MicroRNA Expression Profiling Involved in Borax-Induced Anti-Tumor Effect Using Gene-Chip Analysis

Ying Wei (57729968@qq.com)
Dongfeng General Hospital
Wenbo Zhou
Dongfeng General Hospital
Jiao Zhou
Dongfeng General Hospital
Jinjin Wang
Dongfeng General Hospital
Xuefei Huang
Dongfeng General Hospital
Jun Chen
Dongfeng General Hospital
Qinhua Chen
Dongfeng General Hospital
Huizhen Li
Dongfeng General Hospital
Fen Sheng
Dongfeng General Hospital
Lun Wu
Dongfeng General Hospital https://orcid.org/0000-0001-9737-6730

Research Article

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Abstract

Background

Borax has been shown to have potential therapeutic benefits. However, the mechanisms underlying borax-induced anti-tumor effect remain to be further elucidated. MicroRNAs (miRNAs) may play key roles in cellular processes such as tumor progression and cell apoptosis.

Objective

The present study aimed to investigate whether miRNAs were involved in borax-mediated anti-tumor effect using gene-chip analysis.

Methods

Total RNA was extracted and purified from HepG2 cells treated with 4 mM borax for 2 or 24 h. The samples underwent microarray analysis using a human miRNA Array. Differentially expressed miRNAs were analysed by volcano plot and heatmap, and were validated using quantitative PCR. PPI network was then established, and hub genes were identified via Cytoscape software.

Results

Exposure to borax for 2 or 24 h significantly altered the expression level of miRNAs in HepG2 cells, and 4 or 14 miRNAs were upregulated, respectively, while 3 were downregulated compared with the findings in the control group (≥2 fold-change, $P<0.05$). Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses revealed that the target genes of the differentially expressed miRNAs in HepG2 cells predominantly participated in the MAPK, TGF-β and NF-κB signaling pathways in the 2-h borax treatment group, while they were involved in the Ras signaling pathway, forkhead box O signaling pathway and cellular senescence in the 24-h treatment group. Construction and analysis of PPI network showed that NACC2, CACNB1 and FZD6, CDK6, BCL-2, IGF1R, BTG2, AGO2, DLGAP3 were recognized as hub genes with the highest connectivity degrees in the 2-h or 24-h, respectively.

Conclusion

The results indicate that the borax-induced antitumor effect may be associated with alterations in miRNAs. Furthermore, we established a potential tumor-related miRNA-mRNA regulatory network, which explores a comprehensive understanding of the molecular mechanisms and provides novel therapeutic targets for liver cancer.

Introduction

Borax, as an important borate compound, is a low-toxicity mineral used for insecticidal, sterilizing and weeding purposes. It is widely employed as a food additive and fertilizer, as well as in the manufacturing and pharmaceutical industries [1, 2]. Previous studies have shown that the anti-inflammatory properties of borax via the suppression of interleukin-8, indicating that borax has therapeutic potential [3, 4]. Furthermore, previous studies suggested that different concentrations of borax affected lymphocyte proliferation, cell survival and cell growth [5–7]. In recent years, extensive research has focused on the application of borax for the prevention of various types of cancer, including liver, prostate, cervical and lung cancer [8–11]. Our previous study revealed that HepG2 cell proliferation was inhibited following 24-h borax treatment, and demonstrated borax induced apoptosis in a concentration-dependent manner [11]. However, the molecular mechanisms underlying the borax mediated antitumor effects and apoptosis of tumor cells remain to be elucidated.

MicroRNAs (miRNAs or miRs) are single-stranded, non-coding, highly conserved small RNAs that are 20-24 nucleotides in length. Approximately 1,000 miRNA sequences are predicted in humans [12]. Each miRNA regulates the expression of its target genes by binding to multiple mRNA, which usually occurs at the posttranscriptional level by binding to complementary sequences located in the 3' untranslated region binding to target mRNA transcripts [13]. Since each miRNA can regulate hundreds of target genes by interfering with the translation of mRNA or by acting directly to degrade mRNA, it has been hypothesized that 20,000-25,000 genes may be regulated by specific miRNAs [14]. Previous studies found a close association between miRNA and liver cancer, with the expression levels of miR-21 and miR-221 being upregulated, while other miRNAs, including miR-145 and miR-223 were decreased in hepatocellular carcinoma (HCC) [15, 16]. Furthermore, previous studies showed that abnormal expression of miRNA in HCC affects several clinicopathological characteristics, such as tumor size, vascular invasion and node metastasis [17, 18]. These findings indicate that miRNA modulation may play an important role in the development and progression of HCC. Recently, numerous studies have demonstrated that environmental chemical minerals affect the regulation of miRNA expression [19] and thus, environmental borax may exert antitumor effects via alteration of the expression levels of miRNAs. Therefore, it is important to a analyze the expression profiles of miRNAs and to illustrate their roles in the regulation of gene expression in human diseases.

Gene-chips are also called biological chips with high-throughput, automated platforms and miniaturized properties based on the theory of hybridization [20]. Microarray technology is applied to identify the differentially expressed genes of a whole genome in a single experiment, and is used to evaluate and characterize of gene expression profiles [21, 22]. The present study explored miRNA expression alterations directly caused by treatments with borax in HepG2 cells with high-throughput gene chips. The biological functions of those differentially expressed miRNAs were investigated by Gene Ontology (GO) and pathway analysis through assessment of the 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 the expression of critical miRNAs that are associated with borax antitumor effect.

