INVESTIGATION OF THE IN VITRO CYTOTOXIC EFFECTS OF BORON ON HUMAN HEPATOCELLULAR CARCINOMA CELL LINE, HEPG2

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ABSTRACT

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Boron is a widely available element found in the earth's crust. It is an essential micronutrient for plants and it is also beneficial for animals. At high concentrations boron is known to have toxic effects on cells, and currently the mechanism of this toxicity is still not documented. Liver, kidney, central nervous system, and gastrointestinal track were the most effected organs. Humans mostly exposure to boron as borates or boric acid. The exposure typically occurs through ingestion of food or water, through use of pesticides containing boron compounds, inhalation of boron-containing powders and dusts, or use of boron from cosmetics or medical preparations. Boron is metabolized into boric acid in the liver, which accumulates mainly in the liver, and in the excretory system organs.

In this study we examined the boric acid metabolism and toxicity in hepatocellular carcinoma, HepG2 cells. In order to find the threshold for the

toxic concentration HepG2 cells were treated between 0,5-40 mM of boric acid for 24 h and IC50 value was calculated as 24 mM. At this concentration, significant increase in DNA fragmentation and micronucleus formation were observed with single cell gel electrophoresis (Comet assay) and cytokines block micronucleus assay (CBMN). The genotoxicity results indicated that the DNA damage participated in the mechanism of boric acid toxicity, also the results of cell survival assay also revealed that, the cell viability were significantly inhibited by boric acid in a dose dependent manner.

Gene expression analysis is performed by using Affymetrix Gene Chip Human Gene 1.0 ST Array platforms. Boric acid at IC50 concentration exposure significantly altered the expression of 828 genes in total, 467 were down-regulated and 361 were up-regulated. Database for Annotation, Visualization and the Integrated Discovery (DAVID) is used for the annotation and biological interpretations of the regulated genes. Pathway Analysis revealed that, the top networks that are altered by acute exposure of boric acid were cell cycle, the DNA replication and the steroid biosynthesis networks. Overall we have shown that at toxic concentrations exposure to boric acid results in DNA damage, effecting the regulation of genes that have role in cell cycle, DNA replication, and steroid biosynthesis. Further investigation of the differentially regulated genes identified in the study is planned for understanding the molecular mechanisms of boric acid toxicity in mammalian cells.

Keywords: Boric acid, microarray analysis, cytotoxicity, genotoxicity, HepG2 cell line.

ÖZ

İNSAN HEPATOSELLÜLER KARSİNOM HÜCRE HATTI, HEPG2 İLE BORONUN İN VİTRO SİTOTOKSİK ETKİLERİNİN İNCELENMESİ

Çöpoğlu, Hülya

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Bor, yerkabuğunda bulunan yaygın bir elementtir. Bitkiler için gerekli olan mikro besin maddesidir ve hayvanlar için faydalıdır. Yüksek konsantrasyonlarda borların hücreler üzerinde toksik etkileri olduğu bilinmektedir ve halen bu toksisite mekanizması belgelenmemiştir. Karaciğer, böbrek, merkezi sinir sistemi ve gastrointestinal yol en etkili organlardır. İnsanlar çoğunlukla bora boratlar veya borik asit olarak maruz kalırlar. Maruz kalma, tipik olarak, bor bileşikleri içeren pestisitlerin kullanımı, bor içeren toz ve tozların inhalasyonu yoluyla veya kozmetik ürünlerden veya tıbbi preparatlardan bor kullanımı yoluyla, yiyecek veya içilen su yoluyla oluşur. Bor, karaciğerde borik asite metabolize olur ve bu da karaciğerde ve biriken dışkı sistem organlarında birikir.

Bu çalışmada hepatocellular karsinom HepG2 hücrelerinde borik asit metabolizması ve toksisitesini inceledik. Toksik konsantrasyonun eşiğini

bulmak için HepG2 hücreleri 24 saat boyunca 0,5-40 mM borik asit ile muamele edildi ve IC50 değeri 24 mM olarak hesaplandı. Bu konsantrasyonda, tek hücre jel elektroforezi (Comet testi) ve sitokin blok mikronükleus tahlili (CBMN) ile DNA parçalanması ve mikronukleus oluşumunda belirgin bir artış gözlendi. Genotoksisite sonuçları, DNA hasarının borik asit toksisitesi mekanizmasına katıldığını, ayrıca hücre canlılığı testinin sonuçlarına da, hücre canlılığının borik asit tarafından doza bağımlı şekilde inhibe edildiğini ortaya koymuştur.

Gen ekspresyon analizi, Affymetrix Gene Chip Human Gene 1.0 ST Array platformları kullanılarak gerçekleştirilir. IC50 konsantrasyonu maruziyetindeki borik asit, 828 genin ekspresyonunu önemli ölçüde değiştirdi, 467 aşağı regüle edildi ve 361 yukarı regüle edildi. Düzenlenen genlerin biyolojik yorumları için DAVID veritabanı kullanıldı.Yolak analiz sonuçları, borik asit maruziyeti ile değişen yolakların özellikle hücre döngüsü, DNA replikasyonu ve steroid biyosentez yolakları olduğunu ortaya koydu. Genel olarak, toksik konsantrasyonlarda borik aside maruz kalmanın, hücre döngüsü, DNA replikasyonu ve steroid biyosentezinde rol oynayan genlerin düzenlenmesini etkileyerek DNA hasarına yol açtığını göstermiştir. Memeli hücrelerinde borik asit toksisitesinin moleküler mekanizmalarını anlamak için, çalışmada tanımlanan farklı olarak düzenlenen genlerin daha fazla araştırılması planlanmaktadır.

Anahtar Kelimeler: Borik asit, mikroarray analizi, sitotoksisite, genotoksisite, HepG2 hücre hattı.

Dedicated to my family and my love

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ABBREVATIONS

ATCC	American Type Culture Collection
BA	Boric Acid
BNCT	Boron Neutron Capture Therapy
CBMN	Cytokinesis Block Micronucleus
CBPI	Cytokinesis-Block Proliferation Index
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
H2O2	Hydrogen Peroxide
HepG2	Liver hepatocellular cells
HMPA	High Melting Point Agarose
IC50	The half maximal inhibitory concentration
LMPA	Low Melting Point Agarose
MMC	Mitomycin C
OECD	Organization for Economic Cooperation and Development
PBS	Phosphate-Buffered Saline
SCGE	Single Cell Gel Electrophoresis
XTT	2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

CHAPTER 1

INTRODUCTION

1.1 Boron and Boric Acid

Boron is the fifth element in the periodic table and its nomenclature is the letter B. Boron concentration of rocks is in the range of 5 to 10 ppm. While seawater contains nearly 4.6 ppm, freshwater has 0.01 to 1.5 ppm [1]. Boron is found in rocks, soil and water. Earth's soil has approximately 10 ppm boron and United States, Kazakhstan and Mediterranean regions have the highest concentrations of boron in the world.

Boric acid is a weak inorganic acid of boron derivatives, which is a water soluble powder in white color. The chemical structure of boric acid is that central boron is connected to three hydroxyl groups (-OH) and they create strong hydrogen bonds [2]. Its chemical formula is H_3BO_3 and molecular weight is 61.83 g/mol.

1.1.1 Application areas of boron and its derivatives

Boron compounds and their derivatives are widely used for manufacturing of glass, ceramics, soap, detergent and gasoline. Boron compounds are also used as anti-fungal, and herbicides in agriculture [3]. Recently boron compounds are being used with medical purposes. Boron and its derivatives can regulate calcium and magnesium metabolism and also help to improve bone health and muscle formation. Also, they are actively used in osteoporosis, arthritis and menopause treatments [4, 5].

A new application area of Boron element has emerged as a new approach for cancer treatment. Boron neutron capture therapy (BNCT) is a treatment approach for many tumors in which boron labeled drugs accumulates in cancer cells. So by targeting boron, BNCT is able to eliminate cancer cells selectively without destroying healthy cells [6]. There are several studies, which investigates the molecular mechanism of action boron on cancer cells. In a study carried out by Lu and coworkers [7] boric acid (BA) - mediated BNCT was administered in hepatocarcinoma rats and after 80^{th} application of BNCT, the tumors in the livers was reduced. Furthermore, lung cancer patients were treated with BNCT and after 7-month treatment, tumor size was decreased and without any acute or adverse effects during the treatment [8]. Hence, these studies showed that boron compound mediated BNCT has potential for as a new approach in cancer treatment.

1.2 Boron in Turkey

The most significant boron reserves in the world are in Turkey, USA and Russia. Turkey retains nearly 72% of boron mineral reserves (68% of the definite reserves) of the world. Boron reserves in Turkey are 563 million tons, which includes 224 million tons of definite and 339 million tons of possible reserves. Eti Holding is the main producer of boron in Turkey and it owns the 31% of total boron production in the world [9]. Eti Holding process boron minerals in Turkey and it has four foundations which are set up in Eskisehir-Kirka, Balikesir-Bigadic, Bursa-Kestelek and Kutahya-Emet. Raw boron mineral has been executed in these facilities [10].

Colemanite, ulexite-probertite and tincal ores are three major areas of boron ore production in Turkey. Tincal deposit are found in Eskisehir- Kirka region. Colemanite are located in the Kutahya-Emet, Balikesir-Bigadic and Bursa-Mustafa Kemalpasa, whereas ulexite is obtained in Balikesir- Bigadic region [11].

1.3 The Importance of Boron for Living Organisms

1.3.1 Bacteria and Fungi

Boron affects bacterial and fungal growth. Boron derivatives are especially used as antibacterial and antifungal agents in daily life, such as in timber industry and forestry to keep conifers away from infections [26]. Boric acid can be also used to control the fungal infection in grapes. According to a research, 475 mg boric acid per ml is enough for the inhibition of fungal growth by 50% [27].

1.3.2 Plants

Boron is one of the essential micronutrient for plants and it has a central role in cell wall synthesis, in transportation of sugars and formation of lignin in higher plants [23]. Fruits, vegetables especially leafy greens and hazelnuts are the source of the boron. For example, legumes, fruits and nuts have 1 mg to 4.5 mg boron / 100 g whereas fresh fruits and vegetables have 0.1 to 0.6 mg / 100 g [24].

Boron deficiency can cause some problems in plants. When plants cannot take enough boron, carbohydrate accumulation takes place in chloroplasts and this results in the increased activity of pentose cycle and also decreased activity of Krebs cycle [25].

1.3.3 Animals

Boron is an essential dietary component for both humans and animals. It can bind biological compounds because of its low atomic weight and effect biological functions [12]. Boron is generally nontoxic but its concentration is very critical. Higher levels of boron intake is known to cause developmental abnormalities in animals such as dogs, mouse and rat [12].

Several studies have been carried out in order to determine the effects of boron intake in animals. For instance, researchers revealed that sodium borate which

is a boron salt, inhibit the development of fatty liver in cows [13]. Also, when animals treated with sodium borate, serum triglyceride and very low density lipoprotein levels of animals were significantly decreased [14].

There are studies that boron can affect the liver function. In one study, rat liver failure was induced chemically and rats were treated with borax. Results showed that after borax treatment liver tissue can recover and abnormal oxidative stress parameters can return to their normal level [15]. In another research the effects of boron on fatty liver status in rabbits was studied. Different boron concentrations were given to rabbits orally for 96 hours. Results suggested that boron has positive effect on hepatocytes and it may help to reduce the oxidative stress and lipid concentration in liver [16].

1.3.3.1 Humans

Human body can take boron and its derivatives via food. Fruits, vegetables, legumes and nuts have high level of boron. In addition, humans may take boron either by absorption through skin [17]. When boron enters the body, it can be excreted by urine without accumulation. Tissues and fluids of human have boron and boric acid, respectively. Health people have 15.3-79.5 ng/g total boron serum concentration, which is 98.4% boric acid and as 1.6% borate anion [18]. In addition, human organs have different boron concentrations. For instance, heart, liver, lung, kidney and brain contains different levels of 28, 2.31, 0.6, 0.6, 0.06 ppm boron, respectively [17].

There are several evidences that boron can influence the functions of brain, and also skeletal and immune functions. In one study it was found that, postmenopausal women who suffered from osteoporosis treated with boron daily, and after seven-week of boron intake their symptoms reduced [19]. Moreover, daily boron intake might help to equilibrate blood serum calcitonin levels and increase calcium levels in blood when postmenopausal women took medicine for osteoporosis [20].

When daily dose of boron (10 mg\ day) was administered to human male for seven days, pro-inflammatory cytokine and steroid hormones in plasma were found to be decreased. Also, free testosterone levels in serum increased with boron intake [21].

1.4 Boron Toxicity

The determination of toxic effect of boron and its compounds such as borates and boric acid have been studied in both human and animals. According to World Health Organization report published in 1998, boric acid and borax have showed similar toxic effects on living organisms [28]. In one study, borax and boric acid were tested and found that borax can be converted entirely into boric acid at physiological pH [29]. Therefore, both borax and boric acid effect the organisms similarly.

