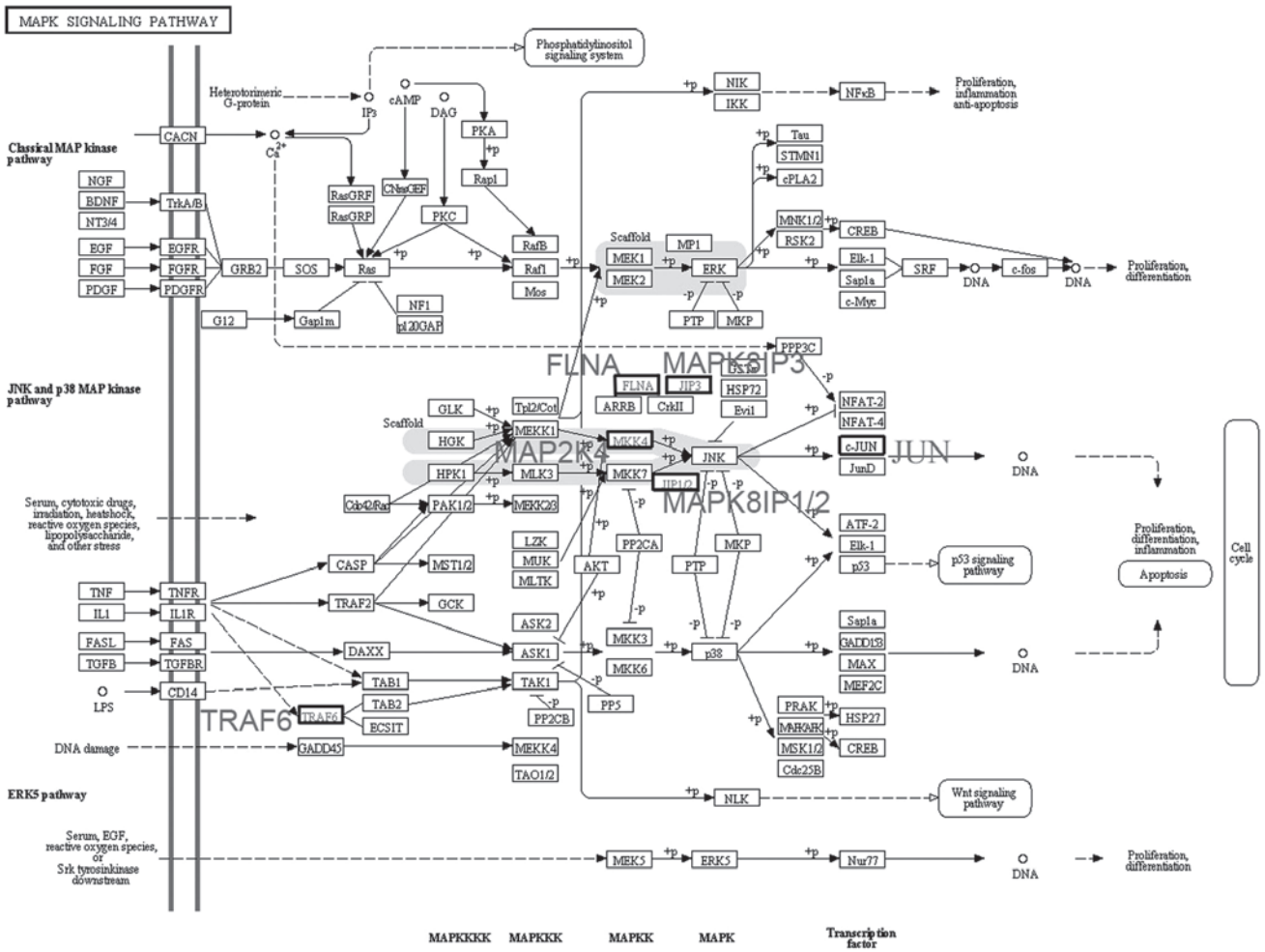

Figure 4. Thumbnail image of mitogen-activated protein kinase (MAPK) signaling pathway. The genes labelled were the genes in module 4 that were mainly enriched in c-Jun N-terminal kinase (JNK) pathway.

