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Original Article

Insights into the molecular mechanisms of methylmalonic acidemia using microarray technology

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Abstract: Methylmalonic acidemia (MMA) is widely considered as an autosomal recessive metabolic disorder that results in accumulation of high levels of methylmalonic acid and eventually brain damage. This study aims to investigate the effects of methylmalonic acid on neurons and analyze various gene expression profiles in rat cortical neurons treated with methylmalonic acid in order to understand the effects of MMA. High concentrations of methylmalonic acid could significantly alter the morphology of rat cortical neurons, attenuate cell viability and aggravate cell apoptosis. Moreover, 564 differentially expressed genes were identified by microarray analysis. A considerable number of these genes were apoptosis-related genes. Enrichment analysis of the apoptosis-related genes revealed that the MAPK and p53 signaling pathways may be involved in the pathogenesis of MMA. Our results together reveal that methylmalonic acid plays a critical role in neuron damage and that the MAPK and p53 signaling pathways may be involved in the mechanism of MMA.

Keywords: Methylmalonic acidemia, microarray data, signaling pathway

Introduction

Methylmalonic acidemia (MMA) is a common autosomal recessive metabolic disorder that was first reported in 1967 [1]. This metabolic disease results from a decrease in the activity of methylmalonyl-coA mutase or its cofactor adenosylcobalamin, a critical Krebs cycle intermediate that is necessary for the metabolism of the branched chain amino acids isoleucine, valine, threonine and methionine [2]. The clinical biochemical hallmarks of this disorder include high levels of methylmalonic acid and homocysteine, as well as decreased methionine production. In our previous study, mass spectrometry was found to be an effective diagnostic technique for identifying this condition [3]. Data from previous reports show that the morbidity of MMA ranges from 1:100000 to 1:169000 [4].

Accumulation of methylmalonic acid in tissues is toxic to all types of body cells. Very few anecdotal reports and case series have documented the organic damage caused by MMA. MMA patients typically present with serious neurologic deficits, metabolic acidosis, vomiting, lethargy, anorexia, respiratory distress, severe ketoacidosis and hypotonia [5, 6]. The clinical manifestations can span the prenatal period and persist through adolescence and adulthood. Moreover, the aberrant accumulation of methylmalonic acid may account for multisystem pathological effects including nervous, renal, skin and hepatic dysfunction [7-9]. However, MMA has more prominent effects in the brain than in other organs [10, 11]. Patients with MMA typically present with serious neuromotor delay, basal ganglia damage and neurological deterioration [12, 13]. To date, no effective therapeutic method has been developed to protect the brain from the damage caused by MMA, and little is known about the mechanisms underlying the neuronal damage caused by this disorder. Li [14, 15] found decreased expression of miR-9-1in five MMA patients by microarray assay of their plasma samples; it was found that miR-9-1 could inhibit neuron apoptosis by targeting the apoptosis-related gene *BCL2L11*. Mass studies have illustrated that methylmalonic acid causes neurons apoptosis via increase in oxidative stress or mitochondrial dysfunction [16, 17]. However, the precise mechanism via

Table 1. The primer sequences of Jun, Txnip and GAPDH

Primers	Sequences (5'-3')	Product length
Jun-F	CCGGGTTGAAGTTGCTGA	163 bp
Jun-R	AATACGCTGCCCAGTGTCA	
Txnip-F	TCCTGCATGTTCATTCCT	239 bp
Txnip-R	GCGCAAGTCCCTGAGATA	
GAPDH-F	GCAAGTTCAACGGCACAG	140 bp
GAPDH-R	GCCAGTAGACTCCACGACAT	

which methylmalonic acid induces neuronal damage has not been elucidated.

Acute and progressive neurological dysfunction is a typical characteristic of patients with MMA [18], but the underlying mechanisms of brain toxicity in this disorder are not entirely established. Recently, microRNA microarray analysis was used to investigate the pathogenesis of MMA and to identify genetic biomarkers in specific target cells or tissues [15]. Microarray is a robust tool for the detection and analysis of differentially expressed genes and can therefore help illustrate the underlying mechanisms of MMA damage, especially neuronal damage. Therefore, in order to investigate the mechanism of MMA in neuronal damage, in this study, we have used a high-density cDNA microarray technique to assess the gene expression profile of normal rat cortical neurons and methylmalonic acid-treated rat cortical neurons. We found that several genes may be involved in the neuronal damage caused by MMA, and these genes may be potential novel therapeutic targets.

Materials and methods

Preparation of cell cultures

Certified timed pregnant rats were deeply anaesthetized on gestational day 17 and fetuses were rapidly removed from the uterus. Fetal brains were then removed and placed in sterile HEPES-buffered saline (HBS) (pH 7.3). The prefrontal cortex was dissected under a stereo microscope in sterile conditions. Cells were dissociated in the presence of trypsin and DNase I (Sigma-Aldrich) and placed in poly-l-lysine-coated dishes in basal DMEM medium supplemented with 10% heat inactivated FBS and 2 mM L-glutamine. The cells were maintained at 37°C in a humidified incubator with 5% CO_2/95% air. After 12 hours in culture medium containing serum was removed from the cells and replaced with a serum-free solution to reduce the proliferation of astrocytes. Serum-free medium was a base of Neurobasal medium (without glutamine, Gibco/BRL, Grand Island, NY), containing L-glutamine and B27 supplement (Gibco/BRL, Grand Island, NY).

All procedures involving animals followed the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques. The study was performed in accordance with the tenets of the Helsinki Declaration of 1975, as revised in 1983. The Ethics Committee of Shanghai Xinhua Hospital approved of the study.

Microarray analysis

The Affymetrix GeneChip Human Genome U133 Plus 2.0 array was used for the microarray analysis. Sample labeling, microarray hybridization, washing, and scanning were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Microarray experiments for the control and methylmalonic acid-treated group were duplicated and, in total, twelve arrays were used. The pre-processing procedure for the cell intensity files (CEL) and the microarray analyses were performed using the GenPlex software (Istech Inc., Goyang, Korea). Data were normalized using global scale normalization. The differentially expressed genes in the methylmalonic acid-treated rat cortical neurons were selected based on the fold change in expression compared with the corresponding controls. Hierarchical clustering was also performed with centered Pearson's correlation analysis of the differentially expressed genes, based on the complete linkage and distance matrix. Differentially expressed genes in the methylmalonic acid-treated rat cortical neurons were imported into Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Redwood, CA, USA), and their biological functions were analyzed. Genes that were deregulated in the MMA-treated rat cortical neurons were analyzed using microarray data for the MMA-treated rat cortical neurons. A lower cutoff threshold (over two-fold change in expression and $P<0.01$) was set for selecting the differentially expressed genes.