Methods

Cell culture and borax exposure

The HepG2 cell line was purchased from Wuhan University. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum in a 5% CO_2 humidified incubator at 37°C. Cells were cultured for 2-3 days. Subsequently, the original medium was discarded, and new medium containing borax (Tianjin Bodi Chemical Co. Ltd., Tianjin, China) solution at concentrations of 0 (control) and 4mM was added to the culture plate. Cells were cultured for either 2 or 24 h. After borax exposure, the cells were washed with PBS to remove borax and then subjected to RNA extraction.

Exiqon Agilent Human miRNA array

Agilent Human miRNA Array (Exiqon A/S; Agilent Technologies, Inc.) contains >2,549 capture probes covering all human microRNAs annotated in the miRBase 21.0 (http://www. mirbase.org/). Exiqon gene chips are designed using Agilent's unique microRNAs detection technology, which allows the detection of specific mature miRNAs and the differentiation of highly homologous miRNAs.

RNA extraction and labeling

After 2 or 24 h borax exposure, total RNA was extracted from cells and purified using miRNeasy Mini Kit (Cat 217004, Qiagen, GmbH) following the manufacturer’s instructions. The RIN number was evaluated to assess RNA integration using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). The miRNAs present in the total RNA were labeled with miRNA Complete Labeling and Hybridization Kit (cat. no. 5190-0456; Agilent Technologies, Inc.) following the manufacturer’s instructions.

Array hybridization

Each slide was hybridized with 100 ng cyanine 3-labeled RNA using miRNA Complete Labeling and Hybridization Kit (cat. no. 5190-0456; Agilent Technologies, Inc.) in a hybridization oven (cat. no. G2545A; Agilent Technologies, Inc.) at 55°C, 20 rpm for 20 h according to the manufacturer’s instructions. After hybridization, the slides were washed in staining dishes (cat. no.121; Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (cat. no. 5188-5327; Agilent technologies, Santa Clara, CA, US). Slides were scanned with Agilent Microarray Scanner (cat. no. G2565CA; Agilent technologies, Inc.) using Feature Extraction software 10.7 (Agilent technologies, Inc.) with default settings. The raw data were normalized by the quantile algorithm using Gene Spring software 12.6 (Agilent Technologies, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR) analysis

Single-stranded cDNA was synthesized using miRNA cDNA Kit (Clontech Laboratories, Inc.). The expression levels of miRNAs in HepG2 cells were determined by qPCR using Real-Time PCR Assay Kit (Takara Bio Inc.) according to the manufacturer’s instructions. The upstream sequences of the miRNA

specific primers, which were designed using miRBase, are presented in Table I. 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. The thermocycling conditions were as follows: 95˚C for 15 min, followed by 45 cycles of 95˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec. The miRNA expression levels were normalized against U6 (which served as an internal control), and were determined using the $2^{-\Delta\Delta Cq}$ method.

Data analysis, target prediction, and GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses

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

Establishment and analysis of protein-protein interaction network

To better understand the relationship among these screened genes, the protein-protein interaction (PPI) network was established using the STRING database (Szklarczyk et al., 2017). The hub genes in the PPI network were identified according to degree using Cytoscape software (version 3.6.1).

Statistical analysis

miRNA data were analyzed with GeneSpring v12.0 (Agilent Technologies, Inc.). Data are expressed as the mean ± standard error of the mean. Differences in mean values were evaluated by Student's t-test (two means comparison). A threshold of fold-change ≥2.0 and P<0.05 was used to estimated differentially expressed miRNAs.

Results

MiRNA expression profile in borax-treated HepG2 cells

Compared with the findings in the 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), while 17 differentially expressed miRNAs (14 up-regulated and 3 down-regulated) were detected in 24-h borax treatment group (fold change ≥2.0, P<0.05) (Table II). Volcano plot analysis indicated that the expression levels of 4 and 17 miRNA were significantly elevated and decreased, respectively, in the treatment group compared with those of the control group (fold change ≥2.0, P<0.05; Fig. 1; red and blue dots). Differentially expressed

genes were stratified by treatment duration and presented as heatmaps either in red (upregulation) or green (downregulation) (fold change ≥2.0, P<0.05; Fig. 2).

Validation of differentially expressed miRNAs by qPCR

The results of validation of miRNAs that were screened by microarray indicated that 4 miRNAs (miR-451a, miR-4749-3p, miR-6763-3p and miR-7845-5p) were upregulated 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 upregulated and 3 miRNAs [Homo sapiens (hsa)-miR-431-3p, hsa-miR-4651 and hsa-miR-629-3p] were downregulated in 24-h borax exposed cells (Fig. 3B). Quantitative measurement of the expression level of each gene was obtained by independent experiments (n=3). qPCR demonstrated that the change tendency of these miRNAs were markedly upregulated or downregulated, which was consistent with the gene-chip results.