Several studies were carried out in order to determine boron toxicity on animals and humans at different doses, exposure times and with different application methods. Animal experiments suggested that, when dietary boron intake exceed 100 μg/g, it might lead to toxicity, effecting the animals. It was also reported that, rats treated with 17.5 mg/kg/day boric acid administration lead to infertility and 9.6 mg/kg/day oral administration of boric acid caused abnormal development of the animals [30].

Excess boron exposure cause some toxic symptoms in humans. When an individual is exposed to high amounts of boron, symptoms such as nausea, vomiting, diarrhea and lethargy can be seen. In one study it was reported that, two infants whom administered accidently borax orally showed scanty hair, anemia, seizures and patchy dry erythema [31]. Another research conducted on mine workers showed that, mean blood boron levels of workers were 223.89 ng/g and this level didn't cause any harmful effects on reproductive systems [32]. Furthermore, boron mine workers' urine analysis revealed that 6.5 mg/day didn't cause adverse effect on their reproductive systems [33]. In conclusion, all evidence suggests that when human exposed daily boric acid or

borax, it is unlikely to cause reproductive toxic symptoms. However, it was reported that, when rats were administered by 400 mg boric acid /kg/day subcutaneously, histopathological changes could be seen in their kidney tissues [34]. In addition, when 1g/kg borax and boric acid were administered for 3 weeks, rats showed some adverse effects after administration such as loss of weight and at molecular level inhibition of DNA synthesis [35].

In chronic exposure studies, borax and boric acid were given daily at concentrations of 117, 350 and 1.170 ppm to rats. Results showed that, at low concentrations of boron has no adverse effects, but high concentrations caused hair loss, swelling, scaly tails and reduced testis weight and tubular size in male rats [36]. In addition, when the same experiment repeated on dogs, which were fed with the same concentration of 1.170-2.000 ppm boron for 60 days showed skin rashes, loss of appetite and gonadal deterioration [36].

There are studies indicating that boron can serve as protector against toxic compounds. One study was carried out in order to detect the protective role of borax on aluminum induced genotoxicity in rat liver. In this study, aluminum chloride and borax were administered into rats for 10 days. Results showed that, rat liver treated with borax (3.25 and 13 mg/kg) showed lower number of micronucleated hepatocytes [37]. In another study boric acid was used as a protector against the adverse effects of the anti-cancer drug. In this study, results indicated that boric acid didn't cause any cytotoxic and genotoxic effects on human lymphocytes and actually it helped to decrease genotoxic and cytotoxic effects which were induced by the anti-cancer drug, paclitaxel (PAC) [38].

1.5 Boron and Cancer

Compounds which are naturally found in nature, can be used as treatment for many cancer types such as liver, breast and prostate cancers [39].Studies showed that, boron and its derivatives can be promising compounds in cancer treatment. Recent *in vitro* and *in vivo* studies revealed the effects of boron and

its compounds on different cancer cells. In one of these studies it was reported that, boric acid decreased tumor size, and also IGF-1 (Insulin- like growth factor- 1) and PSA (prostate specific antigen) levels in a nude model of prostate cancer [40]. In another study, 1 mM of boric acid inhibited the proliferation of prostate cancer cell lines, DU-145 and LNcAP, with a little influence on the cell cycle and mitochondrial function [41].

In rat models, thioacetamine- induced hepatocellular carcinoma, borax reduced the proliferation cell nuclear antigen index and modulated the oxidative stress [42]. Growth of hepatocarcinoma cells were also inhibited in time and dose dependent manner *in vitro* by borax treatment. In this study, borax treatment found to be inducing the apoptotic pathway that through up-regulation of p53 and Bax and down-regulation of Bcl-2, and also altered mitochondrial membrane potential [42].

All these results suggest that boron and its derivatives like borax have great potential to be used as an effective anticancer agent.

1.6 Aim of the study

Boric acid is the soluble form of boron element and it's found at higher concentrations in human circulation. Excess amount of boric acid can be excreted through kidneys and it doesn't accumulate in tissues.

Boron is found in Turkey at high amounts, and both mine workers during its extraction and also people working in industries using this element are exposed to boron. Recent studies also showed the beneficial effects of boron. So in this study, our aim is to find the cytotoxic and genotoxic effects of boric acid on hepatocellular carcinoma cell line HepG2 in dose dependent manner. Also in literature there is no detailed study about the mechanism of the action of boron. So, we further aimed to study the gene expression profile of boron treatment at IC50 concentration emphasizing on the affected pathways through microarray experiment technologies.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

All chemicals which were used in cell culture and other assays, were analytical and cell culture grade. In the cell culture experiments, HepG2 cells were obtained from ATCC (American Type Culture Collection), DMEM with low glucose with L-glutamine with sodium pyruvate and 25 mM Hepes (Biowest), Pen-strep solution (Biological Industries), heat-inactivated fetal bovine serum (Biological Industries), 10x trypsin-EDTA (Biowest), dimethyl sulfoxide (DMSO) (Applichem), phosphate buffered saline without Mg and Ca (Biological Industries) and trypan blue (Biological Industries) were used. Boric acid (Sigma, USA) which is cell culture grade was a gift of Dr. Serap Kolukısa from BOREN Institute.

In cytotoxic and genotoxic experiments, XTT cell proliferation kit (Biological Industries), Low melting agarose (Sigma), high melting agarose (VMR), Hydrogen peroxide 30% (Merck, Millipore), TritonX-100 (Merck, Millipore), NaCI and Trizma Base (Sigma), NaOH (Merck, Millipore), Na2EDTA (Sigma) and propidium iodine (Sigma), KCI (Merck, Millipore), 99.8 % Methanol (Sigma), Acetic acid (EMD, Millipore), Formaldehyde (Applichem) and Giemsa (Sigma) Mitomycin C (MMC) and Cytochalasin B (Serva) were used.

For total RNA isolation RNeasy mini kit was obtained from Qiagen (Germany). In microarray experiments, GeneChip® Human Genome U133 Plus 2.0 Array,

GeneChip IVT Plus Kit and GeneChip Hybridization Wash and Stain Kit were purchased from Affytmetrix (Thermo Fisher Scientific).

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell culture and treatments

Human hepatocellular carcinoma cell line HepG2 was grown in DMEM low glucose with L-glutamine with sodium pyruvate and 25 mM HEPES which was supplemented with 10 % FBS and 1% pen-strep solution. Cells were maintained at 37^{o} C temperature, 95% air and 5% CO_2 culture conditions.

Boric acid was dissolved in complete cell culture medium at room temperature and stock solution was diluted for all assay purposes.

2.2.1.2 Cell Seeding

Cryofrosted cells were first melted at 37^{o} C water bath. Dissolved cells were added into 3-5 ml complete medium in a 15 ml falcon tube. Cells were centrifuged at 1000 rpm for 5 minutes in order to get rid of the DMSO. After that, supernatant was discarded and pellet was dissolved in 15 ml complete medium. These cells were seeded into T75 cell culture flask and incubated at 37^{o} C temperature, 95% air and 5% CO_2 culture conditions.

One day later, medium was discarded and renewed with 15 ml complete medium. After approximately 48 hours, HepG2 cells were duplicated and cell confluence reached to 80%. According to assay procedure, HepG2 cells were seeded into other culture flask.

2.2.1.3 Cell Passaging

When cell confluence reached around 80%, these cells were separated into other culture flask. In the cell culture, old medium was discarded and cells were washed two times with PBS containing no Mg^{+2} and Ca^{+2}. After washing step, 2x rewarmed Trypsin (Stock 10x diluted with PBS) was added onto the cells and they were placed into the CO_2 incubator at 37° C, 95% air and 5% CO_2 culture conditions for 3 minutes.

Then, 6-8 ml complete medium was added into T75 cell culture flask in order to inhibit trypsin. Detached cells were collected into 15 ml falcon tubes. In the meantime, new complete medium nearly 10 ml was added into new T75 flask and 5 ml cell suspension was added into T75 cell culture flask. For HepG2 cell cultures, it was recommended that sub-cultivation can be done as 1:4 to 1:6.

2.2.1.4 Cell Freezing

Before the cell freezing, freezing medium which consists of 95% complete medium and 5% DMSO, was prepared and stored at 4°C. In the cell culture, old complete medium was discarded and cells were washed with PBS. Cells were trypsinized for 3 minutes and detached cells were collected into 15 ml falcon tube. These tubes were centrifuged at 1000 rpm for 5 minutes. And then, supernatant was discarded and pellet was resuspended in cold freezing medium. Immediately, around 1 ml of cell suspension was transferred into cryovials. All cryovials were put into Mr. Frosty freezing container which contained isopropanol at -80° C. This system provides the cooling of cell suspension -1°C for every minute. After one-day incubation at -80° C, all cryovials were transferred into liquid nitrogen tank for long term storage at about -196 °C.

2.2.1.5 Cell Counting

HepG2 cells were seeded onto 6 well plates at a density of $3x10^5$ cells\ ml and was incubated at 37°C. One day later, old complete medium was discarded and each well was washed with 1 ml of PBS. After washing step, 500 µl trypsin was

added into each well and incubated for 3 minutes at 37ºC. Detached cells were collected into 2 ml cell culture tubes.

Cells were counted in BioRad TC20 (USA) cell counter. First of all, 20 µl cell suspension and 20 µl 0.4 % trypan blue were mixed, and then 10 µl of mixed sample was uploaded into counter slide and this slide was inserted into the device. After counting, cell counter gave total cell count as alive or dead. Trypan blue was used in order to see the dead cells since it stained only non-alive cells.

2.2.2 Cytotoxicity assay

2.2.2.1 XTT based Cell Proliferation Assay

XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carbox-anilide) based cell proliferation assay was carried out based on the manufacturer's instructions. Briefly the tetrazolium salt XTT was reduced to orange colored formazan compounds by the activity of mitochondrial enzymes of the metabolically active cells [65]. The formed product was water-soluble and can readily be observed with ELISA reader. According to the assay conditions, $1x10^5$ cells/ml cells were seeded on to 96 wells. One day later, boric acid was added at appropriate concentrations and incubated for 24 hours. For this assay, boric acid concentrations between 0.5-40 mM was used in order to find the half maximal inhibitory concentration (IC50). Then, XTT solution which consists of 0.1 ml activator and 5 ml XTT reagent, was added to each well and cells were incubated for further 4 hours at 37ºC. Color changes were measured with ELISA reader (Thermo Fisher Scientific) at 450 nm. Also, absorbance of cells was measured at 630 nm in order to eliminate unspecific wavelength. For analysis, absorbance at 450 nm was subtracted from the absorbance readings at 630 nm. In this assay, 3 biological replicates and 3 technical replicates were used for the detection of the correct concentration.

The absorbance gives information about cell viability. In order to calculate % cell viability, **Equation 1** is used.

Equation 1: Formula of % cell viability

% cell viability: ($Abs_{(450-630)}$ of treated well (with cells) - $Abs_{(450-630)}$ of treated well (without cells) / ($Abs_{(450-630)}$ of control well (with cells) - $Abs_{(450-630)}$ of control well (without cells)

% cell viability could be calculated for each boric acid concentration. By means of % cell viability curve, the half maximal inhibitory concentration (IC50) could be obtained.

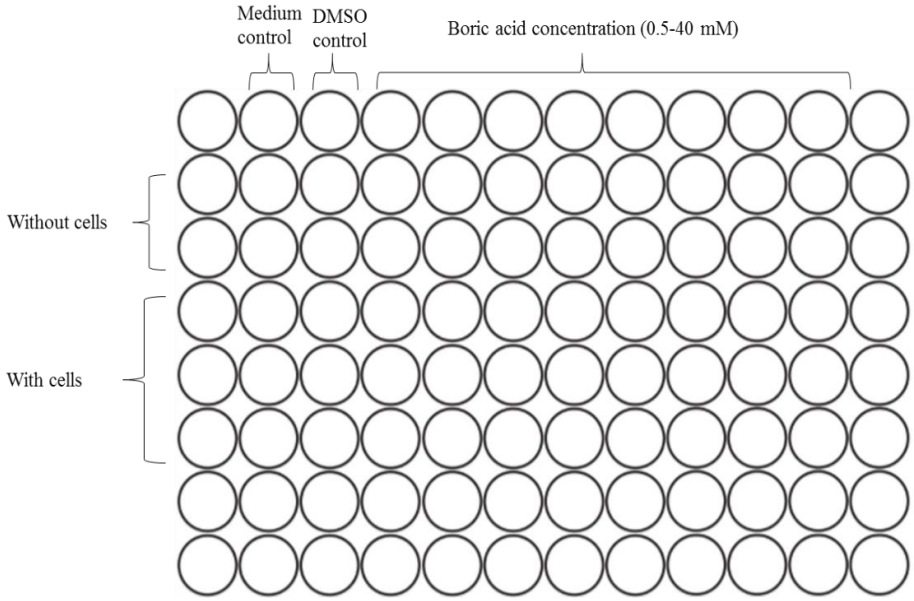


Figure 1. 96 well plate experimental set-up for XTT analysis. In DMSO control, wells contain 50 µl complete medium and 50 µl 0.2% DMSO. In boric acid treatment groups, each wells contain 50 µl complete medium and 50 µl of boric acid concentration.