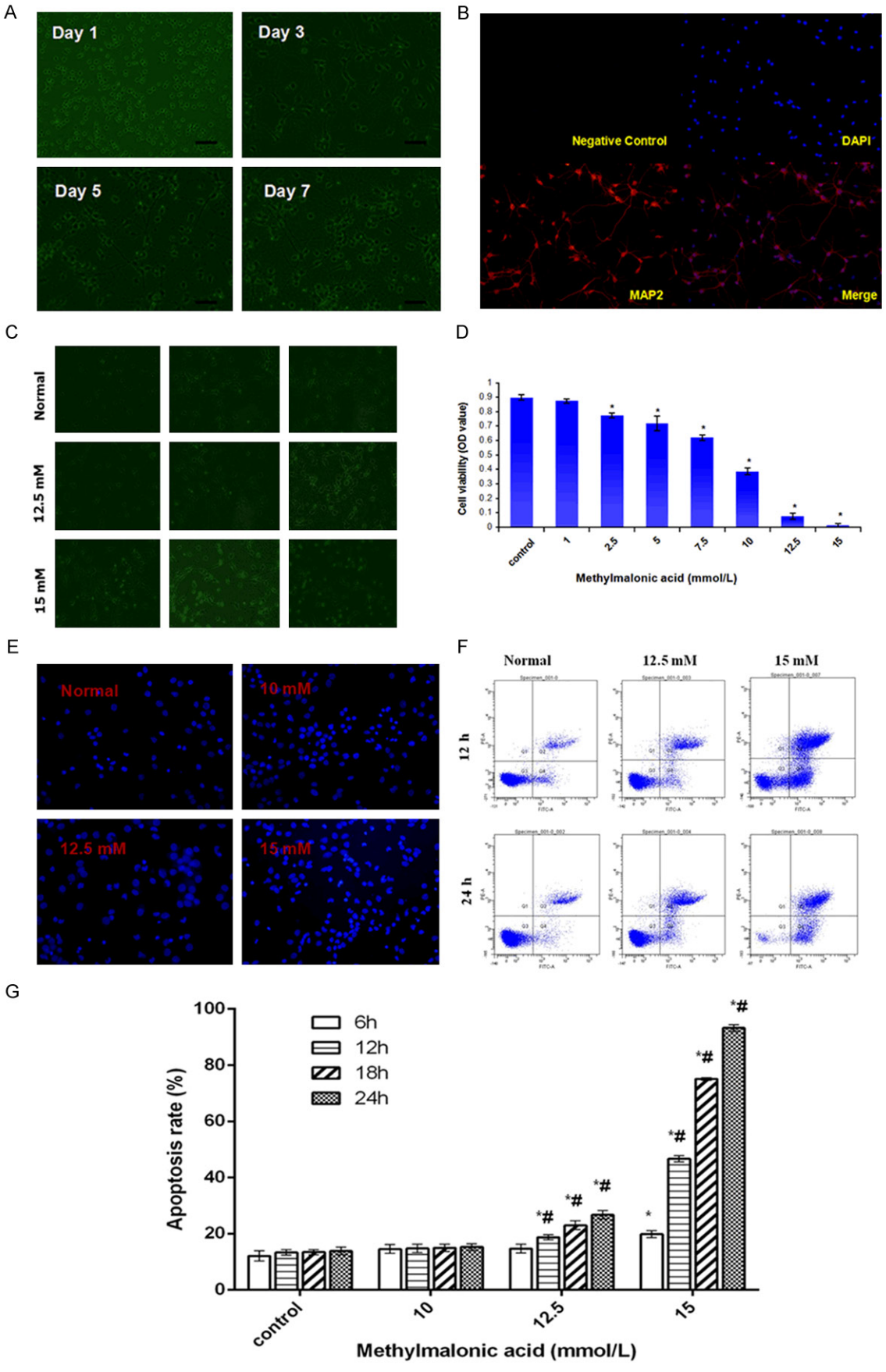

A
Day 1
Day 3
Day 5
Day 7
B
Negative Control
DAPI
MAP2
Merge
C
Normal
12.5 mM
15 mM
D
Cell viability (OD value)
control
1
2.5
5
7.5
10
12.5
15
Methylmalonic acid (mmol/L)
E
Normal
10 mM
12.5 mM
15 mM
F
Normal
12.5 mM
15 mM
12 h
24 h
G
6h
12h
18h
24h
Apoptosis rate (%)
control
10
12.5
15
Methylmalonic acid (mmol/L)

Figure 1. Evaluation of primary cultured rat cortical neurons and effect of methylmalonic acid. A. The maturity of primary cultured rat cortical neurons from different stages (×400). Phase-contrast images of the colonies derived from primary cultured rat cortical neurons on day 1, 3, 5 and 7. After culture for 14 days, the primary cultured rat cortical neurons were transformed into mature rat cortical neurons. Scale bar =550 μm. B. Evaluation of primary cultured rat cortical neurons. After culture for 7 days, immunostaining of MAP2 and DAPI showed that more than 90% of the primary cultured rat cortical neurons were MAP2-positive cells that adopted a neuronal morphology. C. The morphology of the neurons showed obvious changes under 10.0 mmol/L or 12.5 mmol/L of methymalonic acid after 12 h. When the treatment time reached 24 h, mass cellular debris were detected. D. The cell viability of primary cultured rat cortical neurons was measured by MTT after treatment with methylmalonic acid of different concentrations, ranging from 1.0 mmol/L to 15.0 mmol/L for 24 h. E and F. The effect of methylmalonic acidon neuron apoptosis was determined by Hoechst staining and flow cytometry assay. G. Bar graph shows the effect of methylmalonic acid on neuron apoptosis. Error bars indicate mean ± SE of three independent experiments. In the same time, different concentrations of methylmalonic acid versus control group, *P<0.05; In the same concentration of methylmalonic acid, different time treatment versus control group. #P<0.05.

Immunocytochemical assay

Cultured cells were rinsed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. Goat serum was used to block non-specific binding sites. The cultured cells were incubated overnight at 4°C with antibodies against MAP2 (1:200 dilution). After washing three times with PBS, the cells were incubated for 1 h with Alexa Fluor-594 chicken anti-mouse IgG (1:100 dilution). After another round of washing with PBS, the samples were incubated for 5 min with DAPI (1:200 dilution). The samples were then rinsed with PBS, mounted, and observed under a confocal microscope (FV 1000 Olympus IX-81). Images were analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics).