GO and KEGG pathway enrichment analysis

To study the biological dysfunctionality associated with the altered target genes of the expressed miRNAs induced by borax treatment, public data from bioinformatics resources (http://www.geneontology.org/) were utilized for GO enrichment analysis (Fig. 4). KEGG pathway enrichment analysis illustrated that the target genes of differentially miRNAs were primarily involved in the MAPK, TGFβ, NF-kB, cyclic AMP (cAMP) signaling pathways in the 2-h borax treatment group, while they were involved in the Ras, forkhead box O (FoxO) and neurotrophin signaling pathways, as well as cellular senescence, in the 24-h treatment group (Fig. 5 and Table III).

Prediction of downstream target genes of differentially expressed-miRNAs

It is well known that miRNAs exert their biological effect through directly targeting 3’ untranslated region of mRNA. Thus, we predicted the downstream target genes of candidate differentially expressed miRNAs (DE-miRNA) by miRNet database. For better visualization, DE-miRNA-target gene network was successively established and presented in Fig. 6A,C, respectively. These target genes were predicted for the upregulated or downregulated, which involved in the development of tumor or regulating the anti-tumor effect caused by borax. The target gene count for each DE-miRNA were also listed in Fig. 6B,D. Aim to access hub genes in the two PPI networks, these node pairs were input into Cytoscape software. For the target genes of DE-miRNAs, the hub genes were NACC2 and CACNB1 in the 2-h, the hub genes were FZD6, CDK6, BCL2, BTG2, AGO2, DLGAP3, and IGF1R in the 24-h. According to the predicted miRNA-mRNA pairs, the candidate miRNA-hub gene regulatory network associated with development of tumor were finally constructed as presented in Fig. 7.

Discussion

In previous researches, borax ore and pure refined borax were cytotoxic to cultured C3H/IOT1/2 cells, V79 Chinese hamster 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]. A previous study also showed that borax has a low toxicity, and is used in manufacturing, pesticides, fertilizers and pharmaceuticals [25]. Currently, there are studies on the therapeutic effect of borax [26, 27].

In the present study, microarray analysis demonstrated that the expression levels of 4 miRNAs were upregulated in HepG2 cells in the 2-h treatment group, such as miR-451a, which may be useful as a predictor of recurrence in patients with cancer. This miRNA was combined with clinicopathological factors, which would allow patient prognoses to be more accurately predicted. Treatment with borax for 24 h upregulated the expression of several miRNAs, including miR-125b-2-3p, which may affect the G_2/M phase of the cell cycle in HCC through the regulation of its target genes, and may have antitumor effects and serve as a novel treatment of HCC [28]. miR-422a acts as a tumor suppressor, and inhibited cell invasion, proliferation and migration by targeting PI3K/Akt signaling pathway. It has been hypothesized that borax exerts its antitumor effects via miR-422a [29]. miR-34a-3p is an independent biomarker of recurrence and a factor to improve cancer prognosis and treatment [30]. Furthermore, to examine the expression of altered miRNAs, the present study applied volcano plot and heatmap analyses to detect the presence of differentially expressed miRNAs in the borax treatment group. We hypothesize that the antitumor effect of borax was revealed through the abnormal expression of these miRNAs.

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 mainly participated in the MAPK, TGF-β, NF-κB and cAMP signaling pathways. The MAPK signaling pathway is generally involved in cell proliferation, apoptosis and differentiation [31]; TGF-β signaling is often activated and not attenuated during HCC progression; thus, TGF-β may be a therapeutic target for the treatment of liver cancer [32]. NF-κB is a ubiquitous transcription factor in mammals that enters the cell nucleus and enhances gene expression by binding to the κB site within the promoter. NF-κB signaling is associated with cell inflammation, proliferation and differentiation. It is often aberrantly activated in numerous types of cancer, including breast cancer, and it has served as a target for treatment [33]; In the cAMP signaling pathway, cAMP acts as a second messenger and plays a key role in mediating numerous cellular responses. Furthermore, a previous study found that cancer cell migration and survival were inhibited by application of cAMP to cancer cell [34].

The Ras, FoxO and neurotrophin signaling pathways, as well as cellular senescence, were involved the 24-h treatment group. The Ras signaling pathway is activated in the majority of advanced HCC cases, and its inhibition could effectively suppress the proliferation, migration and invasion of HCC cells [35]. FoxO proteins, including FoxO1a and FoxO3a, are involved in multiple fundamental cellular activities, and participate in transcriptional activities associated with cell stress response, proliferation and apoptosis. Cell cycle arrest and apoptosis of tumor cells are induced through activation of FoxO transcription factors

[36]. Cellular senescence is a cell fate triggered by oxidative stress, oncogene activation and DNA damage, and is an important tumor-suppression mechanism [37]. The neurotrophin signaling pathway exerts a range of effects in the control of cell migration and proliferation in non-neuronal cells, including cancer cells. Furthermore, binding of neurotrophin to neurotrophin receptor p75 activates the c-Jun N-terminal kinase 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 observed to be overexpressed in ovarian, breast, liver and pancreatic malignancies [38]. In the present study, the target genes of differentially expressed miRNAs induced by borax were also involved in alterations of those pathways, indicating that borax-mediated antitumour effect may involve changes in these pathways, particularly in 24-h borax exposed HepG2 cells.