2.2.3 Genotoxicity assays

2.2.3.1 Single Cell Gel Electrophoresis Assay (SCGE, Comet Assay)

The single cell gel electrophoresis assay (SCGE) was performed based on procedure of Singh et. all [43] with minor modifications.

2.2.3.1.1 Slide preparation

One side fully frosted slide was coated with 1% high melting point agarose (HMPA) in dH_2O. Before the assay, slides were prepared. Then, one side fully frosted slides was dipped into hot 1% HMPA and other side of slide was cleaned with tissue paper. All slides were left to dry at room temperature.

2.2.3.1.2 Sample preparation

HepG2 cells were seeded on to 6 well plates as $3x10^5$ cell/ml. After one-day growth, cells were treated with IC25 and IC50 concentrations of boric acid for 24 hours. Also, some cells were treated with 1% DMSO and H_2O_2 (40 µM and 100 µM) as positive controls. At the end of treatment period, all cells were washed twice with PBS and trypsinized for 3 minutes. Then, approximately 1 ml of complete medium was added into each well to collect detached cells. All cells were spun down at 200xg for 3 minutes at 4^oC and supernatants were removed as much as possible. Pellet was washed with PBS one more time and centrifuged again. Clear pellet was resuspended with 0.5 % low melting point agarose at 37^oC. Immediately, cell suspension was spread over 1% HMPA coated slides and covered with 22x22 mm coverslip. In this assay, embedded cells in agarose was quantified immediately because agarose quickly melts. All slides were kept in fridge for 5 minutes. After that, all coverslips were removed from the slides.

2.2.3.1.3 Lysis of cells

All slides were dipped into light protected freshly prepared cold lysis buffer containing 2.5 M NaCl, 0.1 M Na2EDTA, 10 mM Tris–HCl, 1% Triton X-100,

10% dimethyl sulfoxide at pH 10, overnight. In the assay, lysis buffer was prepared freshly and it was maintained always at dark.

2.2.3.1.4 Alkaline treatment and Electrophoresis of slides

Alkaline buffer at pH 13 was prepared freshly and stored at cold. Alkaline buffer consists of 0.3 M NaOH, 1 mM Na_2EDTA. After lysis treatment, all slides were placed into electrophoresis tank in which there was no gap between the slides. Cold alkaline electrophoresis buffer was poured into tank and gels were covered with the buffer. All slides were pretreated with alkaline solution to unwind DNA for 40 minutes at dark and in cold.

Electrophoresis was carried out with all slides for 30 minutes at 25 V (Thermo EC250-90). As an important note, both electrophoresis and alkaline treatments should be done in cold and should be kept at dark place in order to prevent additional DNA damage.

2.2.3.1.5 Neutralization and Staining of slides

The slides were washed three times for 5 minutes with 0.4 M Tris–HCl, pH 7.5 neutralizing buffer at 4ºC. Then, the slides were stained with 60µg/ml propidium iodine for 20 minutes. All slides were kept at dark until they were viewed under microscope.

2.2.3.1.6 Quantitation of slides

Stained slides were viewed with fluorescence cell image microscope (Floid, Thermo Fisher). Approximately 50 cells per slide was taken for image analysis. All image was uploaded into OpenComet software which provided fully automated comet analysis and plugins in ImageJ software. By means of this software, tail length, tail moment and % DNA in tail were calculated. In the assay, 3 biological and 3 technical replicates were used and two slides were prepared for each sample.

2.2.3.2 Cytokinesis-blocked micronucleus (CBMN) assay

In vitro cytokinesis-blocked micronucleus assay was carried out according to protocol of Fenech et. all [44] and Organization for Economic Co-operation and Development (OECD) Guideline 487 [45]. HepG2 cells were plated in 6 well plate as $3x10^5$ cells/ml and cultured for 24 hours. The cells were treated with boric acid at IC25 and IC50 concentrations for 24 hours, as well as 1µg/ml mitomycin C (MMC) as positive control. At the end of treatment, cells were washed twice with PBS and 6 µg/ml cytochalasin B was added over the cell culture for additional 24-hour incubation in order to interrupt cell division after mitosis. After treatment, the cells again were washed twice with PBS and trypsinized for 3 minutes. The cells were centrifugated at 1000 rpm for 5 minutes at 4^oC and pellet were resuspended in cold 7.5 mM hypotonic KCI solution slowly for 5 minutes at 4^oC. And then the cells were spun down at 1000 rpm for 5 minutes at 4^oC. The cells were fixed in fresh cold 10 ml of fixative solution which consists of 9 ml methanol, 3 ml acetic and 150µl formaldehyde for 10 minutes at room temperature. In the assay, fixative solution must be cold and it should be added slowly onto the cells. After fixation, the cells again were centrifuged at 2200 rpm for 10 minute at 4^oC. Cell suspension was spread through cold microscope slide and they were allowed to air-dry for 10 minutes. Air-dry process shouldn't exceed 10 minutes, in case shape disruption of cells could happen. All slides were stained with Giemsa stain before the slides were viewed under light microscope (Olympus). Slides were examined blindly under a light microscopy with 400x magnification. Micronucleus formation was calculated in total of 2000 binucleated cells which was 1000 binucleated cells for each replicate. The cytokines block proliferation index (CBPI) was calculated by using **Equation 2**.

Equation 2: Calculation of cytokines block proliferation index (CBPI)

CBPI = (Number of mononucleated cells + 2 x Number of binucleated cells + 3 x Number of multinucleated cells)/Total Number of cells.

2.2.4 Microarray experiment

In order to carry out microarray experiments, GeneChip® Human Genome U133 plus 2.0 Array, GeneChip IVT plus Kit and GeneChip Hybridization Wash and Stain Kit have been purchased from Affytmetrix (Thermo Fisher Scientific). Also, Applied Biosystems thermal cycler, GeneChip® Scanner 300, Hybridization Oven 640 and Fluidics Station 450 were used for microarray analysis. All the experiments were carried out in Assoc.Prof.Dr. Bala Gür's laboratory at the Ankara University.

2.2.4.1 Total RNA isolation

Total RNAs from HepG2 cells were isolated by Qiagen RNeasy total RNA isolation kit (Qiagen, Germany). In brief, for RNA isolation HepG2 cells were plated in 35 mm cell culture dishes as $3x10^5$ cells/ml seeding density prior to boric acid treatment. Cells were treated with 0.1 % DMSO as negative control and IC50 boric acid concentration as test sample for 24-hour incubation. At the end of the treatment, total RNAs were isolated according to manufacturer's protocol.

After isolation, the amount and quality of RNA was detected by Biodrop Nanodrop (United Kingdom). In these measurements, absorbance at 260 nm showed nucleic acids and absorbance at 280 nm gave information about proteins. Absorbance ratio of 260/280 value gave information about the RNA integrity. For microarray analysis, RNA integrity number should be minimum of 1.8-2.0. Nanodrop calculates RNA concentrations automatically according to **Equation 3**.

Equation 3: Calculation of total RNA concentration

RNA concentration (µg/ml) = (OD260) x (dilution factor) x (40µg RNA/ml)/ (OD260)

2.2.4.2 Whole Transcript expression arrays

In this experiment, RNAs which were isolated from 0.1% DMSO and IC50 boric acid treated cells, were labelled according to Affymetrix whole transcript expression array protocols. In this assay, as shown in **Figure 2**, there are several steps in order to analyze the alterations of gene expressions after boric acid treatment.

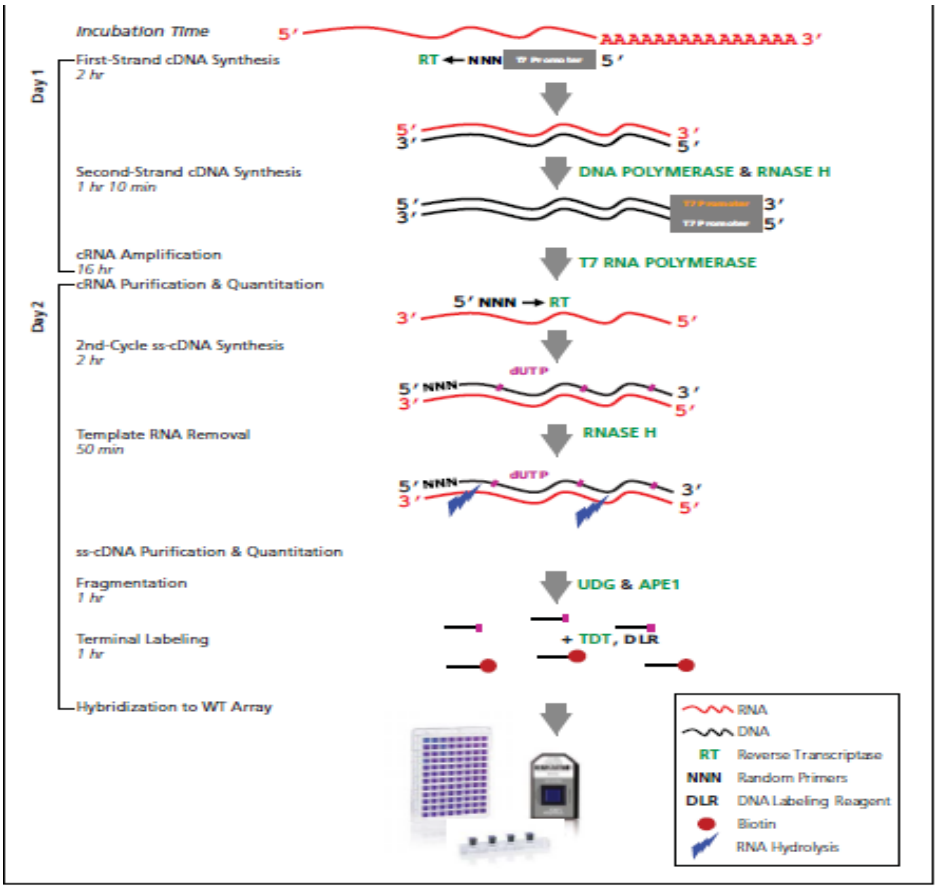


Figure 2. Workflow of microarray assays. This figure schematically explains whole transcript amplification and labeling process. (Adopted from Manual Target Preparation for GeneChip® Whole Transcript (WT) Expression Arrays).

2.2.4.2.1 Preparation of Total RNA/Poly-A RNA Control Mixture for first-strand cDNA synthesis

In order to prepare total RNA/Poly-A control, maximum of 5 µL mixture should be prepared as shown in **Table 1**.

Table 1. Total RNA/Poly-A RNA Control Mixture

Component	Volume for one reaction
Total RNA Sample (50-500 ng)	variable
Diluted Poly-A RNA Controls	2 µl
Nuclease-free Water	variable
Total volume	5 µl

2.2.4.2.2 Synthesis of first-strand cDNA

In order to prepare first-strand cDNA, first of all, first-strand master mix in nuclease free tube should be prepared on ice. As seen **Table 2**, all components were mixed in given amounts and 5% excess volume could be added in order to correct pipetting errors.

Table 2. Components of First strand master mix

Components	Volume for one reaction
First-Strand Buffer	4 µl
First-Strand Enzyme	1 µl
Total volume	5 µl

All components were mixed gently with vortex and 5 µl of mixed solution was added onto 5 µL of the total RNA for a final volume of 10 µl. All tubes were incubated for 1 hour at 25°C and then for 1 hour 42 °C, finally for at least 2 minutes at 4°C. Immediately, after incubation all tubes were centrifuged to collect first-strand cDNA at the bottom of tubes. Then, first-strand cDNA was transferred into new nuclease free tubes and placed on the ice for 2 minutes. Finally, first-strand cDNA was ready to synthesize second-strand cDNA.

2.2.4.2.3 Synthesis of Second- strand cDNA

In this procedure, single stranded cDNA was converted into double stranded cDNA. This double stranded cDNA acted as template for *in vitro* transcription. In this assay, DNA polymerase and RNase H were used to degrade all RNA and to synthesize second-strand cDNA.

All components were mixed to synthesize second strand cDNA based on **Table 3**.

Table 3. Second-Strand Master Mix

Components	Volume for one reaction
Second -Strand Buffer	18 µl
Second -Strand Enzyme	2µl
Total volume	20 µl

All components were mixed very gently by vortex. 20 µl of the second-strand Master Mix was added onto 10 µl first-strand cDNA for a final reaction volume of 30 µl on ice. Again all tubes were mixed very gently and centrifuged very briefly to precipitate all components at the bottom of the tubes. All tubes then were incubated for 1 hour 16 °C, for 10 minutes 65 °C and for at least 2 minutes 4 °C. After the incubation periods, all tubes were centrifuged very briefly to collect second-strand cDNA and placed on the ice for next step of the experiment.

2.2.4.2.4 Synthesis of complementary RNA

GeneChip IVT plus Kit was used to synthesize and amplify complementary RNA. In this assay, second strand cDNA was used to synthesize cRNA by using T7 RNA polymerase. At room temperature, IVT master mix should be prepared as shown at **Table 4**.