Western blot analysis

Cells were lysed with Laemmli sample buffer and boiled for 5 min. Protein lysates were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes, and then the following primary antibodies at the given dilutions were used: Jun (1:1000, Abcam), GAPDH (1:1000, Abcam), Bbc3 (1:1000, Cell Signaling Technology), Gadd45b (1:1000, Cell Signaling Technology), Gadd45g (1:1000, Cell Signaling Technology), and Txnzp (1:1000, Cell Signaling Technology).

Hoechst staining

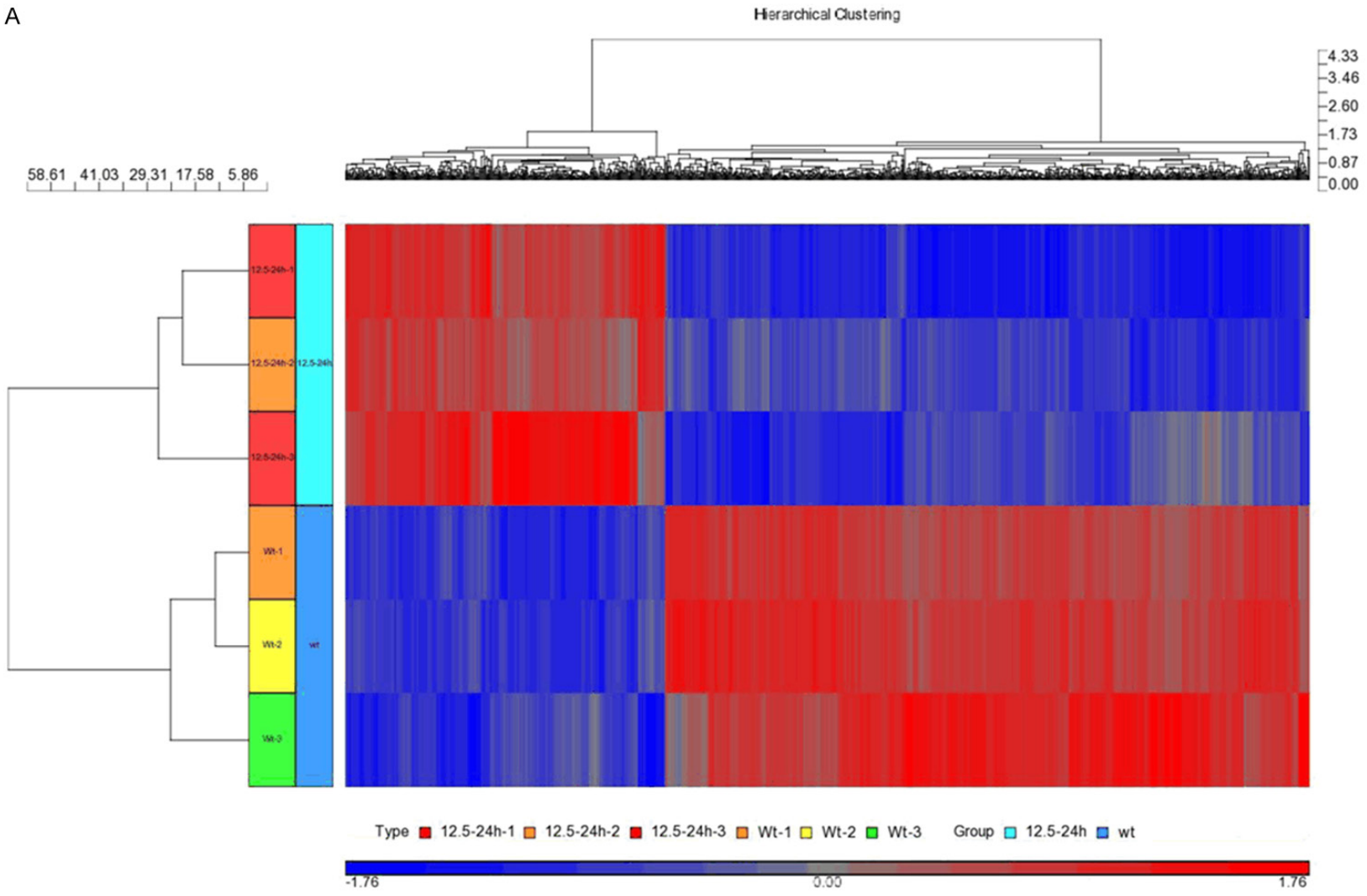
The effect of methylmalonic acid on cell apoptosis was determined by staining the cells with the cell-permeable fluorescent dye Hoechst. After methylmalonic acid treatment, the medium was removed and cells were washed twice with PBS. The control and treated cells were incubated with 10 μg/ml Hoechst 33342 dye for 10 min at 37°C.

MTT assay

The cytotoxic effects of methylmalonic acid were determined by the MTT uptake method. Briefly, the cells were incubated in four replicates in a 24-well plate and then treated with methylmalonic acid for different time periods at 37°C. MTT solution was added to each well and incubated at 37°C for 4 h. The formazan crystals formed were solubilized by adding Dimethyl sulfoxide (DMSO) and incubated with shaking for 15 min. The absorbance was measured at 570 nm using a 24-well multiscanner (Chameleon Multi-detection Platform, Finland). Lactate dehydrogenase (LDH) activity in the culture medium was measured spectrophotometrically to determine plasma membrane damage and loss of membrane integrity, by using a commercial kit (Sigma) according to the manufacturer's instructions.

Flow cytometric analysis of cell apoptosis

Normal and methylmalonic acid-treated cells were harvested and fixed at 8, 16, 24, 36 and 48 h with 70% ethanol at 4°C and stored at 20°C for 1 week. Following this, the fixed cells were stained with PI staining buffer containing RNaseA and PI in 0.05% Triton X-100 and PBS for 30 min in the dark. The fluorescence of individual nuclei was measured using a CyFlow space flow cytometer (Partec, Germany), and 20,000 events were collected for each sample. The annexin V-FITC apoptosis detection kit was used to determine the percentage of cells that underwent apoptosis. Apoptosis caused by methylmalonic acid exposure was confirmed by


A
Hierarchical Clustering
4.33
3.46
2.60
1.73
0.87
0.00
58.61
41.03
29.31
17.58
5.86
12.5-24h-1
12.5-24h-2
12.5-24h-3
Wt-1
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Wt-3
12.5-24h
wt
Type 12.5-24h-1 12.5-24h-2 12.5-24h-3 Wt-1 Wt-2 Wt-3
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-1.76
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1.76

