# MicroRNA expression profiling involved in Borax-induced anti-tumor effect using gene-chip analysis

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**Research**

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# Abstract

Background

Borax, a boron compound, which is becoming widely recognized for its biological effects, including antioxidant activity, cytotoxicity, and potential therapeutic benefits. However, the specific molecular mechanisms underlying borax-induced anti-tumor effect still remain to be to further elucidated. MicroRNAs (miRNAs) may play key roles in cellular processes including tumor progression, cell apoptosis and cytotoxicity. Thus, this study aimed to investigate, whether miRNAs were involved in the borax-mediated anti-tumor effect using miRNA profiling of a human liver cancer cell line (HepG2) using gene-chip analysis.

Methods

Total RNA was extracted and purified from HepG2 cells that were treated with 4 mM borax for either 2 or 24 h. The samples underwent microarray analysis using an Agilent Human miRNA Array. Differentially expressed miRNAs were analysed by volcano plot and heatmap, and were validated using real-time fluorescent quantitative PCR (qPCR).

Results

Among this, 2- or 24-h exposure to borax significantly altered the expression level of miRNAs in HepG2 cells, 4 or 14 were upregulated and 3 were downregulated compared with the control group, respectively (≥2-fold; $P<0.05$). GO enrichment analysis and KEGG pathway enrichment analysis revealed that target genes of differentially expressed miRNAs in HepG2 cells predominantly participated in MAPK signaling pathway, TGF-beta signaling pathway, NF-kappa B signaling pathway, etc; in 2-h borax treatment group, while Ras signaling pathway, FoxO signaling pathway, Cellular senescence, etc; involved in 24-h treatment group.

Conclusions

Result indicates that borax-induced anti-tumor effect may be associated with alterations in miRNAs.


# Introduction

Borax, as an important borate compound, which is also known as sodium tetraborate decahydrate ($Na_2B_4O_710H_2O$) [1] and is a low toxicity mineral used in insecticidal, sterilizing, weeding and so on. Borax is widely used in food additive, manufacturing, fertilizers and pharmaceuticals [2]. The studies also have shown that the mechanism underlying the anti-inflammatory properties of borax involved the suppression of interleukin -8, indicating that borax is potentially applicable for therapeutic potential [3, 4]. Furthermore, previous studies suggested that different concentrations of borax affected lymphocyte proliferation, cell survival, and cell growth [5–7]. Thus, it demonstrated that those alterations were ascribed to certain cellular toxicities and genetic defects by borax. In recent years, numerous studies have

focused on the application of borax for various types of cancer prevention, including liver cancer, prostate cancer, cervical cancer, and lung cancer [2, 8, 9]. Our previous results revealed that HepG2 cell proliferation was inhibited following 24-h borax (4 mM) treatment and demonstrated borax-induced apoptotic in a concentration-dependent manner. Meanwhile, the 50% inhibitory concentration of borax was estimated as 4 mM [10]. However, the molecular mechanisms underlying borax mediated anti-tumor effect and apoptosis of tumor cells remain to be elucidated.

MicroRNAs (miRNAs), single-stranded, non-coding highly conserved family of small RNA that are 20 to 24 nucleotides in length and are predicted approximately 1000 of these sequences in the human [11]. Each miRNA regulates the expression of its target genes by binding to multiple mRNA, this is usually appeared on the posttranscriptional level by binding to complementary sequences located in the 3' untranslated region binding to a target mRNA transcripts [12]. As each miRNA can regulate hundreds of target genes by interfering with the translation of mRNA or acting directly to degrade mRNA, it is speculated that the largely of the 20,000–25,000 genes may be regulated by specific miRNAs [13]. In the current study, it found a closely relationship between miRNA and liver cancer, such as, levels of miR-21, miR-221, etc. expressions were upregulated, other miRNAs, including miR-145, miR-223, etc were decreased in HCC [14, 15]. Furthermore, the study has shown that, abnormal expression of miRNA in HCC, affects several clinical features (tumor size, vascular invasion, and node–metastasis, etc) [16, 17]. These studies indicate that the modulation of miRNA may play a significant part in the development and progression of HCC. Recently, numerous studies have proved that environmental chemical minerals affect the regulation of miRNA expression [18] and thus, the probably have that borax present in the environment may bring into play the anti-tumor effect through alterations in miRNAs expression levels [19]. Therefore, it is important to analysis expression profiles of miRNA and illustrate their roles in the regulation of gene expression in human diseases.

Gene-chips are called DNA microarrays or biological chips with high-throughput, automated platforms and miniaturized properties based on the theory of hybridization [20]. The Microarray technology is applied to realize the differentially expressed genes of a whole genome in a single experiment, thus it is as a global tool, which is used to evaluate and characterize of gene expression profiles [21, 22]. Therefore, in the present study, we explored miRNA expression alterations directly caused by treatments with doses of borax (4 mM) in HepG2 cells for either 2 or 24 h with high-throughput gene-chips. The biological functions of those differentially expressed miRNAs were investigated by gene ontology (GO) analysis and pathway analysis through assessment of Agilent Human miRNA Gene Chip data. The aim of the current study was to investigate potential factors involved in liver cancer progression, as well as critical miRNAs expression that were associated with borax anti-tumor effect.