Table 4. Components of IVT master mix

Components	Volume for one reaction
IVT buffer	24 µl
IVT enzyme	6µl
Total volume	30 µl

After adding all components, all tubes were mixed by vortex gently. At room temperature, 30 µl of the IVT master mix was combined with 30 µl of second strand cDNA. Then, all sample tubes were mixed and centrifuged briefly and incubated for 16 hours at 40 °C, then at 4°C. After incubation, all tubes were placed on ice immediately to proceed next step of experiment.

2.2.4.2.5 Purification of complementary RNA (cRNA)

In this procedure, cRNA was prepared for second cycle single stranded cDNA synthesis. At the same time, all enzymes, salts, inorganic phosphates and unincorporated nucleotides were removed.

In order to bind cRNA and purification beads, purification beads were mixed and resuspended with magnetic particles. 100 µl of purification beads and 60 µl cRNA sample were mixed thoroughly by pipetting and transferred into U bottom plate. Mixed samples were incubated for 10 minutes. During this incubation, the cRNA and purification beads bound to each other very well. Then the plate was moved to magnetic capture area so in this way beads were captured by magnetic area. When capture was completed, the mixture became transparent. In addition, supernatant was discarded without disturbing the pellet and pellet was washed three times with 80% ethanol. All pellet was left to air-dry. 27 µl of the preheated nuclease free water was added to each tube and mixed by pipetting. Again, plate was transferred over magnetic stand for 5 minutes to remove purification beads. At the end of this process, supernatant

contained the cRNA, and it was transferred to new nuclease free tubes and stored at -20 °C. Finally, concentration of cRNA was determined by Nanodrop.

2.2.4.2.6 Second cycle sense stranded cDNA synthesis

In this procedure, second cycle primers were used in order to synthesize sense strand cDNA with the reverse transcription of cRNA. The sense strand cDNA consists of dUTP at a fixed ratio relative to dTTP.

15 µg of cRNA and 4 µl of second cycle primers were combined on ice and mixed gently. All tubes were incubated for 5 minutes at 70 °C, then 5 min at 25°C, then 2 min at 4°C. After incubation were took place on ice.

In order to prepare the second cycle sense strands, all components were mixed according to **Table 5**.

Table 5. Second cycle sense strand cDNA master mix

Components	Volume for one reaction
2nd-Cycle ss-cDNA Buffer	8 µl
2nd-Cycle ss-cDNA Enzyme	4 µl
Total volume	12 µl

12 µl of the second cycle single strand cDNA master mix was combined with 28 µl of cRNA/2nd-Cycle primer sample on ice and they were mixed gently. Then, all tubes were incubated for 10 min at 25°C, then 90 min at 42°C, then 10 min at 70°C, then for at least 2 min at 4°C. After incubation process, all tubes should be placed on ice until the next step of the experiment.

2.2.4.2.7 RNase H treatment

In this procedure, RNase H was used in order to hydrolyze cRNA and to leave sense strand cDNA. 4 µl of the RNase H was mixed with 40 µl of second cycle sense strand cDNA sample and they were mixed very gently. All sample tubes were incubated for 45 min at 37°C, then for 5 min at 95°C, then for at least 2

min at 4°C. After incubation, 11µl of nuclease free water was added to each sample tube on ice and mixed very gently. Then, all tubes were frozen immediately at -20°C for storage.

2.2.4.2.8 Purification of second cycle single stranded cDNA

After RNase H treatment, the second cycle sense stranded cDNA should be removed from enzymes, salt, and unincorporated dNTPs. This step is important for fragmentation and labelling steps.

Hundred µl of purification beads were added into 55 µl of second cycle sense stranded cDNA sample and they were mixed by pipetting and transferred to U-bottom plate. Then, 150 µl of 100% ethanol was added onto each sample well, and all solution was incubated for 20 minutes in order for purification beads to bind to second cycle sense strand cDNA. After incubation, plate was moved to a magnetic stand so that magnetic area could capture purification beads. Supernatant was discarded carefully without disturbing pellet which contained second cycle sense strand cDNA. In this step, while the plate was kept on magnetic stand, pellet was washed at least three times with 200 µl of 80% ethanol for 30 seconds. Then, pellet was left to air dry on the magnetic stand for 5 minutes. For the elution of second cycle sense strand cDNA, plate was removed from magnetic stand and 30µl of nuclease free water was added onto each sample, and incubated for 1 minute. Again, plate was transferred to magnetic stand to capture purification beads for 5 minutes. In addition, supernatant which contained second cycle sense strand cDNA, was carefully transferred to new nuclease free tubes. At this time, sense strand cDNA was obtained and their yield and size was determined by using Nanodrop.

2.2.4.2.9 Fragmentation and labelling of single stranded cDNA

In this procedure, uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) were used in order to break DNA strand which contain

unnatural dUTP residues. Then, fragmented cDNA was labeled with terminal deoxynucleotidyl transferase (TdT).

At first, fragmentation master mix should be prepared in a nuclease free tube which is kept on ice. All components of the master mix are given at **Table 6**.

Table 6. Fragmentation master mix components

Components	Volume for one reaction
Nuclease-free Water	10 µl
10X cDNA Fragmentation Buffer	4.8 µl
UDG, 10 U/µl	1 µl
APE 1, 1,000 U/µl	1 µl
Total volume	16.8 µl

All components were mixed very gently and 16.8 µl of this mix were added onto 31.2 µl of purified single stranded cDNA (5.5 µg). Then, all sample tubes were incubated for 1 hour at 37°C, then for 2 minutes at 93°C, then for at least 2 minutes at 4°C. Finally, 45 µl of fragmented single stranded cDNA were transferred to new nuclease free tubes kept on ice. At this time, cDNA was ready for labelling process.

In labelling process, first of all, labelling master mix should be prepared on ice as given at **Table 7**.

Table 7. Labelling master mix preparation

Components	Volume for one reaction
5X TdT Buffer	12 µl
DNA Labeling Reagent, 5 mM	1 µl
TdT, 30 U/µL	2 µl
Total volume	15 µl

All components were mixed very gently and 15 µl of mixture was added onto 45 µl of fragmented single stranded cDNA on ice. Mixture was incubated for 1 hour at 37°C, then for 10 minutes at 70°C, then for at least 2 minutes at 4°C. After incubation, all sample tubes should be stored at –20°C.

2.2.4.2.10 Eukaryotic target hybridization

In this procedure, first of all, hybridization master mix should be prepared at room temperature as given at **Table 8**. In the experiment, 49 format were used for hybridization.

Table 8. Hybridization master mix preparation for 49 format

Components	49 format	Final concentration
Fragmented and Labeled ss-DNA	5.2 µg	23 ng/µL
Control Oligo B2 (3 nM)	3.7 µl	50 pM
20X Hybridization Controls (bioB, bioC, bioD, cre)	11 µl	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	110 µl	1X
DMSO	15.4 µl	7%
Nuclease-free Water	19.9 µl	
Total volume	160 µl	

After the preparation of the hybridization master mix, hybridization cocktail was prepared at room temperature as given at **Table 9**.

Table 9. Hybridization cocktail for a single array

Components	49 formats
Hybridization Master Mix	160 µl
Fragmented and labeled ss-cDNA	60 µl (5.2 µg)
Total volume	220 µl

All components were mixed very gently and they were incubated for 5 minutes at 99°C for tubes format, then for 5 minutes 45°C.

Then sample was applied onto the array. As seen in **Figure 3**, there were several parts of cartilage array. In this procedure, yellow pipette tip was inserted into upper right septum for venting and 200 µl of sample was uploaded into the array through one of the septa. Meanwhile, yellow pipette tip was removed from

upper right septum of the array and the arrays were placed into hybridization oven. Finally, they were incubated for 16 hours at 45°C with 60 rpm rotation.

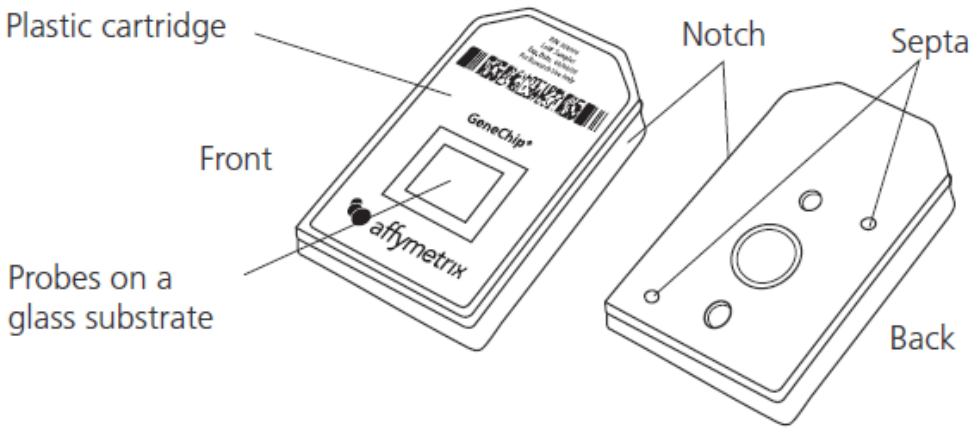


Figure 3. GeneChip® Cartridge Array (Adopted from Manual Target Preparation for GeneChip® Whole Transcript (WT) Expression Arrays).

2.2.4.2.11 Washing, staining and scanning process

Finally, hybridization cocktail mixture was discarded from each array and each array was washed with Wash buffer A. Meanwhile, vials were placed into sample holders on fluid system.

Fluidic system had different vials for different samples. For example, one vial contained 600 µl of stain cocktail 1 for sample 1 holder and one vial had 600 µl of stain cocktail 2. In addition, another vial contained 800 µl of array holding buffer.

At this part, air bubbles had to be checked during sample washing and staining steps. At the end, all arrays were scanned with GeneChip Scanner 3000.

2.2.5 Microarray data normalization, visualization and biological interpretation

In this study, first of all, Array Analysis tool was used for normalization and filtering the microarray data. CEL file of 6 arrays were uploaded into this tool. After a while, normalized microarray data was obtained. Secondly, raw data was uploaded data into BRB tool. In this way, up and down regulated genes were observed and statistical analysis of those of six microarray gene expression were done. Thirdly, after selecting up and down regulated genes, DAVID (The Database for Annotation, Visualization and Integrated Discovery) which provides set of functional annotation tools to understand biological meaning, was used to examine affecting pathways. Finally, GENEMANIA database was used to predict to function of selected genes (up and down expressed genes set) and network between selected genes and other genes.

2.2.6 Statistical analysis

XTT cell proliferation assay, single cell gel electrophoresis and cytokines blocked micronucleus assay were repeated at least three times independently. Statistical analysis was done by student's t-test or one-way ANOVA Graphpad Prism (GraphPad Software Inc., USA) to assess significance of results ($p<0.05$ considered with significance).

Microarray results were carried out by three biological replicates for each group. Results were analyzed with BRB- array tools and statistical analysis were done with two sample t-test (with random variance model). In microarray results, number of genes were significant at the nominal 0.001 level of the univariate test with the fold change 2. Data were presented as mean ± standard error of the mean.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Inhibitory effects of boric acid on HepG2 cell viability

The cytotoxic effect of boric acid on HepG2 cell line was determined by XTT cell proliferation assay method. In this assay, cells were treated with various boric acid concentrations between 0.5-40 mM for 24-hour incubation. XTT is a tetrazolium salt which can penetrate into the cells in the cell culture and used for the determination of the viable cells [46].

The results are given in Figure 4 and according to these results different concentrations of boric acid ranging between 0.5-40 mM can inhibit cell viability of HepG2 cell line for 24-hour interval. When the treatment groups were compared to medium control group, percent cell viability decreased with increasing boric acid concentrations. According to our results, after 1 mM of boric acid concentration cell viability started to decrease. IC50 value was calculated by using **Equation 1** and different inhibitory concentrations were calculated as given at **Table 10**.

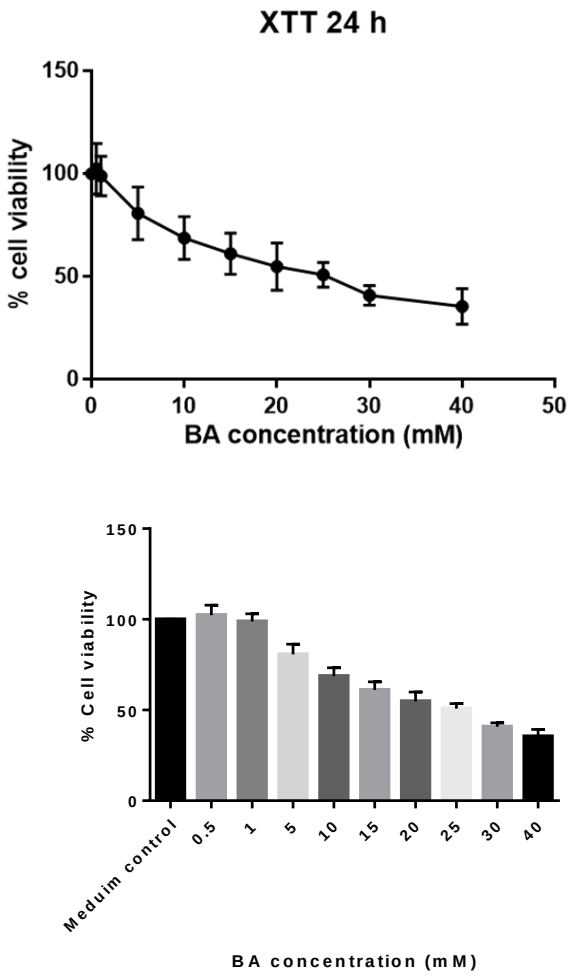


Figure 4. Inhibitory effects of boric acid on HepG2 cell growth. The inhibitory and cytotoxic effect of boric acid was tested by using XTT cell proliferation assay. The viability of cells is represented by % cell viability as means ± SEM.