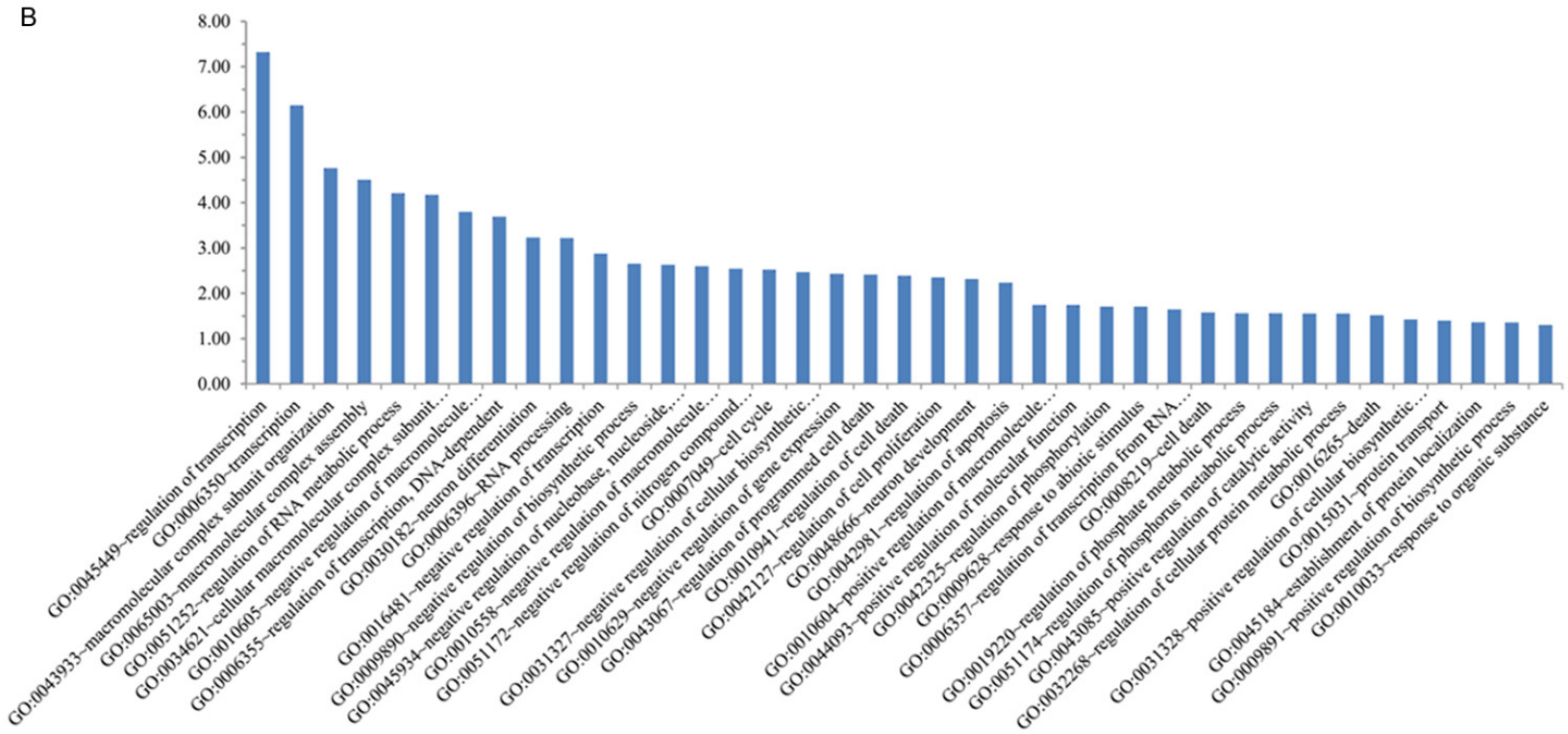


Figure 2. A. Microarray cluster analysis of neurons treated with methylmalonic acid and control neurons. The hierarchical cluster analysis showed distinguishable gene expression profiles between the samples. “Red” indicates relatively high expression, and “blue” indicates relatively low expression. B. GO enrichment analysis of differentially expressed genes that were enriched in the biological processes.

staining the treated cells with annexin V-FITC and PI double-staining according to the manufacturer's protocol. Briefly, after the adherent cells were harvested, they were resuspended in binding buffer (10 mM HEPES/NaOH, pH=7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). Five microliters of annexin V-FITC and 10 μl PI were added to the cell samples. Early apoptotic cells were specifically stained with annexin V-FITC, whereas late apoptotic cells were doubly stained with both annexin V-FITC and PI. The mixtures were incubated for 15 min in the dark at room temperature and then analyzed by flow cytometry (CyFlow space, Partec, Germany) and the FloMax software.

RNA isolation and quantitative real-time PCR

Cell samples were homogenized in TRIzol reagent (Invitrogen, CA). Total RNA was isolated according to the manufacturer's protocol (Invitrogen). The quantity and quality of RNA were determined using NanoDrop ND-1000, and RNA integrity was assessed using standard denaturing agarose gel electrophoresis. First-strand cDNA was prepared using 1 μg of RNA and the PrimerScript Reverse Transcriptase Kit (Takara, Dalian, China). *GAPDH* was used as an internal control. The expression of *JUN and Txinp* was analyzed. The primers used are listed in **Table 1**. SYBR Green quantitative PCR amplification was performed on the ABI 7500 Real-Time PCR System. Reactions were carried out in a 25 μl reaction mixture containing 12.5 μl of 2×SYBR Premix Ex TaqTM (Takara, Dalian, China). The conditions for real-time PCR were as follows: 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s. All the reactions were run in triplicate. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. The average ΔCt of each group was calculated using the following formula: ΔCt=Ct gene-Ct reference RNA. ΔΔCt was calculated using the formula ΔΔCt=ΔCt for the experimental group and ΔΔCt=-ΔΔCt for the negative control group. The relative expression level of the genes was calculated using the formula $2^{-\Delta\Delta Ct}$.

Statistical analysis

All values are expressed as mean ± standard deviation. The Student's *t*-test was used to evaluate statistical significance. A *P* value of < 0.05 was considered to indicate statistical significance. All the statistical analyses were performed using the SPSS 16.0 software (SPSS, Chicago, USA).

Results

Isolation of rat cortical neurons derived from rat embryos

Rat cortical neurons derived from pregnant rats on the 17th gestation day were cultured in neuronbasal medium containing B27 and L-glutamine. On the 1th, 3th, 5th, and 7th day, the apparent dynamic changes in cell morphology were determined. We observed that the rat cortical cells showed rapid morphological changes in these cultures. Notably, as early as day 5 of cultivation, the soma was significantly bigger, and simultaneously, the neuritis showed a remarkable increase in their number and length (**Figure 1A**). At the 5th day, primary cultured rat cortical cells were stained with immunofluorescent anti-MAP2 antibody: more than 90% of the cells were MAP2-positive (**Figure 1B**).