## Materials And Methods

## Cell culture and borax exposure

HepG2 cell line was purchased from the China Center for Type Culture Collection (Wuhan University, Wuhan, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) containing 10% fetal bovine serum (FBS; cat. no. 10099-141; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 5% $CO_2$ humidified incubator at 37 °C. Cells were cultured for 2–3 days, the original medium was discarded and new medium containing borax (Tianjin Bodi Chemical Co. Ltd., Tianjin, China) solution at concentrations of 0 (Control) and 4 mM added to the culture plate. Cells were cultured for either 2 or 24 h. After 2 or 24 h borax-exposure, cells were washed with PBS to remove borax and then utilized for RNA extraction.

# Exiqon Agilent Human miRNA

Agilent Human miRNA Array (Exiqon A/S, Agilent, USA) contains > 2549 capture probes, covering all human microRNAs annotated in the miRBase 21.0 (http://www. mirbase.org/). The Exiqon gene-chips are designed using Agilent's unique microRNAs detection technology, which are able to detect specific mature microRNAs and distinguish highly homologous microRNAs.

# RNA extraction and labeling

After 2 h or 24 h borax-exposure, total RNA was extracted from cells and purified using miRNeasy Mini Kit (Cat 217004, QIAGEN, GmbH, Germany), following the manufacturer’s instructions and checked for a RIN number to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). MiRNA molecular in total RNA was labeled by miRNA Complete Labeling and Hyb Kit (Cat # 5190 – 0456, Agilent technologies, Santa Clara, CA, US) followed the manufacturer’s instructions, labeling section.

# Array hybridization

Each slide was hybridized with 100 ng Cy3-labeled RNA using miRNA Complete Labeling and Hyb Kit (Cat # 5190 – 0456, Agilent technologies, Santa Clara, CA, US) in hybridization Oven (Cat # G2545A, Agilent technologies, Santa Clara, CA, US) at 55 °C, 20 rpm for 20 hours according to the manufacturer’s instructions, hybridization section. After hybridization, slides were washed in staining dishes (Cat # 121, Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Cat #5188–5327, Agilent technologies, Santa Clara, CA, US). Slides were scanned by Agilent Microarray Scanner (Cat # G2565CA, Agilent technologies, Santa Clara, CA, US) and Feature Extraction software 10.7 (Agilent technologies, Santa Clara, CA, US) with default settings. Raw data were normalized by Quantile algorithm, Gene Spring Software 12.6 (Agilent technologies, Santa Clara, CA, US).

# RT-qPCR

The single-strand cDNA synthesized using miRNA cDNA Kit (Clontech, USA). The expressions of miRNAs in HepG2 cells were determined by qPCR using the Real-Time PCR Assay Kit (Takara Bio Inc., Otsu, Japan) according to manufacturer's instructions. The upstream sequences of miRNA specific primers were designed from miRbase as presented in Table 1. The downstream primers were obtained from the Real-Time PCR Assay Kit. Primer sequences targeting specific miRNAs were designed using Primer Express 3.0®software (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The cycling conditions were as follows: 95˚C for 15 min, 45 cycles of 95˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec. miRNA expression levels were normalized against U6 (an internal control) and determined using the $2^{-\Delta\Delta Cq}$ method.

Table 1
Specific Primers Used for the qPCR in This Study

| miRNA name | Primer (5′-3′) |
|---|---|
| hsa-miR-451a | CGCGAAACCGTTACCATTACTGAGTT |
| hsa-miR-4749-3p | TGCGGGGACAGGCCAGG |
| hsa-miR-6763-3p | CTGGGGAGTGGCTGGGG |
| hsa-miR-7845-5p | AAGGGACAGGGAGGGTCG |
| hsa-miR-125b-2-3p | TCCCTGAGACCCTAACTTGT |
| hsa-miR-1275 | TGGGGGAGAGGCTGTC |
| hsa-miR-1307-5p | CGTGGCGTCGGTCGT |
| hsa-miR-132-3p | ACCGTGGCTTTCGATTG |
| hsa-miR-193b-5p | GGTTTTGAGGGCGAGAT |
| hsa-miR-195-5p | GCAGCACAGAAATATTGG |
| hsa-miR-215-5p | TGACCTATGAATTGACAGA |
| hsa-miR-3138 | TGTGGACAGTGAGGTAGAGG |
| hsa-miR-34a-3p | GGCAGTGTCTTAGCTGG |
| hsa-miR-34b-5p | GGCAGTGTCATTAGCTG |
| hsa-miR-422a | ACTGGACTTAGGGTCAG |
| hsa-miR-431-3p | CTTGCAGGCCGTCATG |
| hsa-miR-450a-5p | TTTTGCGATGTGTTCCTAA |
| hsa-miR-4651 | CGGGGTGGGTGAGGTCGG |
| hsa-miR-550a-3-5p | AGTGCCTGAGGGAGTAAG |
| hsa-miR-5701 | TTATTGTCACGTTCTGATT |
| hsa-miR-629-3p | GGGTTTACGTTGGGAGA |
| U6 | GCTTCGGCAGCACATATACTAA |
| | GCTTCACGAATTTGCGTGTCAT |