Table 10. Inhibitory concentrations of boric acid on HepG2 cell growth

Inhibitory concentrations of boric acid for 24-hour treatment		
IC25 (mM)	IC50 (mM)	IC75 (mM)
12	24	36

As seen from Table, 12 mM of boric acid was enough to initiate the inhibition of cell growth by 25%. IC values were calculated as 24 and 36 mM, for 50 and 75% inhibition of cell growth, respectively.

Our preliminary experiment results showed that, inhibitory effect of boric acid on HepG2 cell viability is quite low, inhibition is seen at very high concentrations at mM levels.

Although there are few medicinal products for anticancer therapies, boron based drug synthesis and research have been gained considerable attention recently. Because of this low cytotoxicity boron compounds such as boric acid and borax can be used for the development of new products in drug industries. Boron is an important nutrient and essential for plant and animal life. There are studies showing that boron could be very helpful for drug synthesis and it could interfere with important pathological pathways [49].

In our study, we showed that boric acid has low inhibition potential on HepG2 cell line. According to our results, boric acid could reduce HepG2 cell viability in a dose dependent manner for 24-hour treatment.

There are several research papers who studied the cytotoxic effects of boron derivatives on different cancer cell lines. In one study, they have examined the effect of boric acid on proliferation of both tumorigenic and non-tumorigenic prostate cancer lines, namely LNcaP, DU-145 and PC-3 as tumorigenic and PWR-1E and RWPE-1 as non-tumorigenic cell lines. They found that, boric acid inhibited DU-145 and LNcaP at 1 mM concentration. However, PWR-1E and RWPE-1 which are non-tumorigenic cell lines, and also tumorigenic PC-3 were inhibited at higher concentrations of boric acid [50].

In another study, they used different breast cancer cell lines in order to show the cytotoxic effects of boric acid. MDA-MB-231 and MDA-MB-435 which are negative estrogen receptor human breast cancer cell lines, MCF-7 and T47-D which are positive estrogen receptor human breast cancer line, and SK-BR-3 and ZR-75-1 which are human breast cancer cell lines were used to detect the inhibition of growth. All breast cancer cell lines were treated with 1mM of boric acid concentration. Results showed that, only the growth rate of SK-BR-3 and ZR-75-1 cell lines were decreased upon 1 mM boric acid treatment for 8 days. However, boric acid treatment did not affect the growth of the other cells lines [50].

In a study carried out by Wei and coworkers [51] it was reported that, borax which is a salt of boron has an inhibitory effect on HepG2 cell viability. In this study, HepG2 cell line was incubated with different concentrations of borax at different time intervals. The results suggested that low borax concentration with longer exposure time could be more cytotoxic than higher drug concentrations with shorter exposure time. Also, borax treatment at 16 mM for 72 hours could drastically inhibit survival rate of HepG2 cell line.

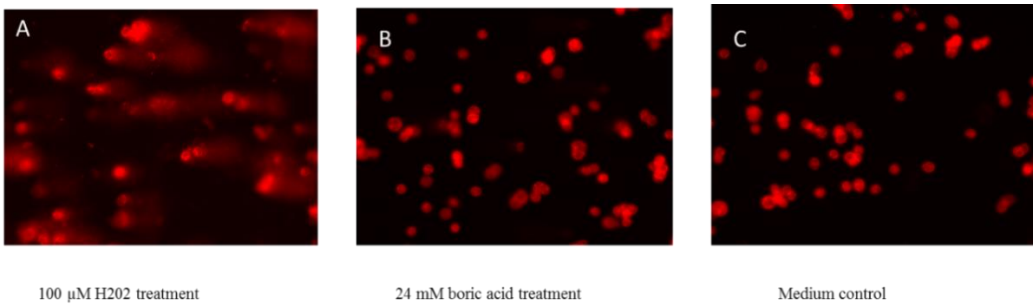
All these results indicated that, cell proliferation could be inhibited by boron compounds at different concentrations depending on cell type, and exposure time. In literature, there is no specific one IC50 concentration for all cancer cell types. Inhibition concentrations of boron derivatives were different for breast, prostate and liver cancers.

3.2 Effects of boric acid on DNA damage in HepG2 cell line

HepG2 cells were treated with both IC25 and IC50 concentrations of boric acid for 24 hours. Also, the cells were treated with various concentrations of H_2O_2 to induce DNA damage as positive control. During experiments, cell viability was calculated as 90% with trypan blue exclusion assay following the different treatments. DNA damage was determined by single cell gel electrophoresis assay, which is also known as Comet assay.

As seen in **Figure 5 A, B, C,** tail moments were changed depending on the type of the treatment. In the positive control image, there was a more comet like structure indicating more DNA damage.

The results showed that, boric acid treatment at IC50 concentration induced DNA damage in HepG2 cells but not as high as positive control (H_20_2 treatment). 0.1% DMSO which is used as negative control showed similar results with medium control whereas, 1% DMSO treatment which is also used as positive control also induced DNA damage but less than the boric acid treatment at IC50 concentration. Boric acid treatment at IC25 concentration had similar effect as medium control (Figure 5D). Therefore, according to our comet results, boric acid at IC50 concentration started to induce DNA damage in HepG2 cell line. Higher concentrations of boric acid most probably would have more genotoxic effects on HepG2 cells causing more DNA damage.



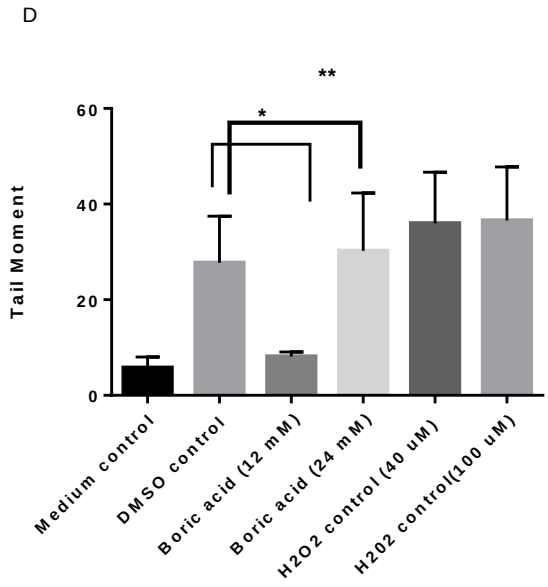


Figure 5. Boric acid-induced DNA damage in HepG2 cell line. (A, B, C) Representative images of assay results, 100 µM hydrogen peroxide treatment as positive control, 24 mM boric acid treatment and medium control, respectively. (D) HepG2 cells treated with boric acid (12 and 24 Mm) and 1% DMSO and hydrogen peroxide (40 and 100 µM) as positive controls for 24-hour incubation. DNA damage was studied by comet assay. Data was represented as mean ± SEM (* $p<0.05$ **$p<0.01$)

Comet assay is a reliable, sensitive and rapid method for detecting genotoxicity of drugs or chemicals. Our results suggested that, boric acid treatment at IC50 concentration could induce DNA damage on HepG2 cell line. In literature, there

were no studies about whether boron derivatives such as boric acid and borax induce DNA damage on cell lines. IC50 concentration of boron though is not a low concentration, still shows genotoxic effects. This is an important finding especially for the use of boron compounds as drugs or as targeting materials like nanoparticles.

Boron genotoxicity was investigated in boron-exposed males [52]. In this study, sperms of boron-exposed males were treated with boron compounds to detect DNA damage. After treatment, very week correlation between boron concentration and DNA strand breaks in sperm cells was observed. Also, there was no big difference between control groups and exposed groups. Hence, according to the results of this research boron compounds did not induce genotoxicity in reproductive cells of males.

In another study, which was carried out in order to determine the genotoxic mechanisms of boron compounds in zebrafish; zebrafish were exposed to different boric acid and borax concentrations, namely, 1, 4, 16, 64 mg/l for 24, 48, 72 and 96 hours. Comet analysis results revealed that 96-hour treatment and high concentrations of boron compounds could induce DNA damage in zebrafish erythrocytes [53].

Kim and coworkers [54] irradiated human lymphocytes with gamma ray to produce DNA damage. Then, these cells were treated with 50 nM and 250 nM boron compound and 50 nM of gadolinium, separately. DNA damage was studied by comet assay. Consequently, the results suggested that, treatment with boron compounds was interestingly reducing further DNA damage in lymphocytes. On the contrary, only gadolinium treatment introduced additional DNA damage. Lymphocytes treated with a mixture of boron and gadolinium compounds have reduced amounts of DNA damages were also found.

Another research about the protective effect of boric acid on oxidative damaged V69 cell line was carried out by Yilmaz and coworkers [55]. In this study, comet assay was used for the determination of genotoxicity of boric acid and

H_20_2. V9 fibroblast cells were treated first with H_2O_2 and then with different concentrations of boric acid (3, 10, 30, 100 and 200 μM). Consequently, the results showed that, the treatment of boric acid has reduced H_2O_2 induced DNA damage significantly.

All these results showed that, boric acid can diminish DNA damage which was caused by other genotoxic chemical. However, in the literature, there were also some contradictory results; boric acid which was applied to non-carcinoma cells showed that at high concentrations and with long exposure of boric acid could induce DNA damage in those cells [66].

Our result also suggested that boric acid treatment can induce DNA damage in HepG2 cells. So furthermore in order to test this genotoxic effect we checked for micronucleus formation another commonly used genotoxicity test.

3.3 Effect of boric acid on Micronucleus, MN formation in HepG2 cell line

HepG2 cells were treated with boric acid at IC25 and IC50 concentrations for 24 hours. Also, some of the cells were treated with 1 μg/ml of Mitomycin C (MMC) as positive control. Mitomycin C is a kind of antibiotic which is isolated from *Streptomyces caespitosus* and in clinical pharmacology, it is used for the inhibition of DNA synthesis. Mitomycin C induced cross linking between guanine and cytosine in DNA, so at high concentrations of MMC, RNA and protein synthesis were also suppressed [74]. Hence, in this assay, MMC selected as a positive control.

After 24-hour treatment, cytochalasin-B was added onto cells, as dividing cells appear as binucleated after blocking the cytokinesis with cytochalasin B. It inhibits microfilament formations which are necessary for the completion of cytokinesis [47].

The results given in Figure 6 showed that, boric acid treatment at IC50 concentration induced MN formation in HepG2 cells. But, boric acid treatment

at IC25 concentration did not cause any significant MN formation (Figure 6D). In addition, there was no change in CBPI value among the treatment groups (Figure 6E). CBPI was used in order to estimate cytostatic activity of the treatments.

As a result, CBMN assay results indicated that, boric acid could be considered genotoxic depending on the concentration. Boric acid treatment of HepG2 cells increased MN formation which was significantly initiated at IC50 concentration.

DNA damage at chromosome level is a critical step of the genotoxicity assessment studies. By means of the micronucleus assay, chromosomal abnormalities can be observed and one can detect the toxic effects of any chemical on the cells. Genotoxic effect of drugs could be determined by MN formation because micronuclei can be formed as an abnormal cell cycle step.

In this study, we have carried out comet assay followed by the CBMN assay in order to detect genotoxic effect of boric acid on HepG2 cell line. In the literature there were few studies about the genotoxic effects of boric acid. Most of the studies emphasized on the protective role of boron compounds on carcinoma and healthy cells [66].

In a research, human lymphocytes were exposed to Aflatoxin B1 which is the causative agent of hepatic carcinoma, and increased DNA damage in human lymphocytes was seen. When lymphocytes were treated with boric acid, further DNA damage was inhibited. So boric acid treatment could create resistance to DNA damage which was induced by Aflatoxin B1 [56].