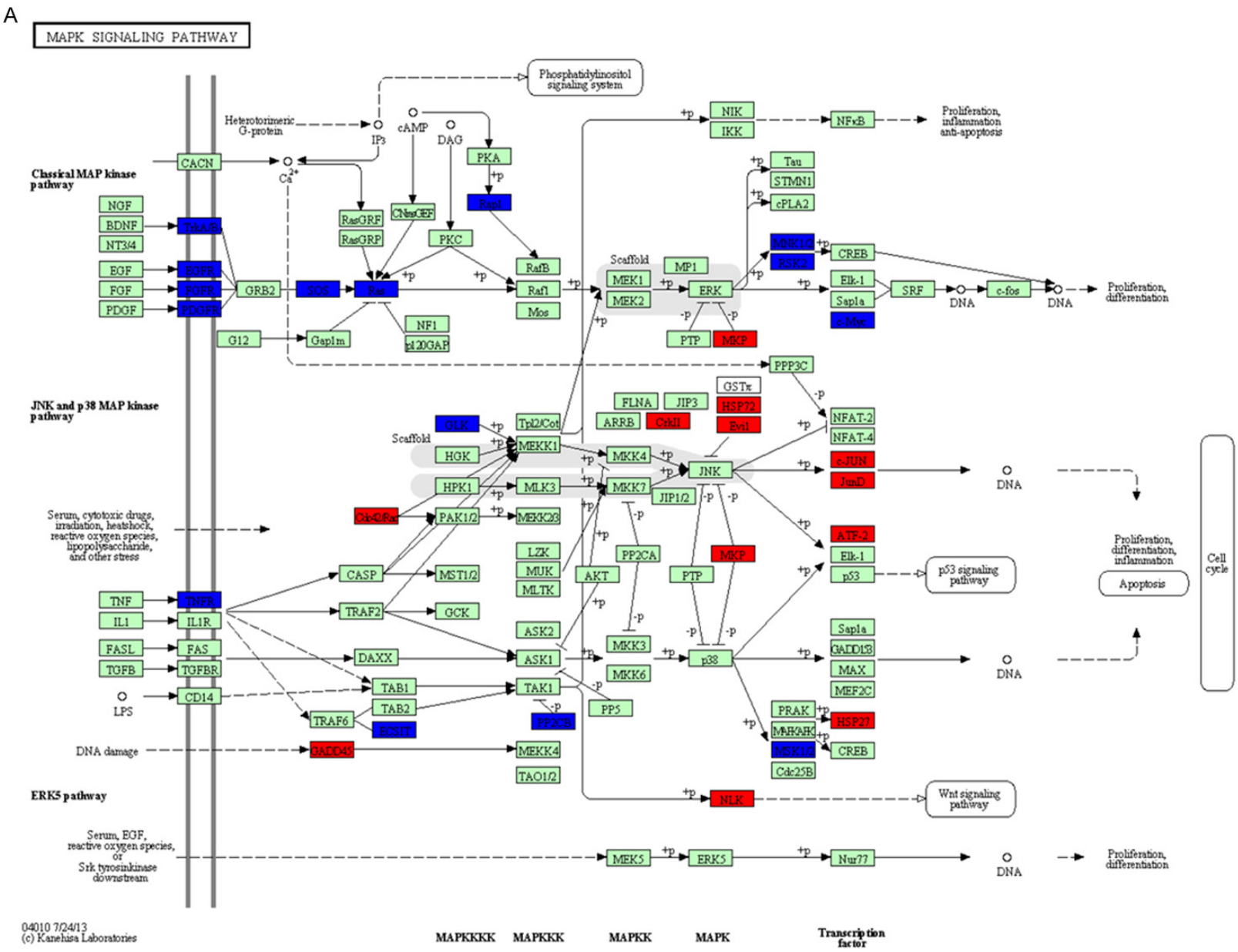
In vitro toxic effects of methylmalonic acid on rat cortical neurons

After five days of culture of the rat cortical neurons in 96-well plates, the neurons were divided into nine groups-the control group and eight groups treated with the following concentrations of methylmalonic acid: 0.1 mmol/L, 1.0 mmol/L, 2.5 mmol/L, 5.0 mmol/L, 7.5 mmol/L, 10.0 mmol/L, 12.5 mmol/L and 15 mmol/L. The treatment time ranged from 6 to 24 h. The neurons in the control group were treated with the same volume of a solution that did not contain methylmalonic acid but had the same pH. We discuss below the effects of methylmalonic acid on cell morphology, cell viability and cell apoptosis.

Cell morphology

At concentrations below 10.0 mmol/L, none of the cells displayed any significant morphological changes after 24 h of treatment. At concentrations equal to and above 12.5 mmol/L, shortening and decrease in the number of neurites were observed from 12 h. With increase in the treatment time and dose of methylmalonic acid, soma shrinkage and mass cell debris were observed under a phase-contrast microscope (**Figure 1C**).

A


MAPK SIGNALING PATHWAY
Phosphatidylinositol signaling system
Heterotorimeric G-protein
IP3
cAMP
DAG
Classical MAP kinase pathway
CACN
Ca2+
PKA
Rap1
NGF
BDNF
NT3/4
EGF
FGF
PDGF
TrkA/B
EGFR
FGFR
PDGFR
GRB2
SOS
Ras
RasGRF
CNrasGEF
RasGRP
PKC
RafB
Raf1
Mos
G12
Gap1m
NF1
p120GAP
Scaffold
MP1
MEK1
MEK2
ERK
PTP
MKP
NIK
IKK
NFκB
Proliferation, inflammation anti-apoptosis
Tau
STMN1
cPLA2
MNK1/2
RSK2
CREB
Elk-1
Sap1a
c-Myc
SRF
DNA
c-fos
DNA
Proliferation, differentiation
PPP3C
JNK and p38 MAP kinase pathway
GLK
HGK
HPK1
Cdc42/Rac
PAK1/2
Tpl2/Cot
MEKK1
MLK3
MEKK2/3
Scaffold
FLNA
JIP3
ARRB
CrkII
GSTπ
HSP72
Evi1
MKK4
MKK7
JIP1/2
JNK
NFAT-2
NFAT-4
c-JUN
JunD
DNA
ATF-2
Elk-1
p53
p53 signaling pathway
Proliferation, differentiation, inflammation
Apoptosis
Cell cycle
Serum, cytotoxic drugs, irradiation, heatshock, reactive oxygen species, lipopolysaccharide, and other stress
CASP
MST1/2
LZK
MUK
MLTK
PP2CA
AKT
PTP
MKP
TNF
IL1
FASL
TGFB
LPS
TNFR
IL1R
FAS
TGFBR
CD14
TRAF2
GCK
ASK2
ASK1
TAK1
PP5C
DAXX
TAB1
TAB2
TRAF6
ECSIT
PP5
MKK3
MKK6
p38
Sap1a
GADD153
MAX
MEF2C
DNA
PRAK
MAPKAPK
MSK1/2
Cdc25B
HSP27
CREB
DNA damage
GADD45
MEKK4
TAO1/2
ERK5 pathway
NLK
Wnt signaling pathway
Serum, EGF, reactive oxygen species, or Src tyrosinkinase downstream
MEK5
ERK5
Nur77
DNA
Proliferation, differentiation
04010 7/24/13
(c) Kanehisa Laboratories
MAPKKKK
MAPKKK
MAPKK
MAPK
Transcription factor