# Data analysis, target prediction, GO, and KEGG pathway analysis

Following normalization, miRNA expression levels were identified via volcano plot and heatmap. The differential expressed miRNAs between borax treated and control were compared using the DEGseq R package (Novogene, Beiing, China). P-value < 0.01and fold change > 2 was set as the threshold for significantly differential expression. The miRanda for humans was used to predicted the target gene of miRNA (http://www.microrna.org/microrna/home.do). GO enrichment analysis was used for the target gene candidates of differentially expressed miRNAs base on public data from bioinformatics resources (http://www.geneontology.org/). KEGG is a database resource for predicting high-level functions at a molecular level (http://www. genome.jp/kegg/).

# Statistical analysis

A computational analysis of microarray data was performed using GeneSpring v12.0 (Agilent Technologies, Inc., Santa Clara, CA, USA). Data are expressed as the mean ± standard error of the mean. Differences in the mean values were evaluated by Student's t-test (two means comparison). Differentially expressed miRNA were filtered through statistical estimation of fold-changes from replicated samples (fold change ≥ 2.0) using a P-value threshold ($P < 0.05$).

## Results

# MiRNA expression profile in borax treated HepG2 cells

Compared with control group, 4 miRNAs were significantly altered (upregulated) in HepG2 cells in the 2-h borax treatment group (fold change ≥ 2.0; $P < 0.05$). Furthermore, Seventeen differentially expressed miRNAs (14 up-regulated and 3 down-regulated) were detected in 24-h borax treatment group, compared with the control group (fold change ≥ 2.0; $P < 0.05$) (Table 2). The volcano plot analysis presented that the expression levels of 4 and 17 miRNA were significantly elevated and decreased, respectively, in treatment group compared to control group (fold change ≥ 2.0; $P < 0.05$) (Fig. 1, red and blue dots). Meanwhile, differentially expressed genes were stratified by treatment duration and presented as heatmaps either in red (upregulation) or green (downregulation) (Fig. 2, fold change ≥ 2.0; $P < 0.05$).

Table 2
The Fold Change for Differentially Expressed miRNAs in HepG2 Cells Following Borax Treatment

| miRNA name | 2 h VS control Fold change p values | 24 h VS control Fold change p values | Regulation after borax treatment |
|---|---|---|---|
| hsa-miR-451a | 464.66 0.00811 | | Up |
| hsa-miR-4749-3p | 16.19 0.03465 | | Up |
| hsa-miR-6763-3p | 11.19 0.02040 | | Up |
| hsa-miR-7845-5p | 3.42 0.01098 | | Up |
| hsa-miR-125b-2-3p | | 29.82 | 0.00000 Up |
| hsa-miR-1275 | | 2.12 | 0.00288 Up |
| hsa-miR-1307-5p | | 11.21 | 0.00195 Up |
| hsa-miR-132-3p | | 2.27 | 0.03802 Up |
| hsa-miR-193b-5p | | 2.13 | 0.02021 Up |
| hsa-miR-195-5p | | 20.31 | 0.00012 Up |
| hsa-miR-215-5p | | 17.08 | 0.00208 Up |
| hsa-miR-3138 | | 3.13 | 0.00011 Up |
| hsa-miR-34a-3p | | 37.48 | 0.00000 Up |
| hsa-miR-34b-5p | | 2.99 | 0.01688 Up |
| hsa-miR-422a | | 48.62 | 0.00000 Up |
| hsa-miR-431-3p | | 0.04 | 0.01772 Down |
| hsa-miR-450a-5p | | 40.62 | 0.00040 Up |
| hsa-miR-4651 | | 0.03 | 0.00935 Down |
| hsa-miR-550a-3-5p | | 28.01 | 0.00016 Up |
| hsa-miR-5701 | | 21.07 | 0.00137 Up |
| hsa-miR-629-3p | | 0.05 | 0.03914 Down |

# Differentially expressed miRNAs validated by qPCR

To validate miRNA that were screened by microarray, the results illustrates that 4 miRNAs (miR-451a, miR-4749-3p, miR-6763-3p and miR-7845-5p) were up-regulated in 2-h borax treatment group (Fig. 3A), while

14 miRNAs (miR-125b-2-3p, miR-1275, miR-1307-5p, miR-132-3p, miR-193b-5p, miR-195-5p, miR-215-5p, miR-3138, miR-34a-3p, miR-34b-5p, miR-422a, miR-450a-5p, miR-550a-3-5p, miR-5701) were up-regulated, but 3 miRNAs (hsa-miR-431-3p, hsa-miR-4651, hsa-miR-629-3p) were down-regulated in 24-h borax exposed cells (Fig. 3B).