Conclusions

In the present study, the expression levels of miRNAs were significantly increased in the 2-h borax treatment group, while the expression levels of 17 miRNAs (14 up-regulated and 3 down-regulated) were obviously changed in the 24-h borax treatment group. To the best of our knowledge, the present study is the first to investigate and report that miRNA alterations may be associated with the antitumor effects exerted by borax. However, the relevance and functions of the differentially expressed miRNAs identified in the current study require further investigation.

Abbreviations

RNA
Ribonucleic Acid
DNA
Deoxyribonucleic Acid
PCR
Polymerase Chain Reaction
MAPK
mitogen-activated protein kinase
TGF-β
transforming growth factor-β
HCC
hepatocellular carcinoma
GO
Gene Ontology
KEGG
Kyoto Encyclopedia of Genes and Genomes
PPI
protein protein interaction

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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Contributions

Lun Wu and Ying Wei designed the study. Jiao Zhou, Jinjin Wang, Xuefei Huang and Jun Chen involved in in cell culture and experimental handling.Wenbo Zhou, Qinhua Chen, Huizhen Li, Wei Wang, Lichao Yao, Zhigang Tang and Fen Sheng participated in data acquisition and analysis. Lun Wu and Ying Wei wrote the manuscript. All authors read and approved the final manuscript.

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Declared none.

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Tables

Table 1-3 are available in the Supplemental Files section.

Figures

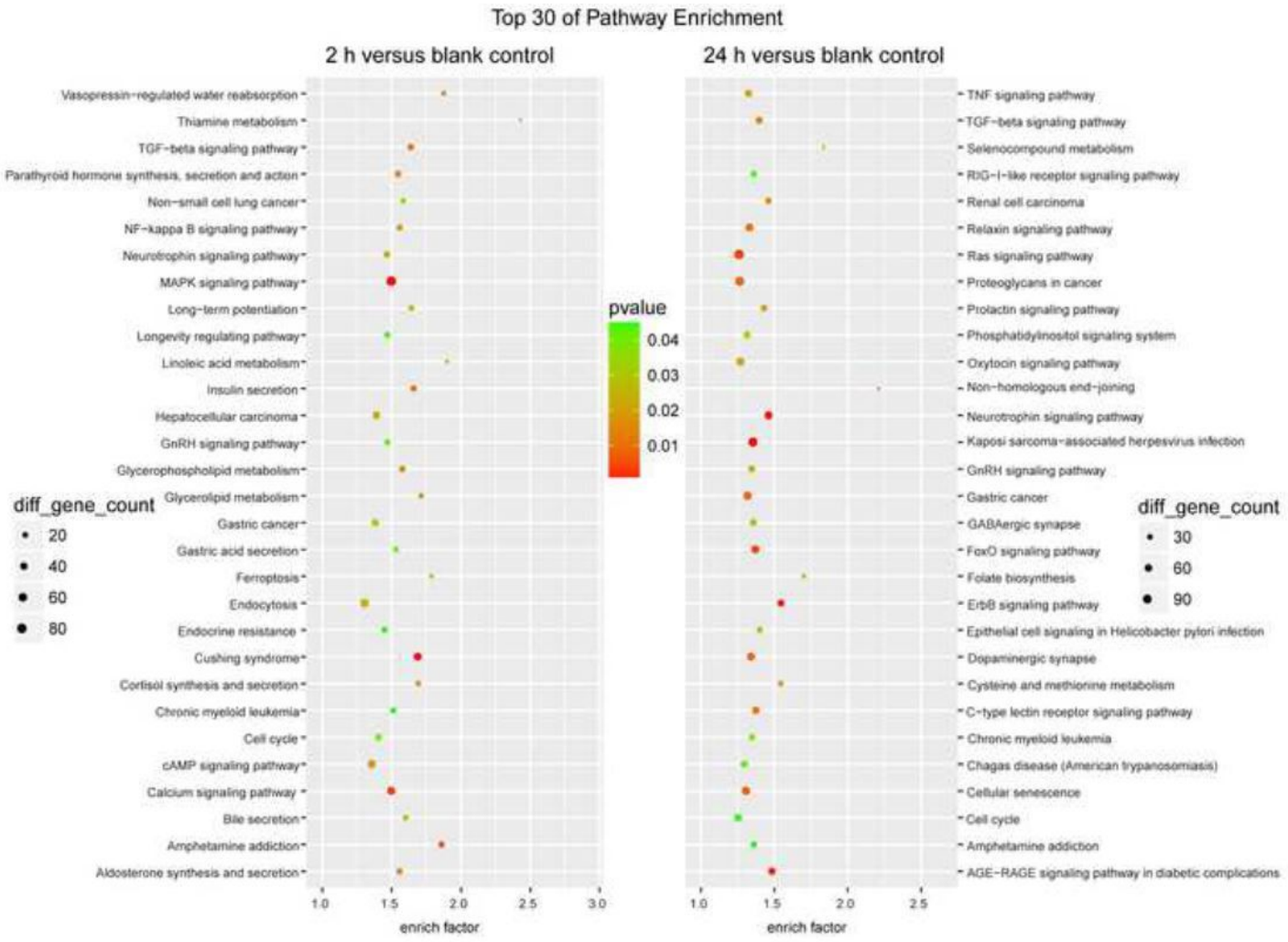
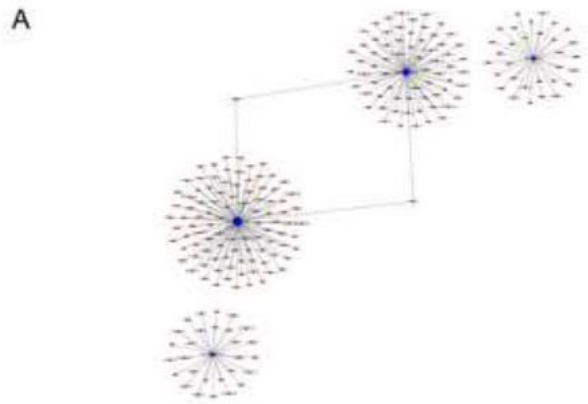