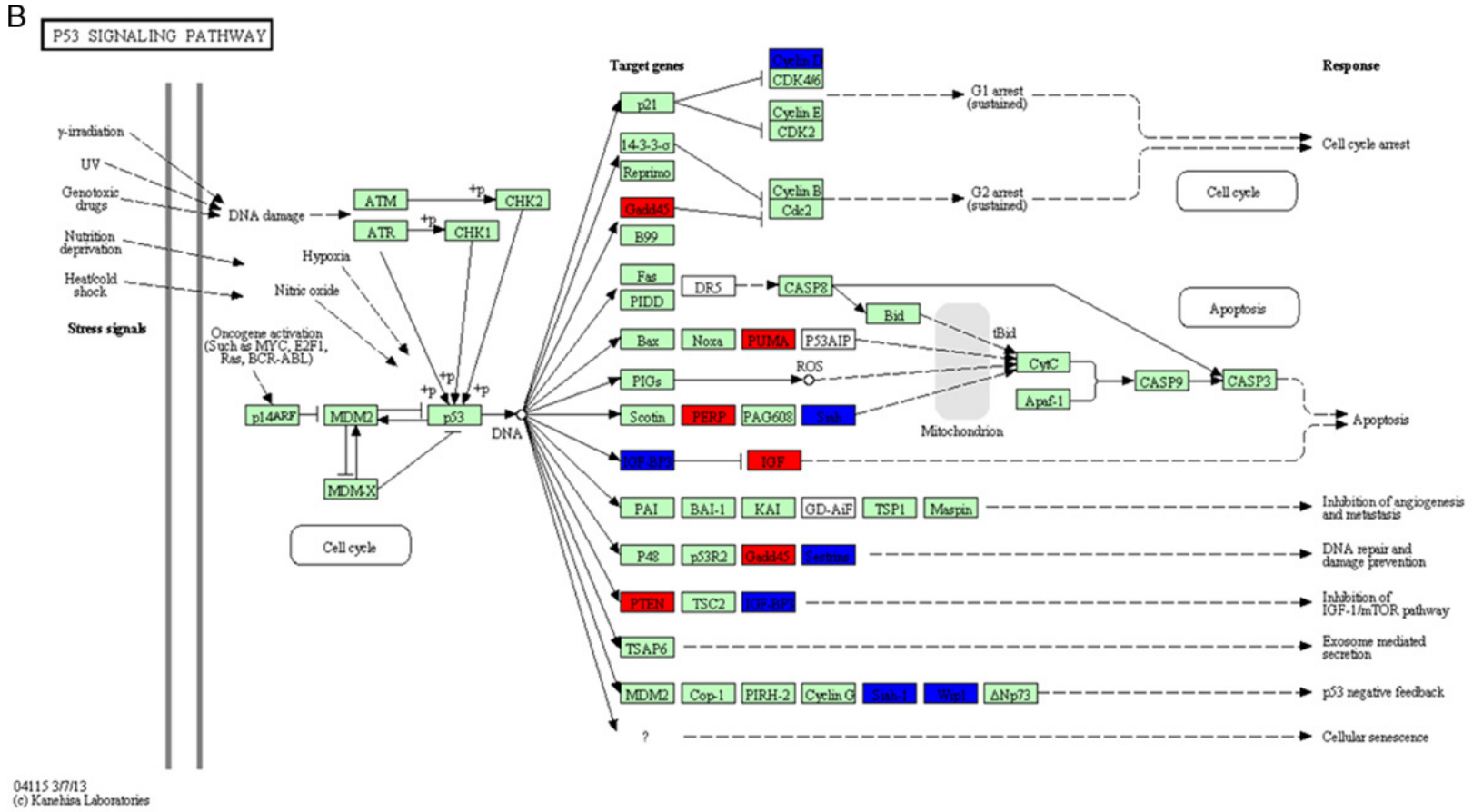


Figure 3. A. Genes with differential expression (fold-change ≥1.5 or ≤-1.5) in the MAPK signaling pathway. B. Genes with differential expression (fold-change ≥1.5 or ≤-1.5) in the p53 signal pathway.

Cell viability

The MTT assay was used to determine the effects of methylmalonic acid on cell viability. As expected, increase in the methylmalonic acid concentration resulted in an increase in cell viability, which suggests that the effect of methylmalonic acid on cell viability is time- and dose-dependent (**Figure 1D**).

Cell apoptosis

Abnormal changes in the shape of the nucleus were observed in rat cortical neurons treated with higher concentrations (10 Mm, 12.5 mM and 15 mM) of methylmalonic acid (**Figure 1E**). Moreover, flow cytometric analysis showed that the apoptotic effect of methylmalonic acid was dose dependent (**Figure 1F** and **1G**).

Differentially expressed genes in the methylmalonic acid-treated rat cortical neurons

To explore the underlying molecular mechanism, the genes involved and their related pathways, Affymetrix Rat Genome 230 2.0 Arrays were performed in triplicate on normal rat cortical neurons and rat cortical neurons treated with 12.5 mM methylmalonic acid for 24 h. Microarray data from the Affymetrix GeneChip Rat Genome 230 2.0 array were normalized with both robust multi-array average 27 (RMA) and Microarray Analysis Suite 5.0 (ref. 28; MAS5). mRNAs that showed a two-fold change and a statistically significant difference in their expression ($P<0.05$) between the methylmalonic acid-treated and control groups were selected for further analysis. We found that 148 and 416 genes were up- and down-regulated in the methylmalonic acid-treated rat cortical neurons, respectively. Unsupervised hierarchical cluster analysis was performed to identify the differentially expressed genes (**Figure 2A**). The results showed that the expression of a large number of mRNAs was dysregulated in the methylmalonic acid-treated rat cortical neurons compared with the control neurons (**Figure 2A**), which implies that methylmalonic acid may have altered some pathways that eventually resulted in MMA.