# GO and KEGG pathway enrichment analysis

To study the biological dysfunctionality associated with the altered target genes of expressed miRNAs induced by borax treatment, public data from bioinformatics resources (http://www.geneontology.org/) were utilized for GO enrichment analysis. The significantly enriched GO terms were also arranged correspondingly based on the biological processes, cellular components, and molecular functions of each gene, (Fig. 4). KEGG pathway enrichment analysis illustrated that the target genes of differentially miRNAs were primarily involved in MAPK signaling pathway, TGF-beta signaling pathway, NF-kappa B signaling pathway, cAMP signaling pathway, cAMP signaling pathway, etc; in 2-h borax treatment group, while Ras signaling pathway, FoxO signaling pathway, Cellular senescence, and Neurotrophin signaling pathway, etc; involved the 24-h treatment group. (Fig. 5).

## Discussion

In the previous researches, borax ore and pure refined borax were all cytotoxic in cultured C3H/lOT1/2 cells, V79 Chinese hamster cells, human HeLa cells, and human foreskin fibroblasts at high (mg/ml) concentrations [23], which was similar to the range of toxic concentrations in animal feeding experiments [24]. It also showed that borax had toxicity, including acute toxicity, neurotoxicity, reproductive, and developmental toxicity. However, borax is a low toxicity, which is used in manufacturing, pesticides, fertilizers, and pharmaceuticals [25]. Currently, more and more research on the therapeutic effect of the borax, mainly including anti-tumor effect and affecting the tumor growth, development and metastasis [26, 27]. A great number of studies have concentrated on the application of borax for tumor prevention and illustrated a strong inverse correlation between borax and various types of cancer, including lung cancer, cervical cancer, prostate cancer and hepatocellular carcinoma (HCC) [2, 8, 9]. But, the underlying mechanisms of anti-tumor effect remain unidentified and borax administered altered the expression of miRNAs in HepG2 cells has been reported less.

In the present study, microarray analysis showed that the expression levels of 4 miRNAs were upregulated in HepG2 cells in the 2-h treatment group. such as, miR-21-5p may be useful as a predictor of recurrence in young gastric cancer and breast cancer patients. This miRNA was combined with clinicopathological factors, which would allow patient prognoses to be more accurately predicted. 24-h treatment with borax upregulated the expression of several miRNAs, including miR-125b-2-3p, which may affect the G2/M phase of the hepatocellular carcinoma cell cycle through the regulation of its target genes and provide early diagnosis and novel treatment of hepatocellular carcinoma [28]. miR-422a, acts as a tumor suppressor, inhibited cell invasion, proliferation, and migration by targeting PI3K/Akt signal pathway [29].

miR-34a-3p is an independent biomarker of recurrence and a factor to improve predictive value of cancer and treatment [30]. Furthermore, to examine the expression of altered miRNAs, we applied volcano plot and heatmaps analyses to uncover the presence of differentially expressed miRNAs in the borax treatment group.

To precision select the target genes affected by miRNAs, GO enrichment analyses showed that the processes significantly altered by borax involved biological regulation, cell junction, metabolism, and protein binding. Furthermore, KEGG pathway enrichment analysis showed that the target genes of differentially expressed miRNAs in HepG2 cells following borax treatment for 2 h major participated in MAPK signaling pathway, TGF-beta signaling pathway, NF-kappa B signaling pathway, cAMP signaling pathway, etc; MAPK signaling pathway, a mitogen-activated protein kinase pathway, which is generally involved in cell proliferation, apoptosis and differentiation [31]; TGF-β signaling (TGF-beta signaling) is often activated and not attenuated during the malignancy of hepatocellular carcinoma cells, so TGF-β could be a therapeutic target for the treatment of liver cancer [32]. NF-kappa B, an ubiquitous transcription factor in mammals, which enters into cellular nucleus and enhances gene expression by binding to κB site within promoter. NF-κB signaling is associated with cell inflammation, growth and differentiation and is aberrant activated frequently in numerous kinds of cancer including liver cancer and it has also been served as a target for treatment. [33]; cAMP signaling pathway, 3'-5'-cyclic adenosine monophosphate (cAMP), as a second messenger, is the key role in mediating many cellular responses. Furthermore, the study found that the cancer cell migration and survival was inhibited by application of cAMP to cancer cell [34].