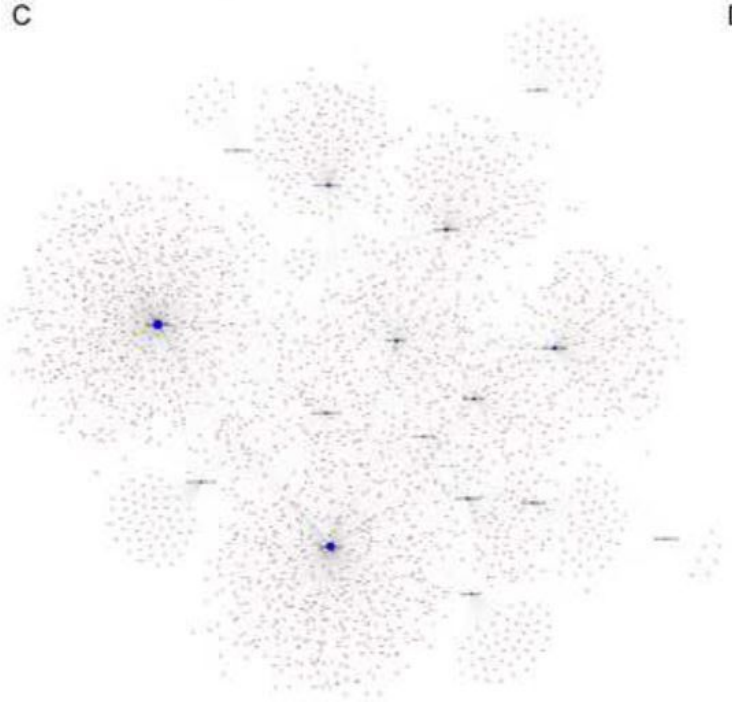
Figure 1

miRNAs included in the clustering analysis by Human miRNA Array were plotted in a volcano plot between the borax treatment group and the control group. (A) A total of 4 miRNAs were upregulated in the 2-h borax treatment group. (B) In total, 14 miRNAs were upregulated and 3 were downregulated in the 24-h borax treatment group. Untreated cells served as the blank control. miRNA, microRNA.



B

miRNA ID	Target gene count
hsa-miR-7845-5p	98
hsa-miR-4749-3p	68
hsa-miR-451a	31
hsa-miR-6763-3p	28



D

miRNA ID	Target gene count
hsa-miR-215-5p	755
hsa-miR-195-5p	640
hsa-miR-132-3p	255
hsa-miR-193b-5p	215
hsa-miR-629-3p	197
hsa-miR-4651	192
hsa-miR-1275	161
hsa-miR-34b-5p	123
hsa-miR-125b-2-3p	112
hsa-miR-550a-3-5p	91
hsa-miR-34a-3p	81
hsa-miR-5701	74
hsa-miR-3138	47
hsa-miR-422a	45
hsa-miR-450a-5p	17
hsa-miR-431-3p	10