Functional classification of differentially expressed genes in the methylmalonic acid-treated groups

GO analysis were performed to determine the molecular mechanisms of the 564 genes (148 up-regulated and 416 down-regulated genes) identified from the methylmalonic acid-treated groups with regard to the biological process, molecular function and cellular component. The results showed that the differentially expressed genes were involved in transcription regulation, high polymer material metabolism regulation, cell apoptosis and proliferation, cell death and neuron differentiation (152 GO terms). 25 GO terms related to cellular components and their enrichment were obtained, and 20 GO terms related to molecular function were obtained. In particular, 32 apoptosis-related genes showed enrichment in biological processes, including *TXNIP, EGFR, BBC3, JUN, NOTCH1, NOTCH2, SON, CASP8AP2, BCL6, HSPA1A, HSPA1B, NDUFAF4,* and *TIMP3*, which suggests that their dysregulation might contribute to MMA (**Figure 2B**). For further study, pathway analysis should be carried out to determine which genes play a vital role in methylmalonic acid-related neuropathy.

Microarray-based pathway analysis

The differentially expressed genes were compared against the KEGG database to identify the pathways involved in MMA. Pathway analysis indicated that the two networks that showed the highest enrichment were the MAPK signaling pathway and the p53 signaling pathway, composed of 14 and 6 of the selected genes, respectively. Even small changes in gene expression can cause remarkable functional changes in neural cells [19]. According to our gene expression profiling data, if 1.5-fold change is set as the criterion instead of 2-fold change, the number of genes in the two networks, the MAPK signaling pathway and p53 signaling pathway, dramatically increases to 31 and 13, respectively. Based on this criterion, the up- and downregulated genes enriched in the two pathways are shown in **Figure 3A** and **3B** respectively. Moreover, the activated MAPK pathway has been reported to mediate neuron apoptosis in Alzheimer disease. These results suggest that the p53 and MAPK pathways may be synergistically involved in the neuron damage caused by methylmalonic acid.

Real-time quantitative PCR and western blot analysis

We randomly selected five up-regulated genes (*Jun, Txnip, Bbc3, Gadd45b,* and *Gadd45g*) and three down-regulated genes (*Aqp4, Slc1a3,*

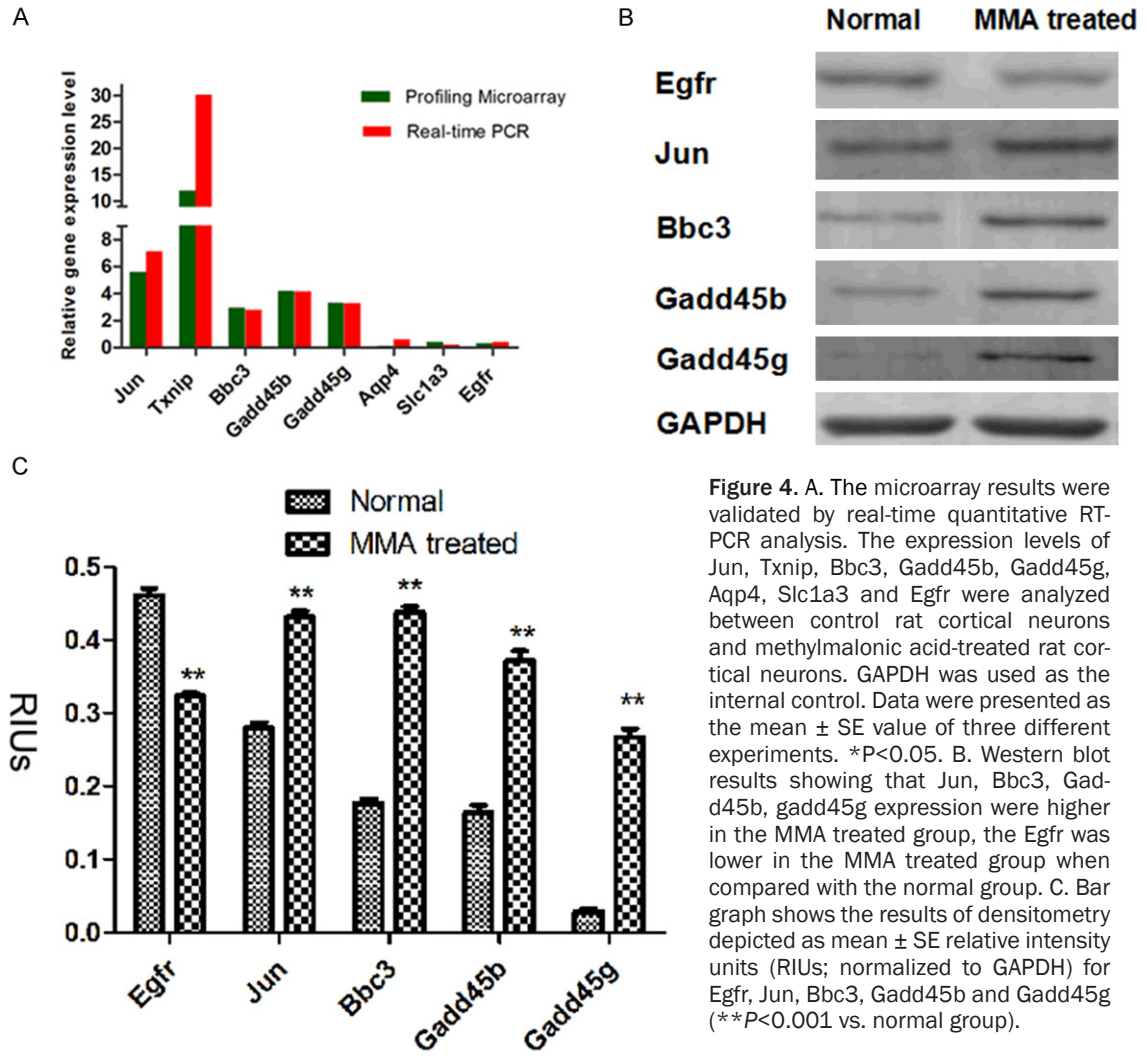


Figure 4. A. The microarray results were validated by real-time quantitative RT-PCR analysis. The expression levels of Jun, Txnip, Bbc3, Gadd45b, Gadd45g, Aqp4, Slc1a3 and Egfr were analyzed between control rat cortical neurons and methylmalonic acid-treated rat cortical neurons. GAPDH was used as the internal control. Data were presented as the mean ± SE value of three different experiments. *P<0.05. B. Western blot results showing that Jun, Bbc3, Gadd45b, gadd45g expression were higher in the MMA treated group, the Egfr was lower in the MMA treated group when compared with the normal group. C. Bar graph shows the results of densitometry depicted as mean ± SE relative intensity units (RIUs; normalized to GAPDH) for Egfr, Jun, Bbc3, Gadd45b and Gadd45g (**P<0.001 vs. normal group).

and *Egfr*) that were significantly dysregulated from the findings of the microarray dataset. Quantitative real-time PCR showed that the expression of these eight genes was consistent with the microarray results (**Figure 4A**). *Jun, Bbc3, Gadd45b, Gadd45g* and *Egfr* were also enriched in the p53 and MAPK pathways; their protein expression levels were validated by western blot analysis (**Figure 4B** and **4C**).