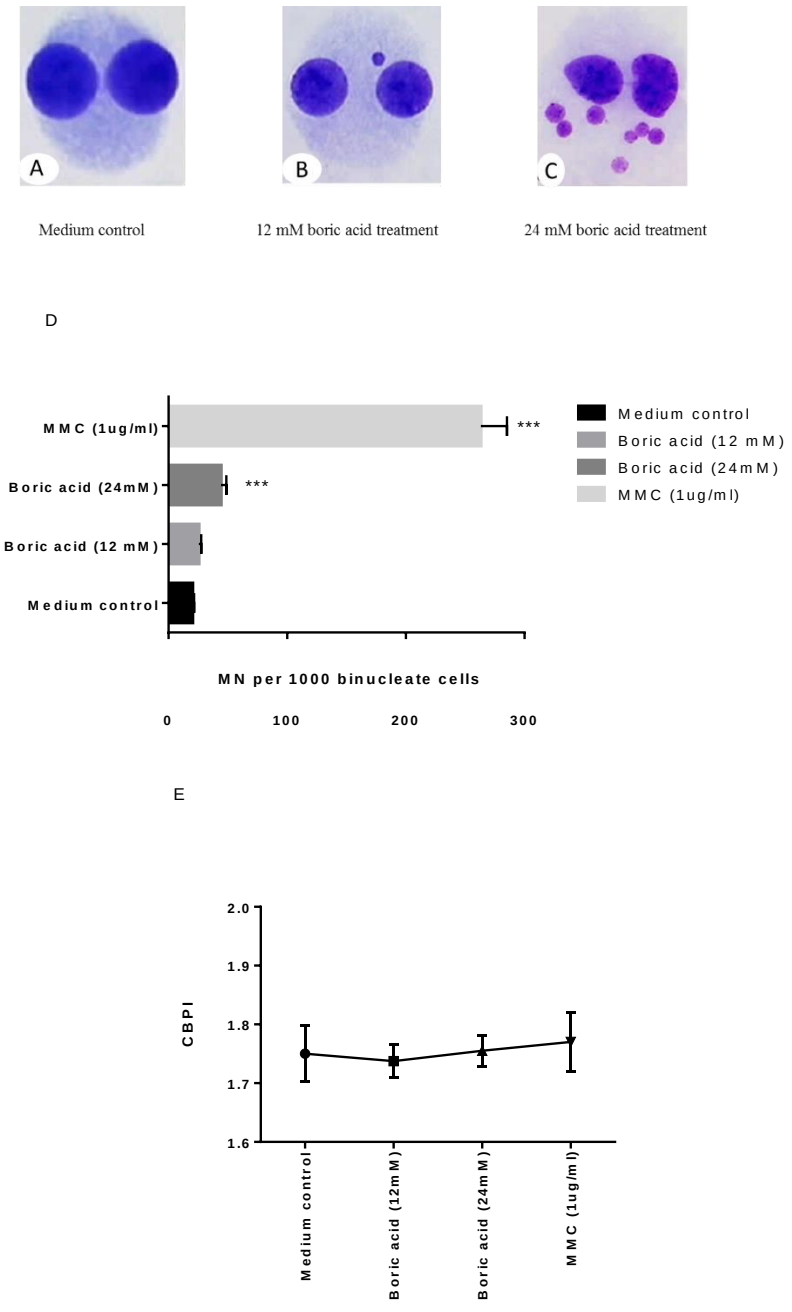


Figure 6. Micronucleus frequency per 1000 binucleated cells of HepG2 cells treated with IC25 and IC50 concentration of boric acid and CBPI (cytokinesis block proliferation index). The data was represented as mean ± SEM (***p < 0.001)

Another study investigated the toxic effects of boron compounds such as boric acid and borax (2.5, 5, 10 μM) on human lymphocytes. In this study, lymphocytes were treated with either titanium dioxide (TiO2) – boron compounds or with only boron compounds. Results showed that, boric acid treated lymphocytes did not increase MN frequency. Also, boric acid and borax treatments further reduced DNA damage, which was caused by titanium dioxide treatment [57].

There was another study which showed the protective effect of the boric acid on normal cells. In this research, genotoxicity was induced by heavy metals such as arsenic trioxide, colloidal bismuth subcitrate, cadmium chloride, mercury chloride and lead chloride in human blood cultures and DNA damaged was detected by CBMN assay. Human blood cultures were then treated with different boron compounds (boric acid, borax, colemanite and ulexite). As a result, it was reported that boron compounds decreased genotoxic effect of the tested heavy metals [58].

In this study, we investigate the effects of boric acid treatment alone on HepG2 cell line. According to our results, boric acid treatment at IC50 concentration can induce MN formation so in this way, boric acid can trigger apoptosis. However, some research results suggested that boric acid could have different effects on normal cell line and carcinoma cell line [66].In normal cell line, boron compounds could protect DNA damage and cell death and it could rescue further cytotoxic and genotoxic effects.

In order to investigate the effect of boric acid on HepG2 metabolic pathways we further carried out microarray analysis.

3.4 Microarray analysis

3.4.1 Normalization and visualization of data

We treated the HepG2 cells with boric acid at IC50 concentration and also with 0.1% DMSO as a negative control. Afterwards, microarray experiments were carried out in order to find differentially expressed gene lists which were affected by boric acid treatment at this concentration. In this assay, biological replicates were used. For example, for both boric acid treatment and 0.1% DMSO treatment three replicates are designed and analyzed accordingly.

Before differentially expressed gene analysis, raw data acquired from arrays were loaded into Array Analysis online software for normalization of the microarray data. By means of this software, sample quality, array cluster and hybridization could be determined. Also, normalization and some correction could be done by pre-processing methods. In this assay, raw data which were boric acid treated arrays (n=3) and 0.1% DMSO control arrays (n=3), were corrected by RMA normalization methods.

The pre-processing report provided information about sample quality. According to density histogram, normalization of sample was well enough because sample density showed same distribution (Figure 7).

As seen in **Figure 8**, all arrays were fit in the same line and their mean and median values were almost same. The quality of the data from all array were sufficient for further analysis. Also result after RMA normalization suggested that there was not any significant variance between boric acid treated and 0.1% DMSO treated array data.

In clustering analysis is done for boric acid treatment arrays (n=3) and 0.1% DMSO treatment arrays (n=3). As seen in **Figure 9**, after RMA normalization, 0.1% DMSO treatment arrays belonged to same group and they were clustered. Likewise, boric acid at IC50 concentration arrays were put into same clustered group.

So, in compliance with pre-processing report, all array results were well normalized with RMA normalization methods. Microarray experiments could now be carried out. Also, as there was no variance between array results, they should be used for visualization of data.

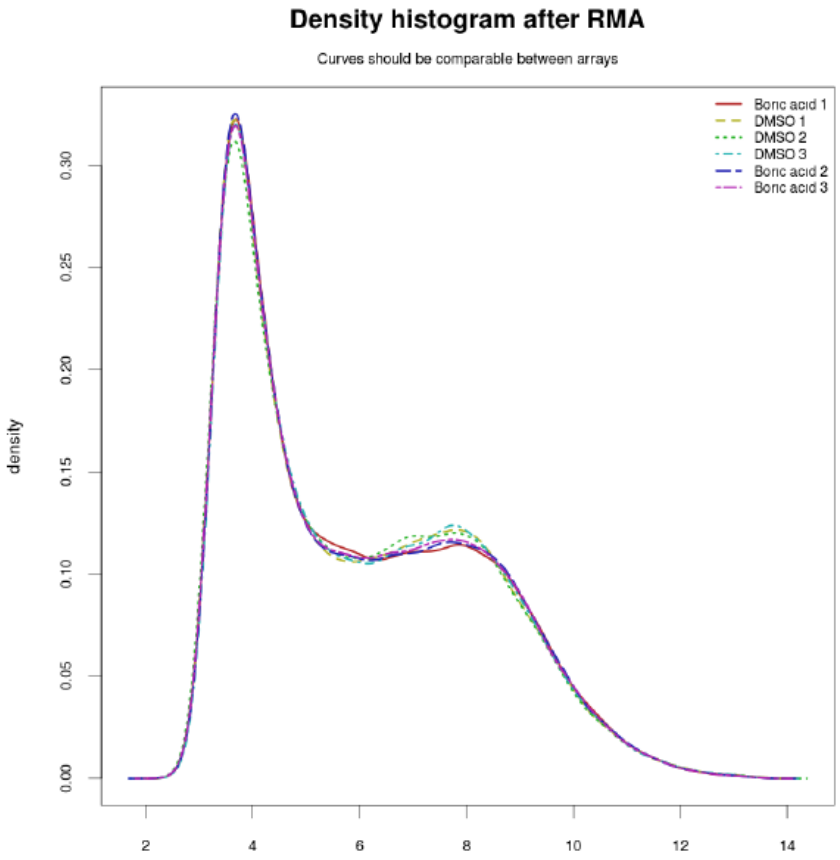


Figure 7. Density histogram after RMA normalization method. Array results were loaded into Array analysis online software (http://www.arrayanalysis.org). DMSO control (n=3) and boric acid treatment at IC50 concentration (n=3) arrays were well normalized. RNA density of control groups and treatment groups show same histogram manner.

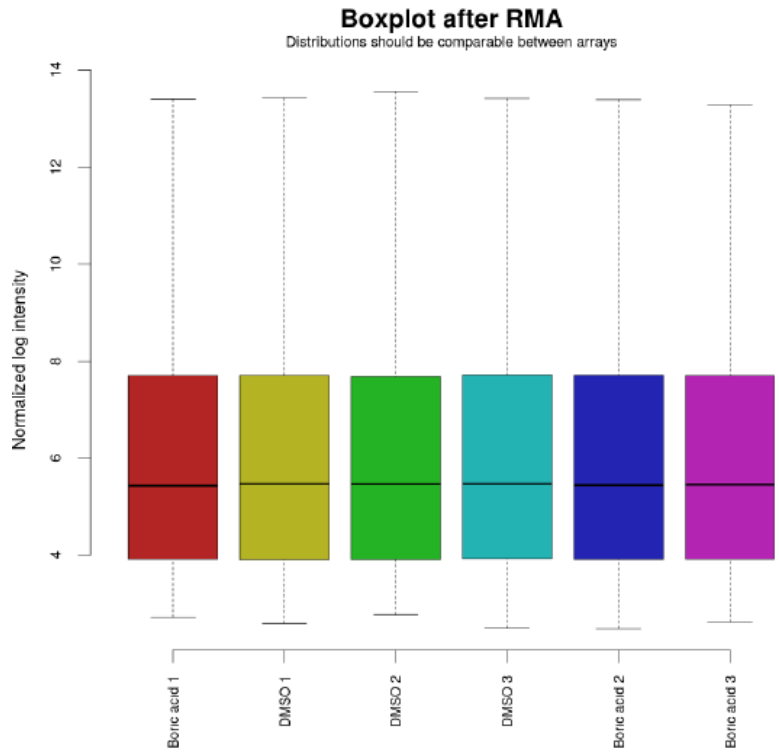


Figure 8. Box plot after Robust Multiarray Average (RMA) normalization. Array results were uploaded into Array analysis online software (http://www.arrayanalysis.org). DMSO control (n=3) and boric acid treatment at IC50 concentration (n=3) arrays were well normalized and mean of array fit in the same line.

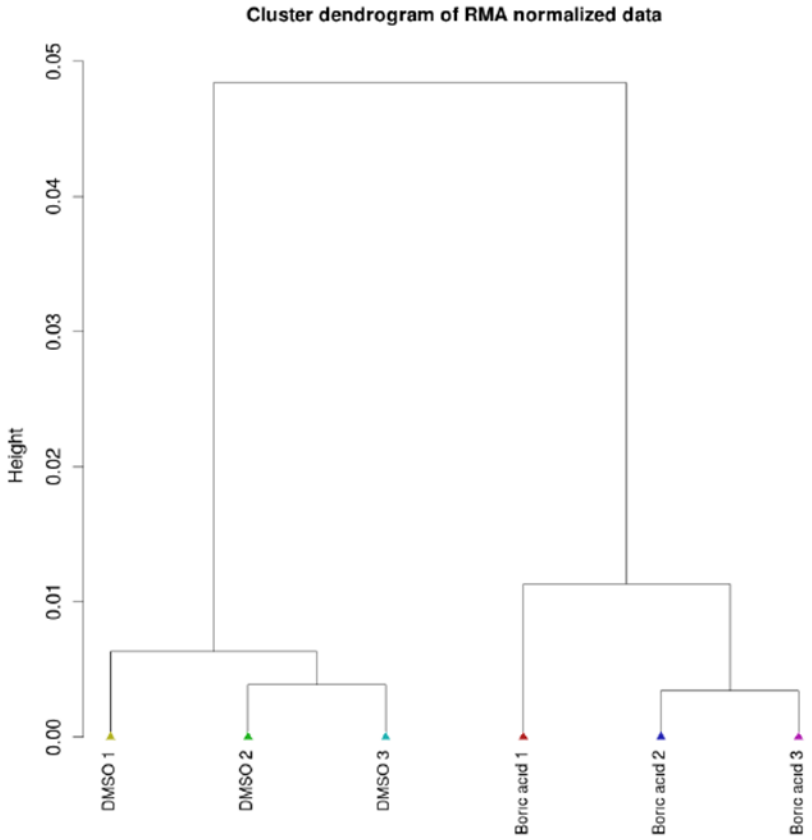


Figure 9. Cluster analysis of array after RMA normalization. Array results were loaded into Array analysis online software (http://www.arrayanalysis.org). DMSO control (n=3) and boric acid treatment at IC50 concentration (n=3) arrays were well normalized. DMSO control groups and boric acid treatment groups were clustered on each other.