Figure 2

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

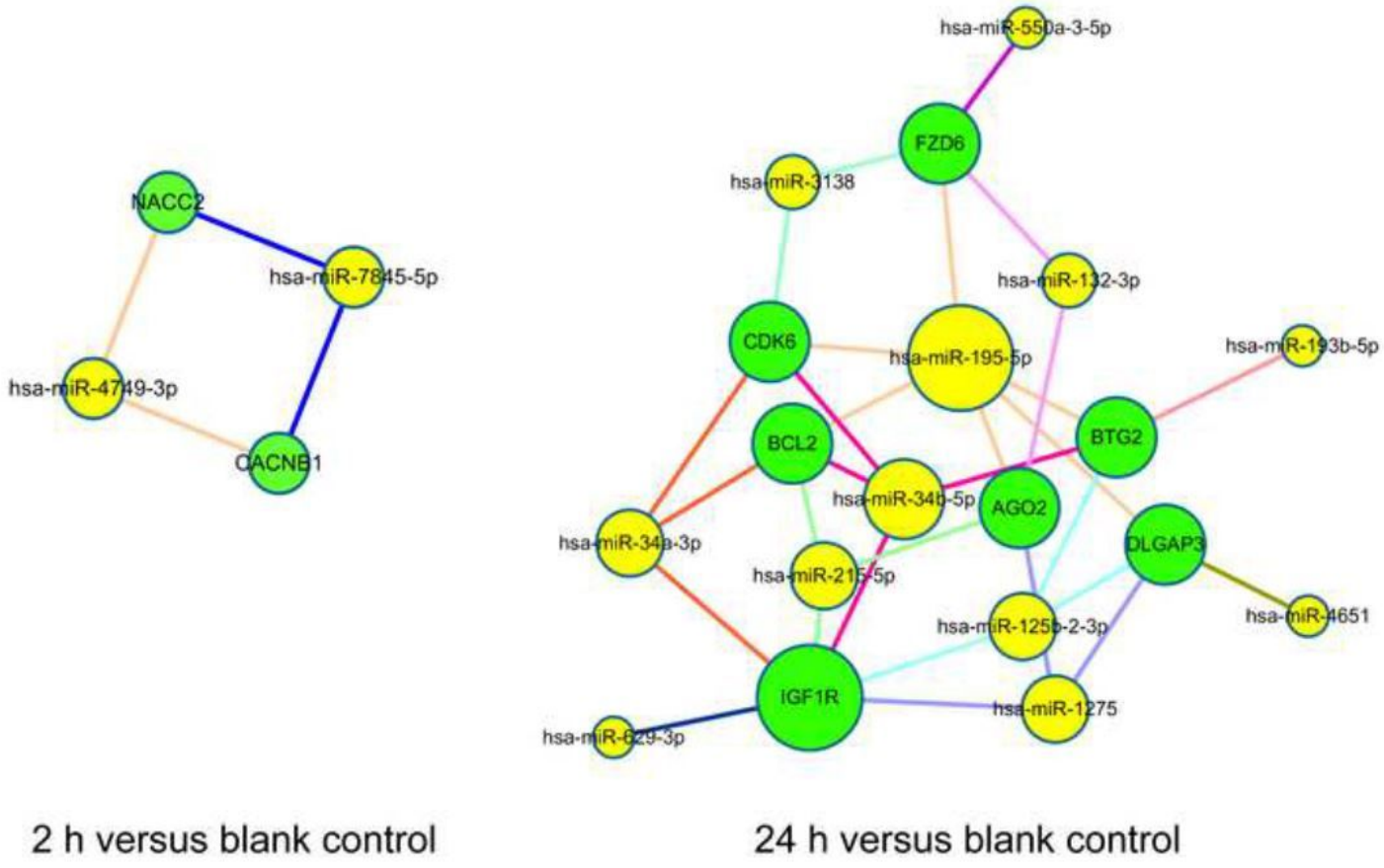


Figure 3

Expression levels of miRNAs examined by quantitative PCR. (A) Expression levels of miRNAs in the 4 mM borax treatment groups compared with those of the control group treated for 2 h. (B) Expression levels of miRNAs in the 4 mM borax treatment groups compared with those of the control group treated for 24 h. qPCR analysis demonstrated that the change tendency of the expression of miRNAs was consistent with the microarray data. ↓, downregulated expression; ↑, upregulated expression. miRNA, microRNA.