Ras signaling pathway, FoxO signaling pathway, Cellular senescence, and Neurotrophin signaling pathway, etc; involved the 24-h treatment group. Ras signaling pathway is activated in the majority of advanced HCC, and if it is inhibited that could effectively suppress the proliferation, migration, and invasion of HCC [35]. FoxO proteins, including FoxO1a and FoxO3a, are involved in multiple fundamental cellular activities and acting in transcriptional activities related to cell stress response, proliferation, and apoptosis, the tumor cells growth arrest and apoptosis are induced through activation of FoxO transcription factors [36]. Cellular senescence is a cell fate that triggered by oxidative stress, oncogene activation, and DNA damage and is an important tumor-suppression mechanism [37]. Neurotrophin signaling pathway, exerts a range of effects in the control of cell migration and proliferation in non-neuronal cells, including in cancer. Furthermore, neurotrophin binding to p75NTR that activates the c- JNK signaling cascade, which results in activation of p53 and expression of pro-apoptotic genes such as Bcl-2. Neurotrophin and their receptors have been investigated in human cancers and were over-expressed in ovarian, breast, liver, and pancreatic malignancies [38]. In present study, target genes of differentially expressed miRNAs induced by borax also involved alterations in those pathways, indicating that borax-mediated antitumour effect might involve changes in these pathways, specially in 24-h borax exposed HepG2 cells. Thus, the study revealed that miRNAs might play an important role in development of liver cancer. The possibility exists that borax induced antitumor effects also involves differential expression of miRNAs based on our results.

# Conclusions

Expression levels of miRNAs were significantly increased in the 2-h borax treatment group, while miRNAs expression levels (14 up-regulated and 3 down-regulated) were obviously changed in 24-h borax treatment group. Furthermore, antitumor effect was obvious base on GO and KEGG pathway enrichment analysis in HepG2 cells exposed to borax at 24-h. To the best of our knowledge, this is the first investigate to report that miRNAs alterations were associated with antitumor effect induced by borax. The significance and functions of the differentially expressed miRNAs identified in the current study require further investigation.

# Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and materials

Not applicable

### Competing interests

The author declares that he has no competing interests.


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### Authors' contributions

You-Shun Zhang and Zhi-Gang Tang conceptualised the study and formulated the study design, Jiao Zhou, Li-Hua Yang, and Qin-Hua Chen collected the data, Wei Wang and Lin Ye, and Li Chao Yao analysed the data, Lun Wu and Wen-Bo Zhou interpreted the results and drafted the manuscript. The author read and approved the final manuscript.

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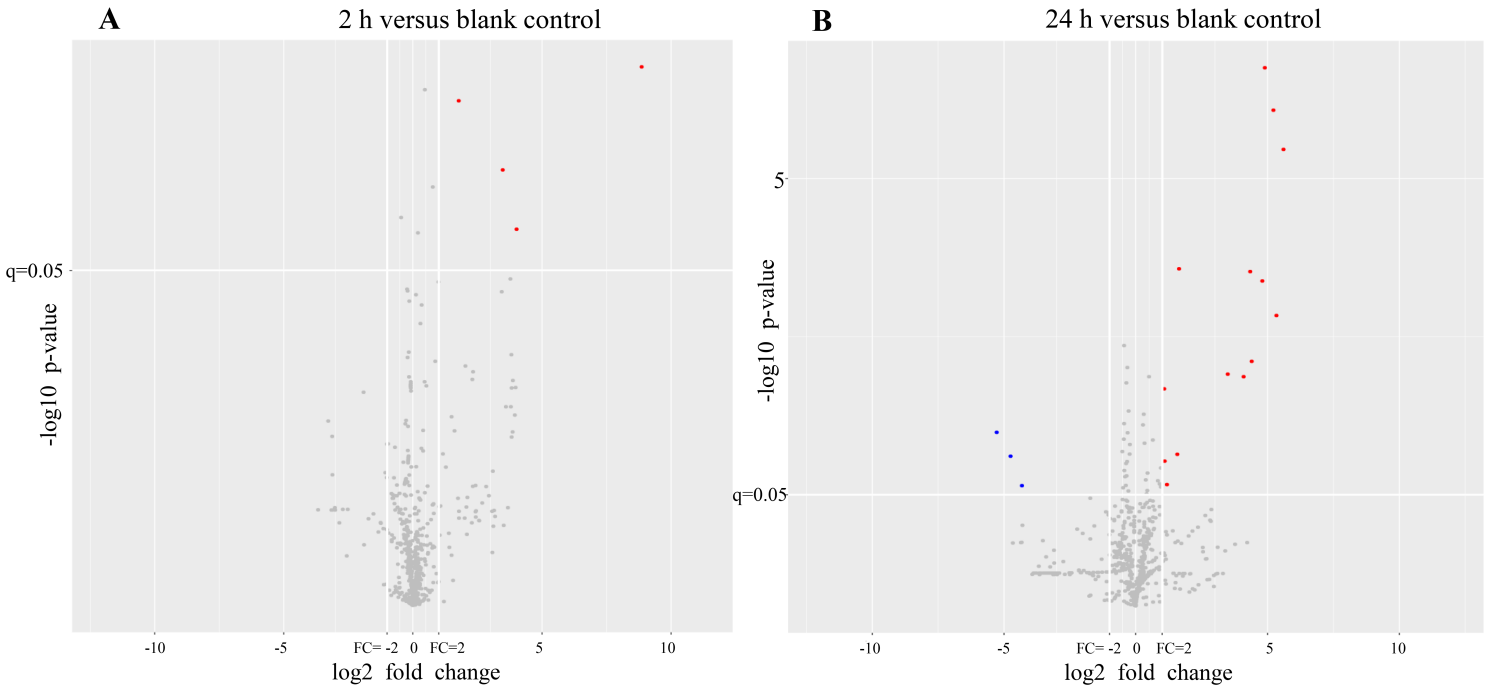
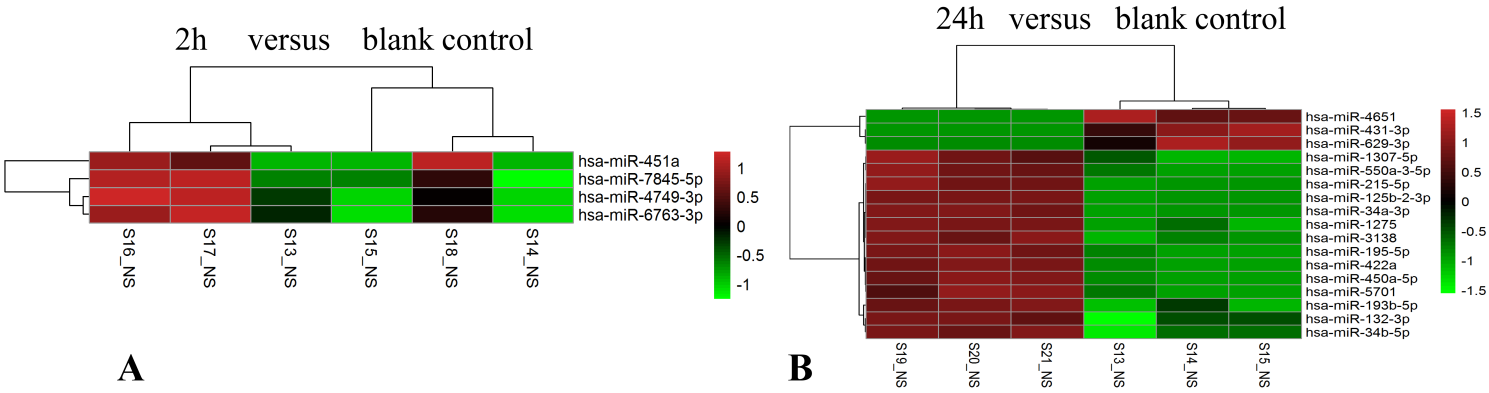
# Figures