After ensuring the quality of the data, BRB array tools which is a package for the statistical analysis and visualization of microarray gene expression, is used. This tool is available as an add-on tool to Microsoft Office Excel. For analysis and visualization of the data, CEL file of the array was uploaded into BRB array tools. 1674 genes were passed through the gene filter with two-sample t-test with random variance model is used as type of univariate test and nominal significance level of each univariate test was 0.001. In class comparison

analysis, two-sample t-test with random variance model is used as type of univariate test and nominal significance level of each univariate test was 0.001. According to statistical analysis, total of 828 genes were significant at the nominal 0.001 level of the univariate test with the fold change 2 (see Appendix A). Among the differentially expressed gene list, 361 and 467 genes were down and up regulated, respectively.

As seen in **Figure 10**, dynamic volcano plot of significant genes show distribution of the up and down regulated genes upon boric acid treatment.

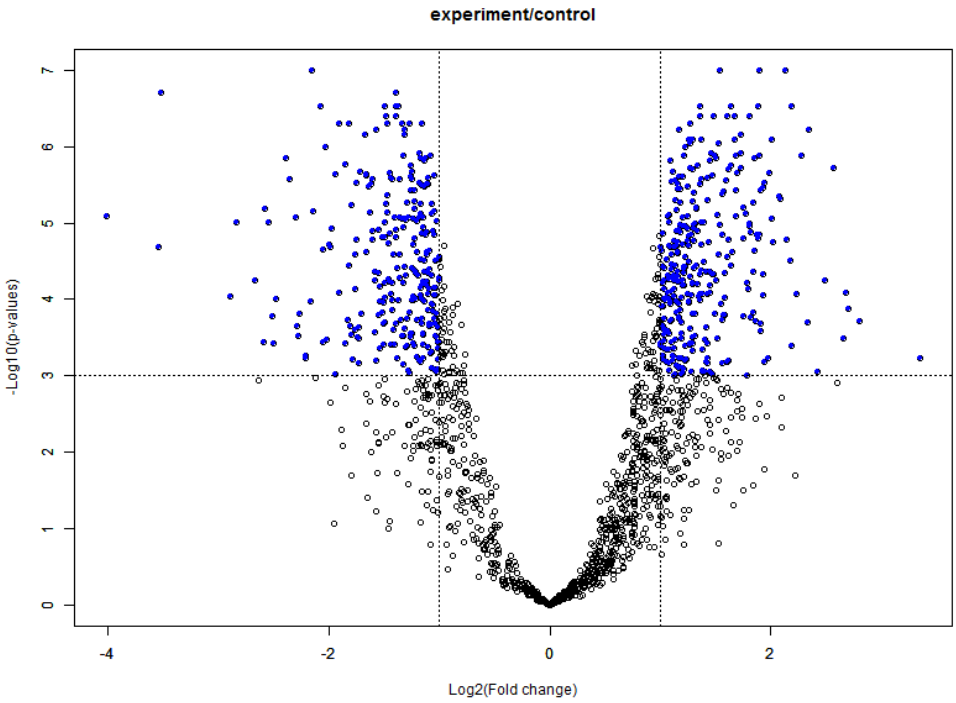


Figure 10. Volcano of differentially expressed genes for boric acid treatment at IC50 concentration. The first 828 genes are significant among the class (class 1: 0.1% DMSO treatment, class 2: Boric acid treatment at IC50 concentration) at the nominal 0.001 level of the unvariate test with the fold change 2 ($p<0.001$)

3.4.2 Pathway analysis of differentially expressed genes

After the determination of the differentially expressed genes, annotation of the genes are performed to reveal the biological functions of the genes. In order to understand the effects of the boric acid treatment at IC50 concentration, up and down regulated genes were uploaded into The Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/).

DAVID is online software and it provides functional annotations. By the DAVID annotation results, up and down regulated genes were found in three pathways which were BBID, BIOCARTA and KEGG pathways (Figure 11). Among the pathways analysis tools, KEGG pathway included the highest number of differentially expressed genes.

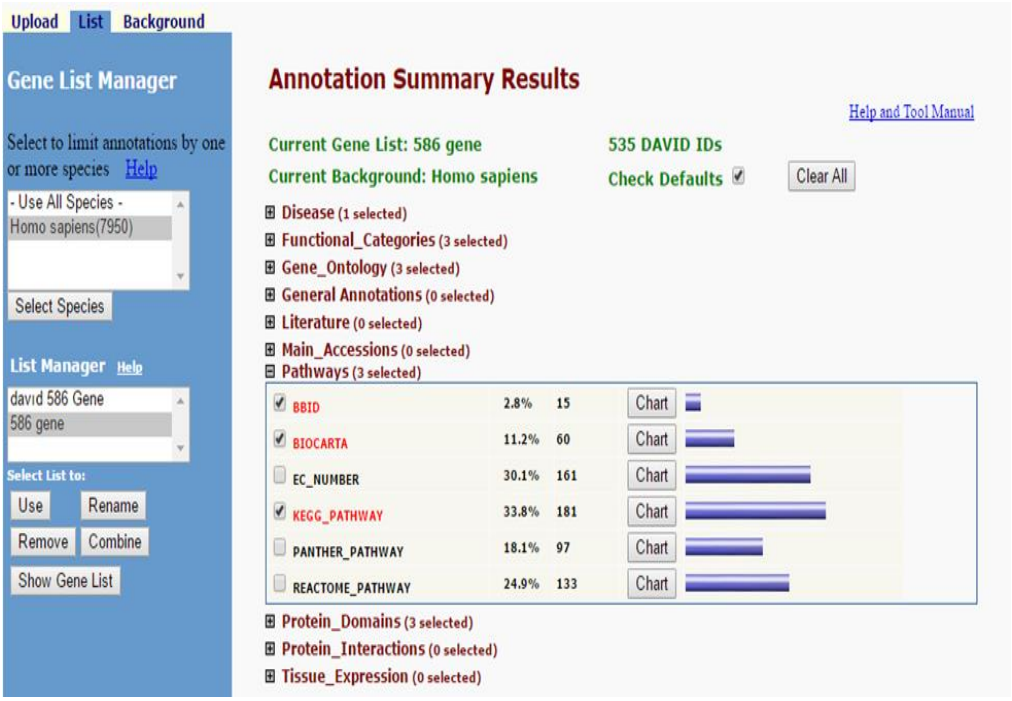


Figure 11. Annotation summary results of the differentially expressed genes

In the KEGG pathway, 18 different pathways were found to be enriched by the differentially regulated genes after boric acid treatment. These pathways were listed in Figure 12, showing unadjusted p-value for the pathway enrichment and adjusted p-value after Benjamini Hochberg correction. According to KEGG pathway analysis, mostly cell cycle, DNA replication and steroid biosynthesis pathways were affected upon boric acid treatment (Table 11).

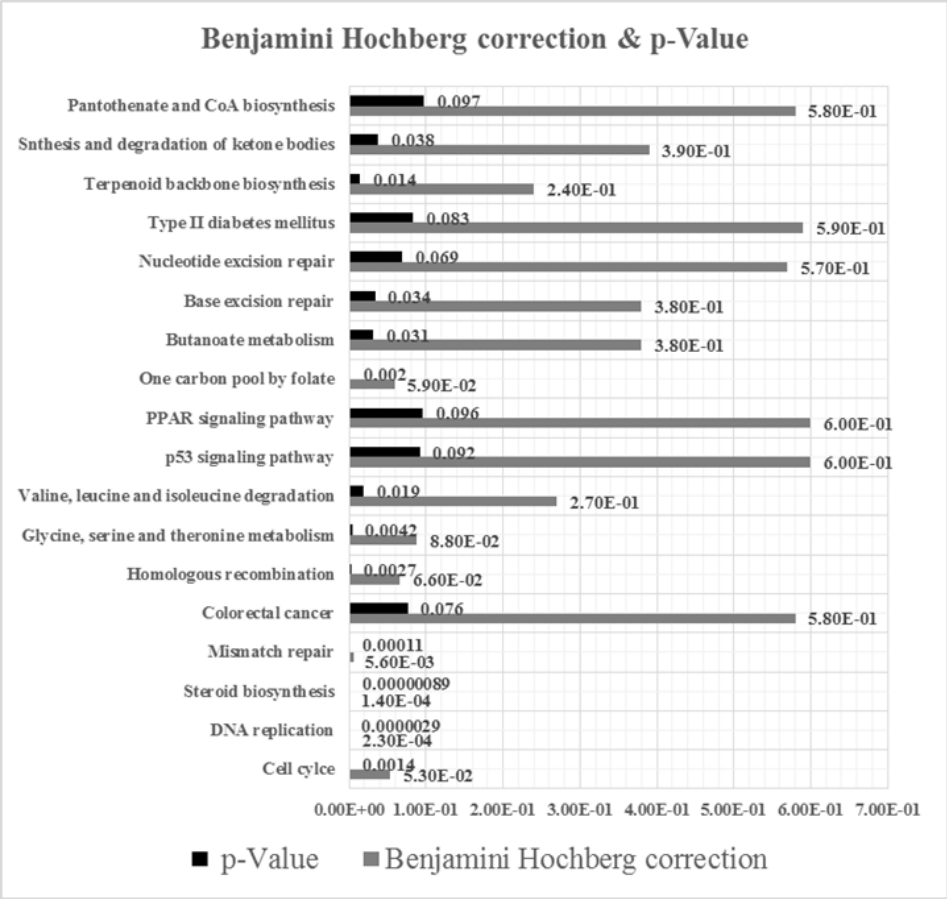


Figure 12. List of enriched KEGG pathways according to the differentially expressed genes identified after boric acid treatment.

Table 11. The top of the three affected pathways by boric acid treatment at IC50 concentration.

Category	Term	Number of genes	p-value
KEGG pathway	Cell cycle	13	1.4E-3
KEGG pathway	DNA replication	10	2.9E-6
KEGG pathway	Steroid biosynthesis	8	8.9E-7

In this study we have observed increase of both cytotoxicity and genotoxicity in HepG2 cells with the increased concentration of boric acid. So the enrichment of both cell cycle and DNA replication pathways where differentially expressed genes are mapped confirmed our findings at the cellular level. Interestingly we have also observed the enrichment of steroid synthesis pathways after boric acid exposure.

3.4.3 Visualization of differentially expressed genes in affected pathways

In BRB array analysis, differentially expressed genes were observed according to gene filter options. After class comparison analysis, differentially expressed genes obtained for 0.1% DMSO control and boric acid treatment at IC50 concentration. For the purpose of pathway analysis, up and down regulated genes are annotated according to their function and related biological pathway.

As seen in **Table 12**, there were 17 genes which were significantly changed in the cell cycle pathway. According to heat map represented in **Figure 13**, among the 17 genes, only 2 of them namely, CDKN 1A and TGFB2 was up-regulated upon boric acid treatment at IC50 concentration. But, their fold changes are small. The other 15 genes were significantly down regulated.

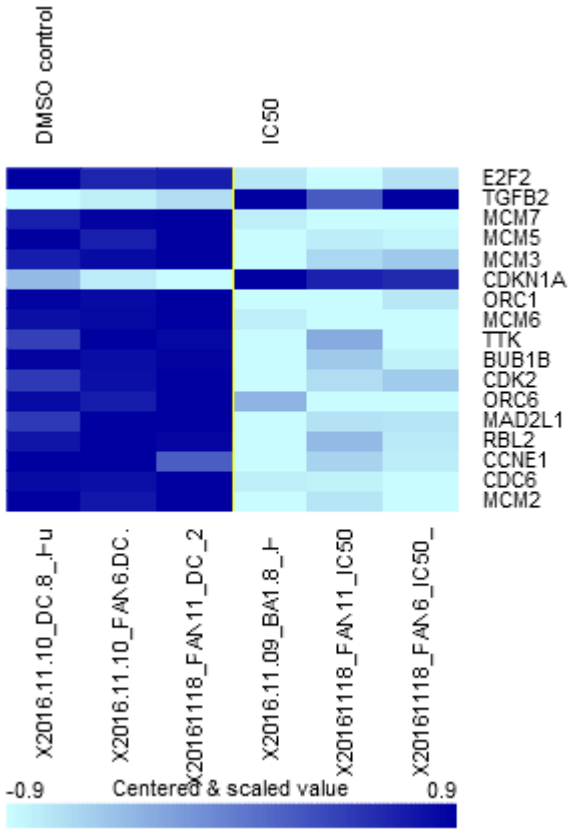


Figure 13. Heat map of significant genes in cell cycle pathway. The first 17 genes were significant at the nominal 0.001 level of the univariate test with the fold change 1.5. The color shows up and down regulated genes.

CDKN 1A is a cyclin-dependent kinase inhibitor 1A (p21, Cip1) and TGFB2 is transforming growth factor, beta 2, both working in the regulation of cell cycle. By activating the cyclin-dependent kinase inhibitor most probably CDK was inhibited affecting the cyclin binding hence the cell cycle [76]. Also CDK2 gene was down regulated, which actually supports this result.