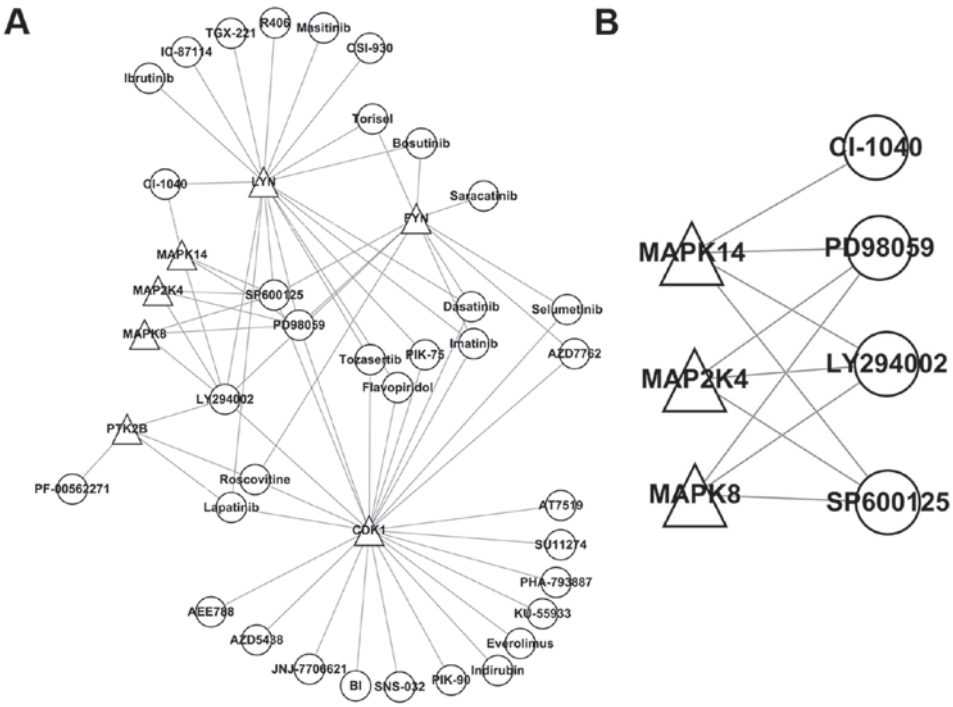

Figure 5. Association network of protein kinases and corresponding kinase inhibitors. (A) Network of trypsinogen activation-associated kinases. (B) Network of kinases in module 4. Elliptical nodes indicate kinase inhibitors, whereas triangular nodes indicate protein kinases. MAPK, mitogen-activated protein kinase.

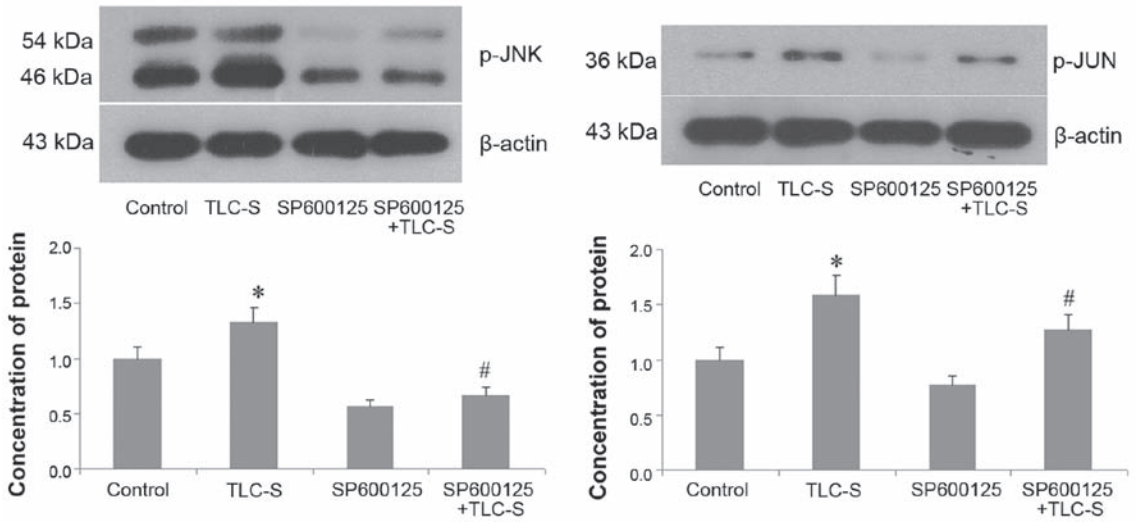

Figure 6. Detection results of p-JNK and p-JUN by using western blot analysis. Data represent the mean of 3 independent experiments. *P<0.05 compared with the control group. #P<0.05 compared with the TLC-S treatment group.

significantly reduced by pre-treatment with SP600125 (JNK inhibitor). p-JUN levels trended with p-JNK levels (Fig. 6).

Results of trypsinogen activation detection. Based on the results of the control and TLC-S groups, the extent of trypsinogen activation was found to be markedly increased in AR42J cells following TLC-S treatment. Based on the results of the TLC-S and SP600125 + TLC-S groups, the amount of trypsinogen activation following TLC-S stimulation was found to be markedly reduced by pretreatment with SP6001255 (JNK inhibitor) (Fig. 7).