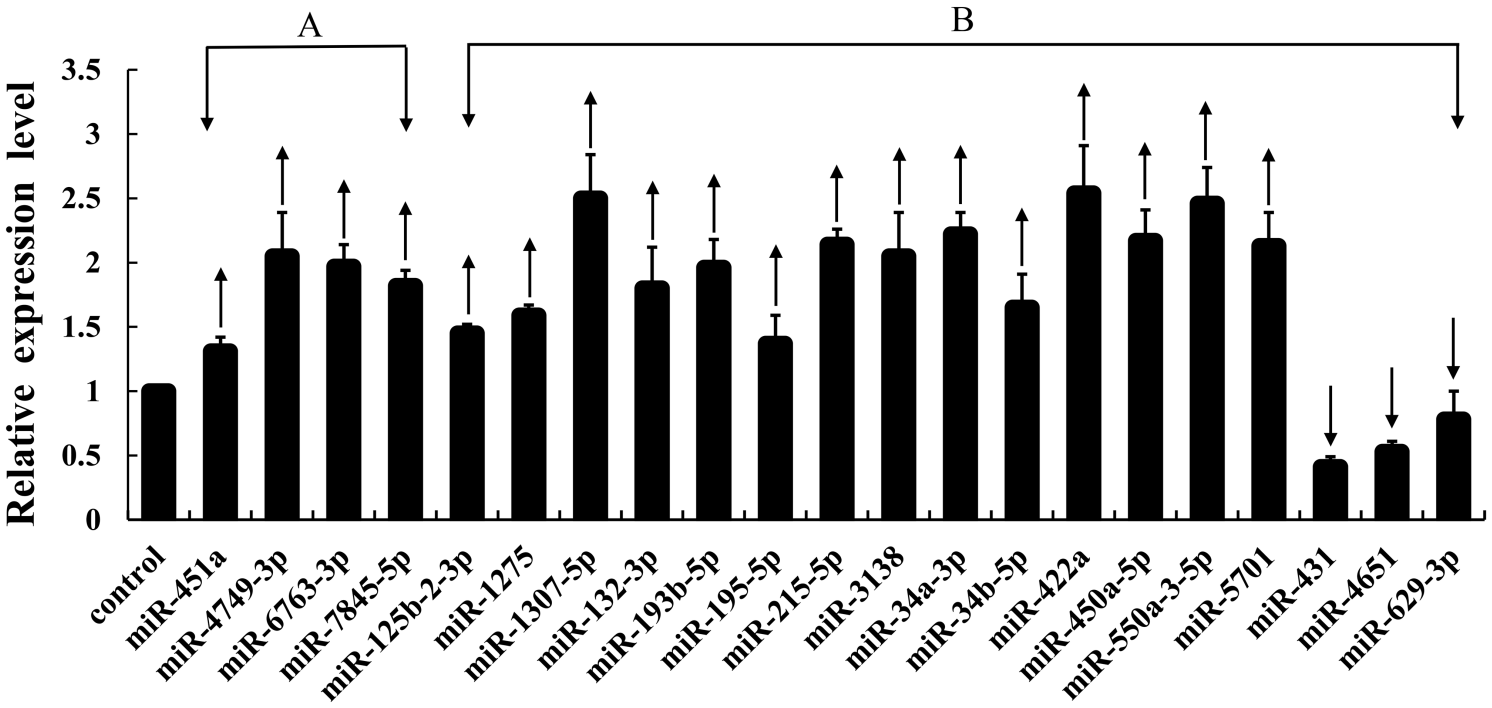

## Figure 1

miRNAs clustering analysis by Human miRNA Array were plotted in the volcano plot between borax treatment group and control group. (A) 4 miRNAs were upregulated in the 2-h borax treatment group. (B) 14 miRNAs were upregulated and 3 were downregulated in the 24-h borax treatment group. Blank control (untreated cells)