Discussion

In this study, we comprehensively analyzed the toxic effects of methylmalonic acid, the main active metabolite of MMA, on neurons. We used the microarray technique to identify the dysregulated genes in the methylmalonic acid-treated neurons compared with the control neurons. To the best of our knowledge, this is the first investigation to use microarray technology to study the role of MMA-related genes in methylmalonic acid-treated rat cortical neurons.

Neurological dysfunction is the most typical and serious clinical symptom in patients with MMA [20, 21]. Recently, several reports have shown that mitochondrial oxidative energy barrier, enhancement of oxidative stress and some other mechanisms may be involved in the brain damage caused by MMA. What all these mechanisms have in common is neuronal apoptosis. A great deal of research has shown that neuron apoptosis plays a role in Alzheimer disease, Parkinson disease and other nervous system diseases [22, 23]. Based on these previous studies, we first investigated the effect of methylmalonic acid on rat cortical neurons. The

results showed that exposure to methylmalonic acid could induce efficient apoptosis of rat cortical neurons in a time- and dose-dependent manner. Thus, elucidating the molecular mechanism of methylmalonic acid-induced neurons apoptosis may help understand the progression of neurological impairment in MMA patients.

Large-scale analysis of gene expression patterns is required. The microarray technique offers the possibility to study the regulation of thousands of genes simultaneously. We focused on the mRNAs of genes that are known to be associated with apoptosis. From the microarray results, 148 up-regulated and 416 down-regulated genes were screened out. These 564 dysregulated genes were subject to GO enrichment analysis to determine their function. Interestingly, 32 of the genes showed a high degree of association with neuron growth and the regulation of cell death, apoptosis and proliferation. Of the 32 genes, *Txnip, Jun* and *Bbc3* were up-regulated and may play a vital role in methylmalonic acid-induced neuron apoptosis. *C-jun* is a proto-oncogene that is encoded by the *JUN* gene. C-Jun combines with c-Fos to form AP-1, an early response transcription factor, which could regulate the transcription and expression of downstream target genes. The transcriptional activation of c-jun plays a vital role in neuron apoptosis. C-jun is generally present at low levels in the nervous system, but its expression is rapid and widespread in response to toxic substances. It is activated on phosphorylation by c-Jun N-terminal kinase (JNK), which enhances the transcriptional activity of JNK and subsequently triggers a series of downstream genes whose expression regulates cell apoptosis [24, 25]. Therefore, c-Jun is the key downstream target molecule in the neuronal apoptosis promoted by JNK [26]. Thioredoxin (Trx) is a small-sized multifunctional protein that is present in a variety of organisms and cells. Trx inhibits oxidative stress and plays an important role in regulating the cellular oxidation-reduction equilibrium. However, thioredoxin-interacting protein, which is encoded by the *Txnip* gene, is an endogenous Trx inhibitor. The thioredoxin-interacting protein could mediate oxidative stress response and regulate cell proliferation and apoptosis by combining with Trx via disulfide bonds [27, 28]. Bcl-2-binding component 3 (Bbc3) was first reported as the target gene of p53 and is called puma (p53-upregulated modulator of apoptosis). Bbc3 can promote cell apoptosis in a p53-dependent or p53-independent manner. These apoptosis-related genes that are dysregulated may play important roles in methylmalonic acid-induced neuron apoptosis.

Currently, there are no reports regarding the pathways involved in methylmalonic acid-induced neuron apoptosis. KEGG pathway analysis showed that the MAPK and P53 pathways were enriched with a higher number of differentially expressed genes compared to other pathways. The MAPK signaling pathway may play a role in the neuron apoptosis induced by MMA, which is consistent with the results of Finnigan and Richard [16]. MAPK is a Ser/Thr protein kinase that mediates multiple signaling pathways involved in cellular activity, regulation of cell proliferation, differentiation and apoptosis. There are three main components of the MAPK pathway: extracellular regulatory proteins (ERK), p38 and JNK. It has been suggested that this signaling pathway contributes to neuron apoptosis in Alzheimer and Parkinson disease [29, 30]. As mentioned before, JNK enhanced apoptosis signaling via phosphorylation of C-Jun to improve the transcriptional activity of C-Jun and downstream target genes such as Puma and Hrk. However, puma interacts with Bcl2 to increase neuron apoptosis in a P53-dependent or P53-independent manner. The interaction between Bcl2 and puma increases the release of cytochrome C into the cytoplasm by weakening the mitochondrial membrane, which triggers the caspase cascade. Therefore, in this study, we have shown for the first time that the expression of Jun and puma is significantly up regulated in methylmalonic acid-treated neurons, which implies that activation of the MAPK/c-Jun/puma signaling pathway may play a key role in the process of MMA-induced neuron apoptosis. In addition, puma could also be a candidate gene for p53, so further studies may be required to confirm whether the P53 signaling pathway and enhanced oxidative stress are involved in the activation of the MAPK and p53 signaling pathways.

All these results together indicate that the apoptosis-related genes *Jun*, *Puma*, and *Txnip* are involved in the MMA-induced neuron apoptosis via activation of the MAPK/c-Jun/puma

signaling pathways. Moreover, the P53 signaling pathway and enhanced oxidative stress may interact with MAPK to regulate neuron apoptosis, but this still needs further validation.

There are also several limitations to this research. Firstly, we did not perform in vivo and in vitro functional analysis of the genes and pathways that were found to participate in the neuron damage induced by MMA. However, the enrichment analysis clarified to some extent the MMA pathogenesis and revealed potential therapeutic targets. Secondly, other than mRNAs, non-protein-coding RNAs such as lncRNA may play important roles in cellular regulation, development, and disease, but these were not examined. Finally, even though in vitro methylmalonic acid-treated neurons can simulate neurons under pathological conditions to a great extent, in vivo experiments are still required to confirm the findings.

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Disclosure of conflict of interest

None.

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