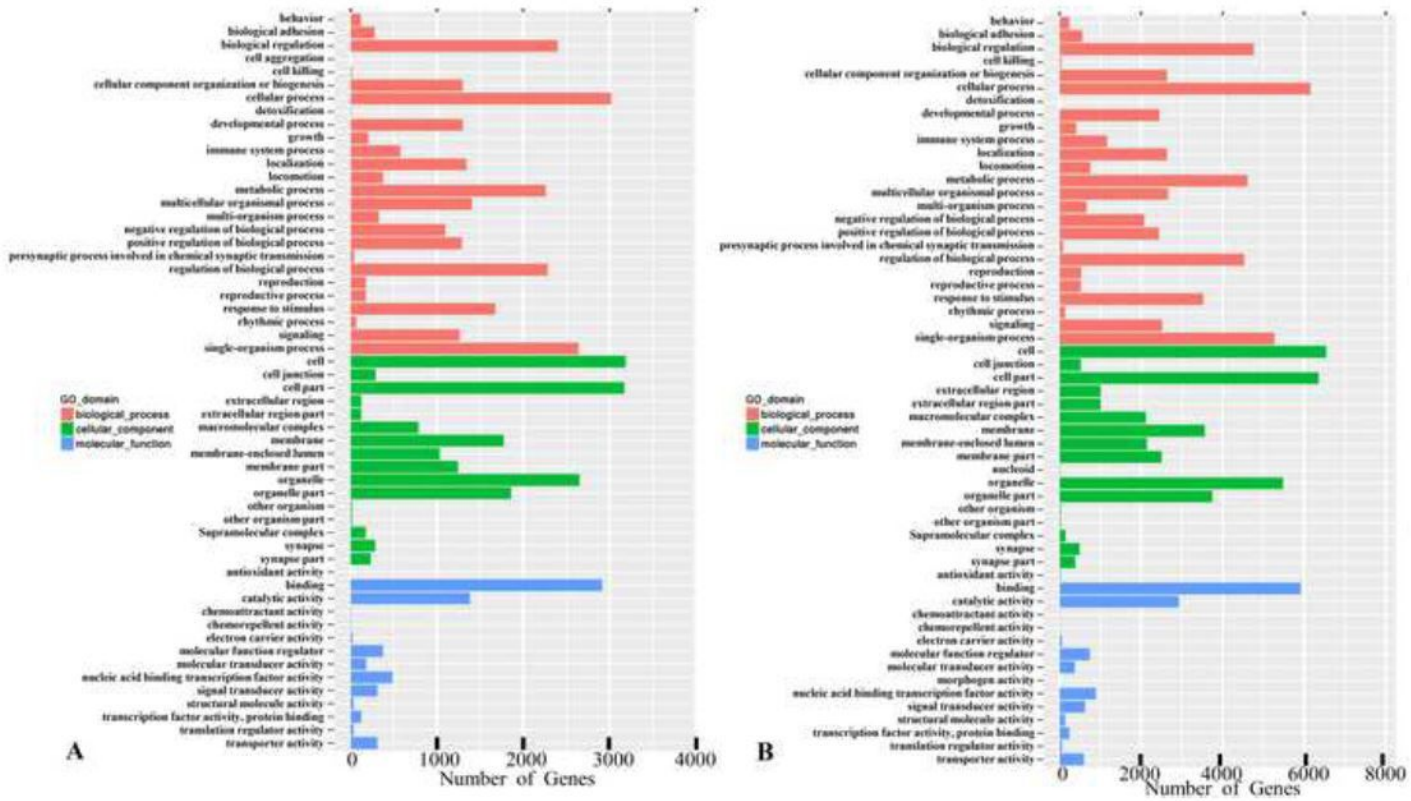


Figure 4

Enriched GO terms according to biological processes, molecular functions and cellular components for borax treatment and control groups. 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) with P<0.05 and fold- change >2.0 were included. A, 2 h vs. control; B, 24 h vs. control. GO, Gene Ontology.

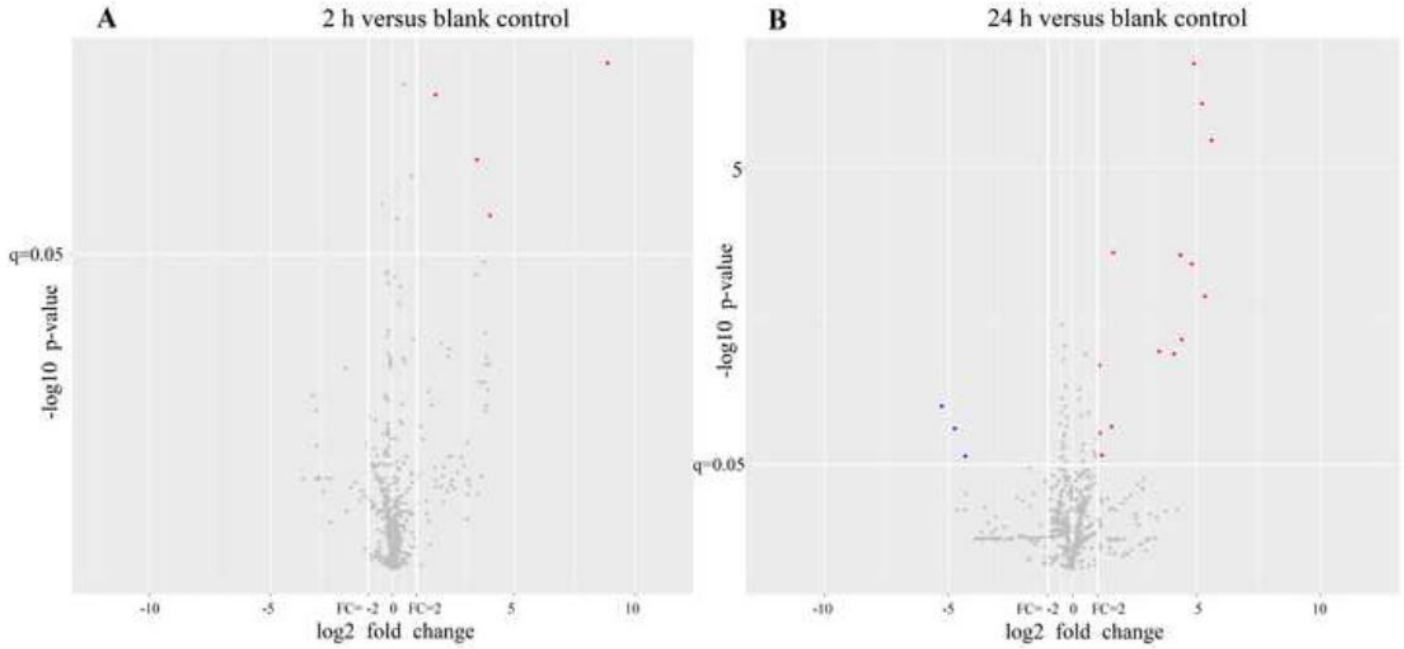


Figure 5

Representative Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis for borax treatment and control. A, 2 h vs control; B, 24 h vs control.