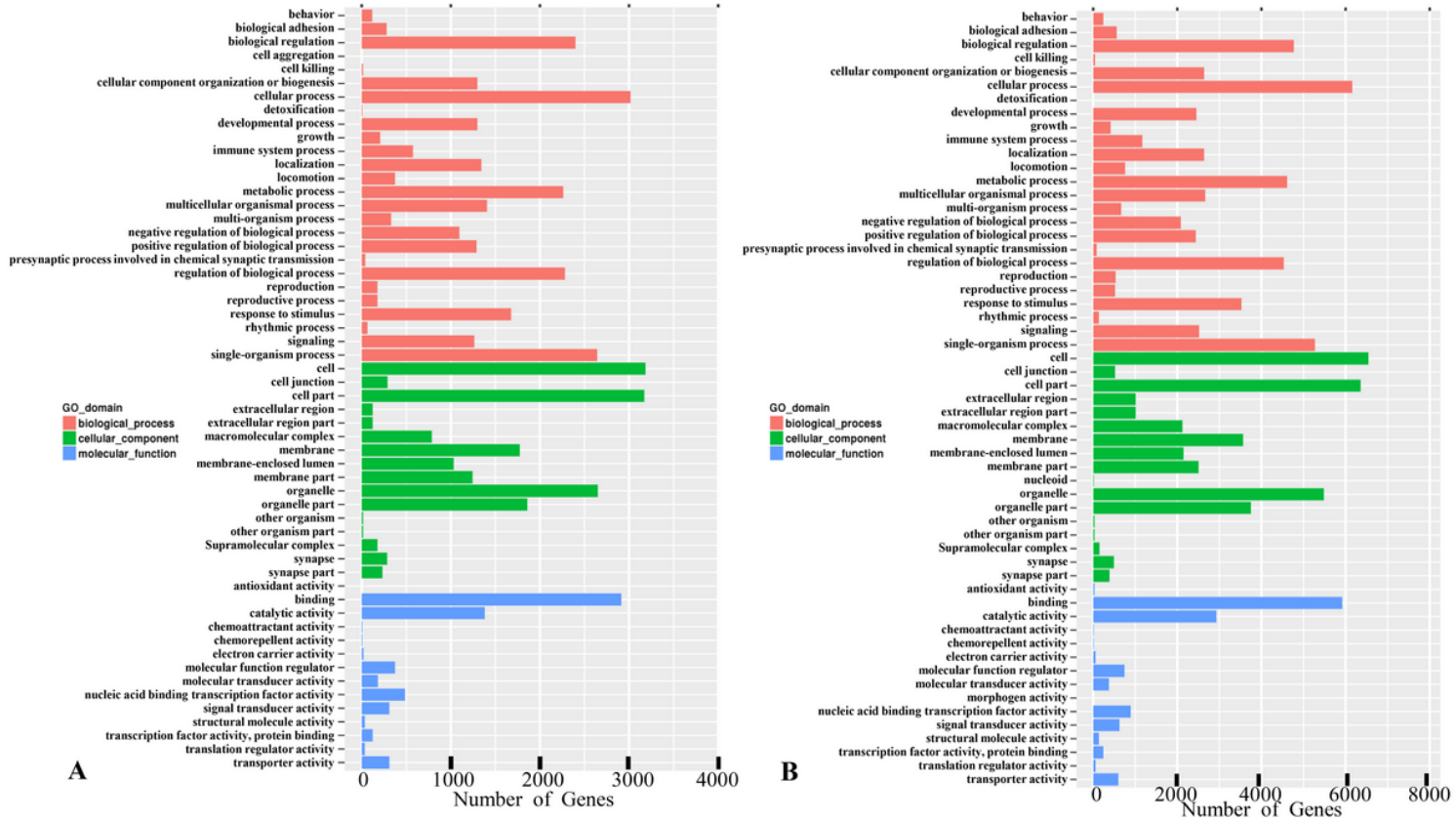

## Figure 2

Heatmaps of differentially expressed miRNAs due to borax treatments in HepG2 cells for 2 h (A) and 24 h (B) (>2-fold change, P<0.05). Red indicates upregulation whereas green indicates downregulation of miRNAs expression relative to control (untreated cells).