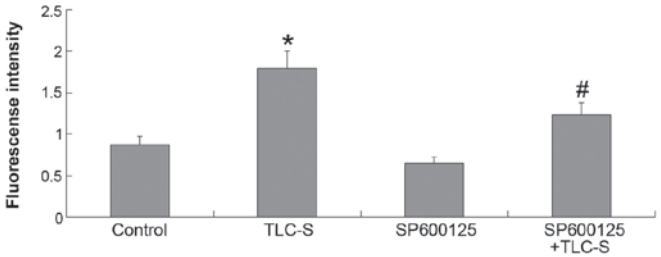

Figure 7. Detection results of trypsinogen activation by BZiPAR labelling and fluorescence intensity measurement using a multifunctional microplate reader. Data represent the mean of 3 independent experiments. *P<0.05 compared with the control group. #P<0.05 compared with the TLC-S treatment group.

Discussion

In the post-genomics era, elucidating the mechanisms that underlie a large number of biological processes has become one of the most important research goals; however, this process is complex and challenging. Therefore, studies focusing on only a few biological molecules (genes or proteins) cannot systematically reveal the mechanisms underlying biological processes. A comprehensive system consisting of a large number of molecules requires a multi-dimensional systemic analysis. If only a complete complex network is taken into account, it is very difficult to screen and identify suitable biological target molecules or relevant pathways. In recent years, researchers have noticed the modularization of genetic functions. Using the network module information, the search scope may be greatly reduced. For example, Liu *et al* (14) used genetic network characteristics to classify cancer and analyze the involved pathways. There is a close association between biological network modules and functions; each module corresponds to a different biological function (15). Wagner and Fell (16) defined metabolic pathway sub-networks with high local connectivity as 'modules'. Segal *et al* (17) defined a group of genes that have the same regulatory programs, functions and expression patterns as a 'regulatory module'. Several other scholars believe that a genetic functional module is a group of genes with high functional relevance; the module must respond to an experimental stimulus consistently, and all component genes must exhibit consistent expression responses. Through investigating the functional modularization of multiple genes, we may be able to understand the regulatory mechanisms of regulatory pathways more definitively and accurately.

The present study attempted to deepen our understanding of the changes in TLC-S-stimulated AR42J cells through a genetic functional modular analysis. Whole-genome expression profile chip arrays were applied to detect genes that were differentially expressed in pancreatic acinar AR42J cells treated with TLC-S for 20 min. Based on the HPRD database, a PPI network was obtained, which was then processed by CFinder software to derive 14 modules. Among these 14 modules, the GO-BP enrichment analysis identified two as modules of interest.

On the basis of GO-BP analysis, it was observed that in modules 4 and 7, the function of differentially expressed genes is mainly enriched in biological processes such as the 'MAPK signaling pathway'. This result suggests that this pathway plays an important role in TLC-S stimulation of AR42J cells to activate trypsin. The MAPK signal transduction pathway is an important intracellular signaling pathway (18). MAPKs are a class of serine/threonine protein kinases that are widespread in mammalian cells and mainly include MAPKK kinases (MAPKKKs), MAPKKs and MAPKs. MAPKs are expressed in all eukaryotic cells, and activated MAPKs convey extracel-

lular stimuli into the cell; moreover, MAPKs phosphorylate transcription factors and various protein kinases at the substrate level, regulate related gene transcription and participate in physiological processes, such as cell proliferation, differentiation, transformation and apoptosis (19). MAPKs are also associated with the pathogenesis of inflammation, cancer and a variety of other diseases. Currently, there are at least four MAPK family members, namely ERK1/2, JNK, p38 and ERK5, which interact with other proteins to regulate cellular functions (20). The existing literature confirms that the MAPK signaling pathway plays a crucial role in trypsinogen activation (21). Dabrowski *et al* (22) and Grady *et al* (23) demonstrated that activation of the MAPK pathway is an early event in AP and leads to further development of acute pancreatic disease.