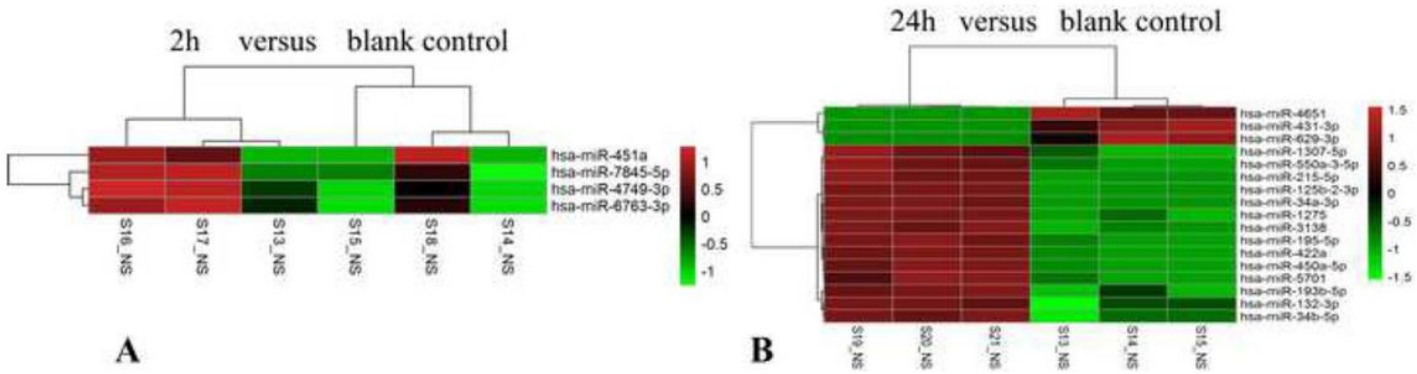


Figure 6

Potential target genes of DE-miRNAs predicted by miRNet database. DE-miRNAs-target genes network constructed using miRNet (A, C); Target gene count for each DE-miRNA (B, D); A, B: 2 h vs control; C, D: 24 h vs control.

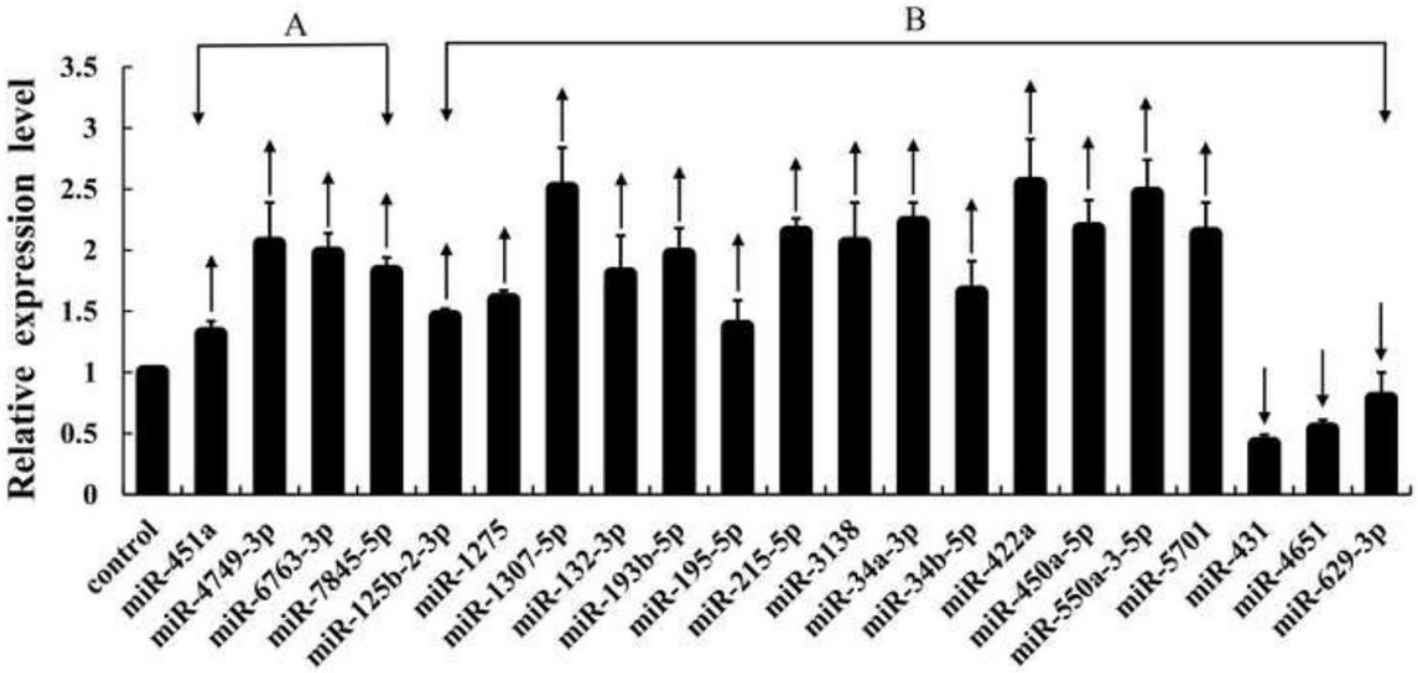


Figure 7

The candidate miRNA-hub gene regulatory network.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.jpg
- Table2.jpg
- Table3a.jpg
- Table3b.jpg
- Table3c.jpg