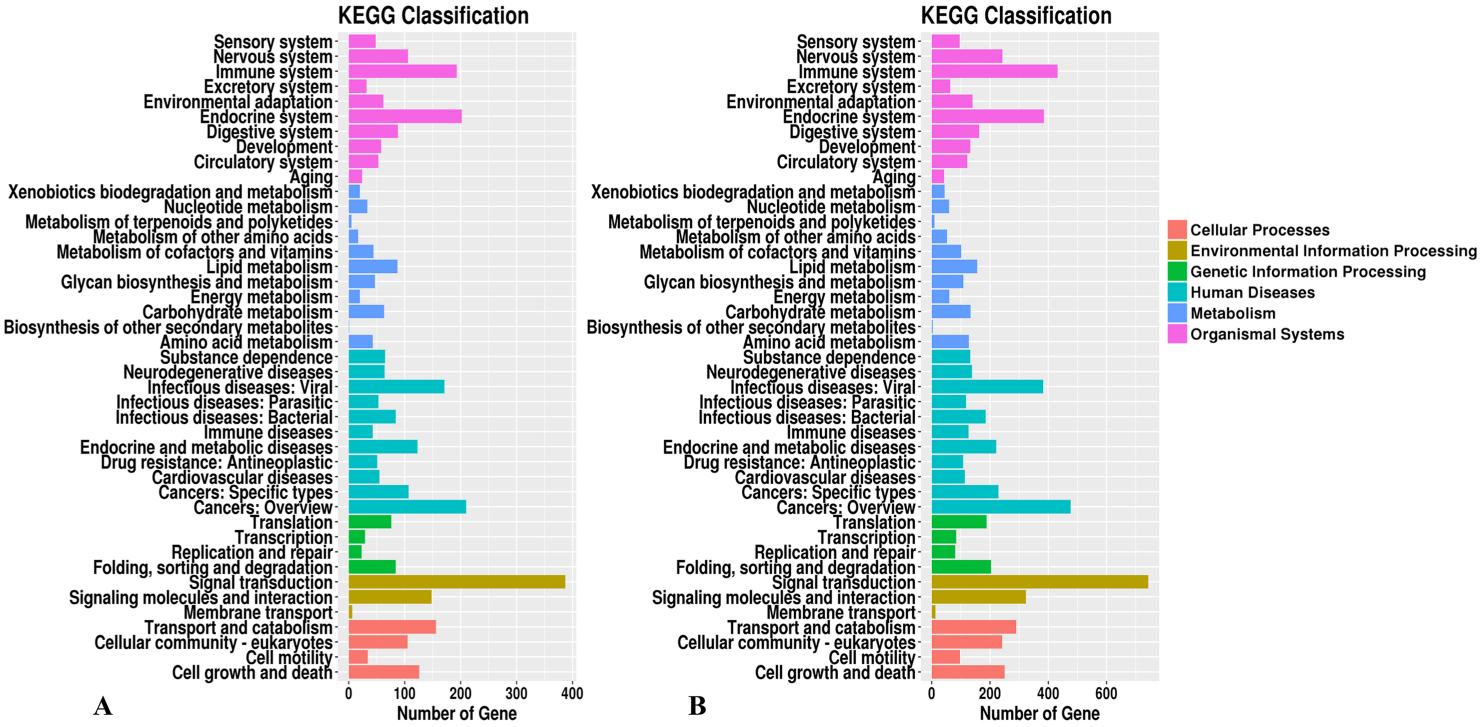

## Figure 3

Expression levels of miRNAs examined by qPCR. (A) Expression levels of miRNAs in 4 mM borax treatment groups compared with the control group for 2-h. (B) Expression levels of miRNAs in 4 mM borax treatment groups compared with the control group for 24 h. arrow " ↓ ": expression level of miRNA is down-regulated, arrow " ↑ ": expression level of miRNA is up-regulated.



## Figure 4

Enriched GO terms according to biological processes, molecular functions, and cellular components for borax treatment and control. GO terms are ordered by enrichment score with the highest enriched term at the bottom of the list. Differentially expressed transcripts involved in the term (count) P<0.05 with and fold change >2.0 were included. GO, gene ontology. A, 2-h vs control; B, 24-h vs control.




## Figure 5

Representative KEGG pathway enrichment analysis for borax treatment and control. A, 2-h vs control; B, 24-h vs control.