In module 4, differentially expressed genes that encode the kinases MAP2K4, MAPK14 and MAPK8 were found. KEGG map analysis revealed that MAP2K4, MAPK8 and FLNA are part of the JNK pathway. JNK was initially identified as a protein kinase that specifically phosphorylates the transcription factor c-JUN in the nucleus (24,25). From the 'MAPK signaling pathway' map (map04010), it may be observed that a MAPKKK activates MAP2K4 through phosphorylation as part of the JNK signaling pathway. MAP2K4 is a MAPKK and may phosphorylate downstream MAPK8 (JNK1), a MAPK (26). JNK is activated by an upstream signal and further phosphorylates the 63rd and 73rd serine residues at the amino terminus of the nuclear transcription factor c-JUN, thereby activating c-JUN and enhancing its transcriptional activity. Stress may activate the JNK signaling pathway, leading to the transcriptional activation of c-JUN by phosphorylation (27). The JNK signaling pathway is part of the MAPK signaling pathway and acts as an important messenger to convey extracellular signals from the cell surface into the nucleus (28). The JNK gene has three subtypes, JNK1 (MAPK8), JNK2 and JNK3; JNK1 (MAPK8) and JNK2 are present in a wide array of tissue types (29). The activation of JNK is closely associated with inflammation, nerve cell degeneration (30) and apoptosis (31). JNK is activated by different stimuli, such as inflammatory cytokines and stress factors (32). Research has revealed that the JNK signaling pathway plays an important role in the pathogenesis of AP. In 2000, Schafer reported that the JNK signaling pathway may produce inflammatory cells, thus playing a key role in the pathogenesis of AP (33). Thereafter, the importance of the JNK signaling pathway in the pathogenesis of AP was demonstrated by a series of research findings (34,35). A specific inhibitor of the JNK signaling pathway exerted a therapeutic effect in a rat model of AP (35,36). In a study of an acute edematous pancreatitis model, Grady *et al* (23) also found that JNK activation is an early event in the pathogenesis of AP. In the present study, it was observed that the JNK pathway was activated in AR42J cells following TLC-S stimulation, and that this pathway plays an important role in trypsinogen activation.

Next, a regulatory network of seven kinase inhibitors was constructed and arranged by the number of kinases regulated: LY294002 (n=6); SP600125 and PD98059 (n=5); and lapatinib, roscovitine, CI-1040, bosutinib, Torisel, selumentinib, imatinib, AZD7762, PIK-75, tozasertib and flavopiridol (n=2). In the regulatory network constructed containing three kinases of module 4, there were LY294002 (PI3K inhibitor), SP600125 (JNK inhibitor) and PD98059 (MEK inhibitor). Thus, SP600125 (JNK inhibitor) was selected for subsequent experiments. This JNK signaling pathway inhibitor (SP600125) is a broad-spectrum inhibitor of serine/threonine kinases capable of inhibiting JNK activation. SP600125 is an ATP-competitive, efficient, selective and reversible inhibitor of JNK (37) that significantly inhibits JNK-mediated MAPK activation. The selectivity of SP600125 for JNK is 300 times that for ERK1 and p38 (37). Irrera *et al* (38) demonstrated that SP600125 improves AP. Nishi *et al* (39) proposed that SP600125 is one of the most effective inhibitors for the treatment of inflammatory diseases involving the MAPK signaling pathway, such as AP.

The results were verified by four sets of experiments. From western blots, it was observed that the phosphorylation of JNK and JUN was significantly increased in AR42J cells following TLC-S treatment. However, pre-treatment with the inhibitor SP600125 led to a significant reduction in phosphorylation in AR42J cells following TLC-S treatment. The same trend was observed by trypsinogen activation assays, further indicating that the JNK signaling pathway plays an important role in trypsinogen activation following TLC-S stimulation of AR42J cells; treatment with the inhibitor SP600125 blocked the JNK signaling pathway and, thus, reduced the amount of trypsinogen activation. This finding further indicates that trypsinogen activation is mediated by the JNK signaling pathway in the pathogenesis of AP.

From the abovementioned experiments and analysis, it may be concluded that the biological process of kinase phosphorylation plays a key role in activating trypsinogen in AR42J cells following TLC-S stimulation. Phosphorylation is an important and universal post-translational modification and is also the most important regulatory modification in prokaryotic and eukaryotic organisms (40). Phosphorylation provides a dynamic means of regulating protein activity (41). During trypsinogen activation, phosphorylation is required for the activation of MAP2K4, MAPK8, MAPK14 and other kinases. MAPK8 activation requires the phosphorylation of threonine and tyrosine residues (26), whereas MAPK14 activation requires the phosphorylation of its serine/tyrosine residues (42). Phosphorylation is required for the activation of the abovementioned kinases, and the kinases are closely associated with trypsinogen activation. Therefore, phosphorylation of kinases participating in the JNK signaling pathway is considered to be necessary for trypsinogen activation.

In conclusion, the JNK signaling pathway is involved in regulating trypsinogen activation in rat pancreatic AR42J cells. The present study provides a useful reference for better understanding the pathogenesis of AP and identifying new targets to regulate trypsinogen activation. Moreover, it provides valuable information for the treatment of AP.

Acknowledgements

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