TGFB2 gene encodes a secreted ligand of the TGF-beta (transforming growth factor-beta) superfamily of proteins and disruption of the TGF-beta/SMAD pathway has been implicated in a variety of human cancers [67].

Among the cell cycle gene list, origin recognition complex (ORC1-6) can bind replication forks and they can initiate DNA replication [72].

Retinoblastoma like complex (Rb) is phosphorylated by cyclin-E-Cdk2 in order to initiate G1 phase of cell cycle. E2F is released by phosphorylated Rb and then they drive S phase of cell cycle. Also, Cyclin- E- Cdk2 phosphorylates Smad3 to inhibit transcription of Smad3. Together with all complex facilitates cell cycle, Smad3 and TGFB2 are the regulatory factors to inhibit cell cycle progression [73].

Table 12 shows the gene symbols and the names of the up and down regulated genes by boric acid treatment. Also, the observed fold changes between the treatment groups given in the table could give information about how these changes in gene expressions could affect the pathway.

Table 12. Genes which are differentially expressed among classes in cell cycle pathway.

Probe set	Gene symbol	Gene name	Fold change
7916167	ORC1	origin recognition complex, subunit 1	3.12
8007071	CDC6	cell division cycle 6	3.18
8055426	MCM6	minichromosome maintenance complex component 6	2.34
8072687	MCM5	minichromosome maintenance complex component 5	3.73
8082350	MCM2	minichromosome maintenance complex component 2	2.45
8141395	MCM7	minichromosome maintenance complex component 7	2.26

Table 12. (Cont'd)

Probe set	Gene symbol	Gene name	Fold change
7982663	BUB1B	BUB1mitotic checkpoint serine/threonine kinase B	2.19
8102560	MAD2L1	MAD2 mitotic arrest deficient-like 1	2.1
7913644	E2F2	E2F transcription factor 2	1.95
7995354	ORC6	Originrecognition complex, subunit 6	2.39
8127031	MCM3	minichromosome maintenance complex component 3	3.36
7995631	RBL2	retinoblastoma-like 2	2.29
7909789	TGFB2	transforming growth factor, beta 2	0.49
8119088	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.35
7956076	CDK2	cyclin-dependent kinase 2	1.85
8027402	CCNE1	cyclin E1	1.72
8120838	TTK	TTK protein kinase	1.83

As seen in **Table 13**, there were 10 genes which were significantly changed in the DNA replication pathway. Significant changes in gene expressions of DNA replication pathway was also observed by BRB array tools. Heat map of these genes in DNA replication pathway indicated that the expression of the first 10 genes were significant at the nominal 0.001 level of the univariate test with the fold change of 2. In boric acid treatment array, only POLD4 gene was up-regulated while the other 9 genes were down-regulated as seen in **Figure 14**.

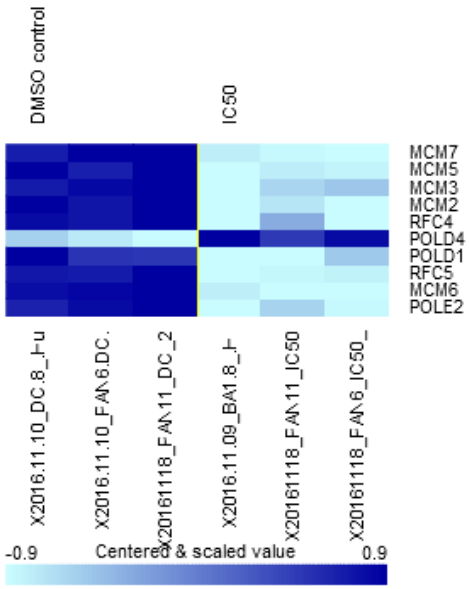


Figure 14. Heat map of significant genes in DNA replication pathway. The first 10 genes were significant at the nominal 0.001 level of the univariate test with the fold change 2. The color shows up and down regulated genes.

Table 13 shows the gene information of the affected genes by giving their names, symbol and fold change among classes. According to expression fold change results, the first 10 gene expressions were changed significantly, and among them, only POLD4 was up-regulated with boric acid treatment. All the other genes were down regulated.

POLD4 is one of the tetrameric DNA polymerase delta complex. It plays role in DNA replication and repair. Under DNA replication stress, its expression increases and the other complex such as POLD1 and POLD2 expression decreases [68].

All the down regulated genes are related to DNA replication process. For example, minichromosome maintenance complex subunit MCM2-7 is required for S phase checkpoint upon DNA damage stress, and it is essential for initiation

and elongation in DNA replication process [69]. So it's down regulation will affect the whole cell cycle.

Table 13. Genes which are differentially expressed among classes in DNA replication pathway.

Probe set	Gene symbol	Gene name	Fold change
8072687	MCM5	minichromosome maintenance complex component 5	3.73
8082350	MCM2	minichromosome maintenance complex component 2	2.45
8141395	MCM7	minichromosome maintenance complex component 7	2.26
7959052	RFC5	replication factor C (activator 1) 5	2.47
7978846	POLE2	polymerase (DNA directed), epsilon 2, accessory subunit	2.05
8127031	MCM3	minichromosome maintenance complex component 3	3.36
8055426	MCM6	Minichromosome maintenance complex component 6	2.34
7949746	POLD4	polymerase (DNA-directed), delta 4, accessory subunit	0.48
8092640	RFC4	replication factor C (activator 1) 4	2.05
8030641	POLD1	polymerase (DNA directed), delta 1, catalytic subunit	2.08

As seen in Table 14, there were 7 genes which were significantly changed in the cell cycle pathway. According to our array results we also found that, steroid biosynthesis pathway was affected by boric acid treatment. Figure 15 shows the heat map of the significant genes observed in steroid biosynthesis pathway. Class comparison analysis revealed that the first 7 genes were significant at the nominal 0.001 level of the univariate test with the fold change of 2.

Interestingly, in steroid biosynthesis pathway, all differentially expressed genes were down-regulated with boric acid treatment.

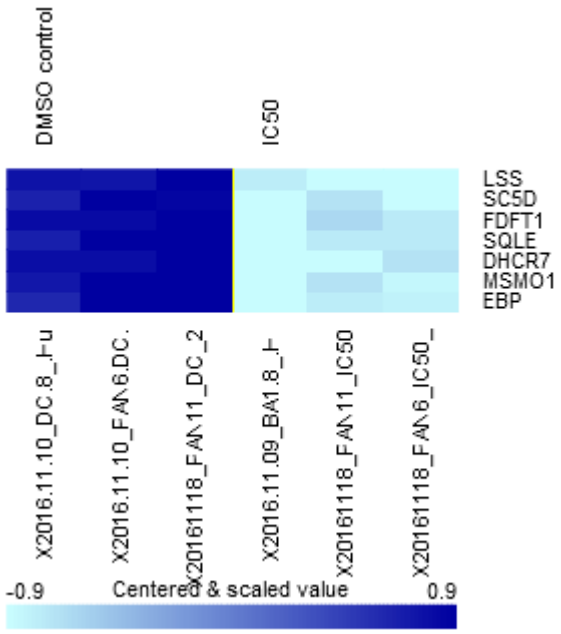


Figure 15. Heat map of significant genes in steroid biosynthesis pathway. The first 7 genes were significant at the nominal 0.001 level of the univariate test with the fold change 2. The color shows up and down regulated genes.

Table 14 shows the names of the genes and the fold changes between the classes. Among them DHCR7, 7-dehydrocholesterol reductase is the enzyme which is responsible for the final step in cholesterol production in many types

of cells. Specifically, 7-dehydrocholesterol reductase converts 7-dehydrocholesterol into cholesterol. Any decrease in the gene expression could affect the cholesterol production [75]. Also, LSS, lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase), plays a role in cyclization of oxidosqualene into lanosterol which is a reaction during steroid synthesis. Lens protein aggregation can be regulated by the production of lanosterol [70].

SQLE is another enzyme in cholesterol synthesis. This gene is especially used for the diagnosis of early stage breast cancer. It is up-regulated in early stage breast cancer [71]. So it can be suggested that boric acid' protective effect might be though down regulation of SQLE gene.

EBP, emopamil binding protein (sterol isomerase), converts delta8-sterols into delta7-isomers which is required for the last step of cholesterol synthesis. It was found that EBP decreased cell viability of human prostate cancer cells and could be used as anticancer drugs [77].

Table 14. Genes which are differentially expressed among classes in steroid biosynthesis pathway.

Probe set	Gene symbol	Gene name	Fold change
7950067	DHCR7	7-dehydrocholesterol reductase	2.82
8098195	MSMO1	methylsterol monooxygenase 1	3.62
8070961	LSS	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	2.57
8167305	EBP	emopamil binding protein (sterol isomerase)	3.22
8148280	SQLE	squalene epoxidase	2.52
8144669	FDFT1	farnesyl-diphosphate farnesyltransferase 1	3.42
7944656	SC5D	sterol-C5-desaturase	2.3

After revealing the differentially expressed genes and top pathways enriched, all genes which were listed in these pathways are analyzed with the Genemania online tool. Genemenia software is able to give information about the functional interaction networks between genes from a gene list. This interaction could be

genetic, physical, co-localization, co-expression or through shared protein domains. Genemania allows users to perform network analysis, where each color represents one functional interaction. This network analysis consists of the gene annotations based on the Gene Ontology (GO) [48].

Genemania analysis showed us the previously known interactions between the differentially expressed genes identified in our study. The results suggested that differentially expressed genes in cell cycle pathway, DNA replication and steroid biosynthesis could be related to each other. Cell cycle network analysis revealed that there was a relationship between 17 significant genes with the others.

As seen in **Figure 16**, these differentially expressed genes could be represented in an interactions network.

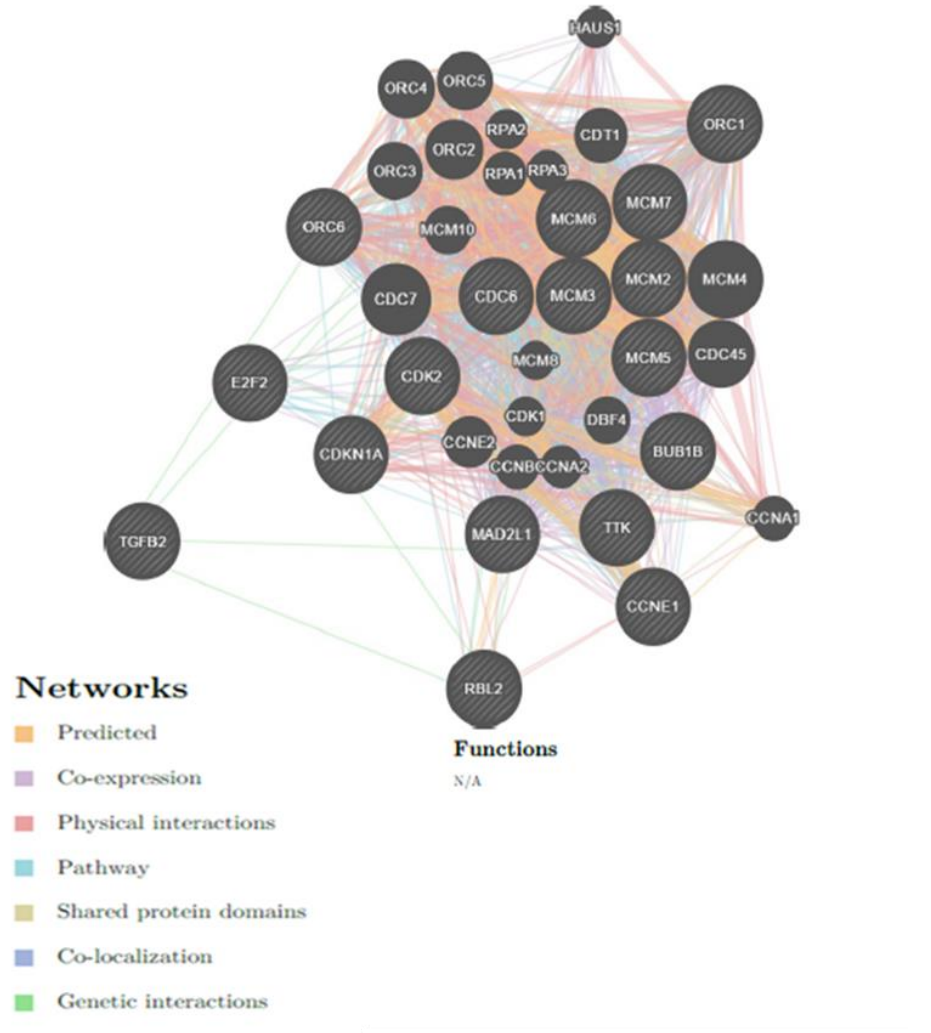


Figure 16. Biological network of significant genes in cell cycle pathway

Similarly, as shown in **Figure 17**, differentially expressed genes in DNA replication pathway could also interact with each other.

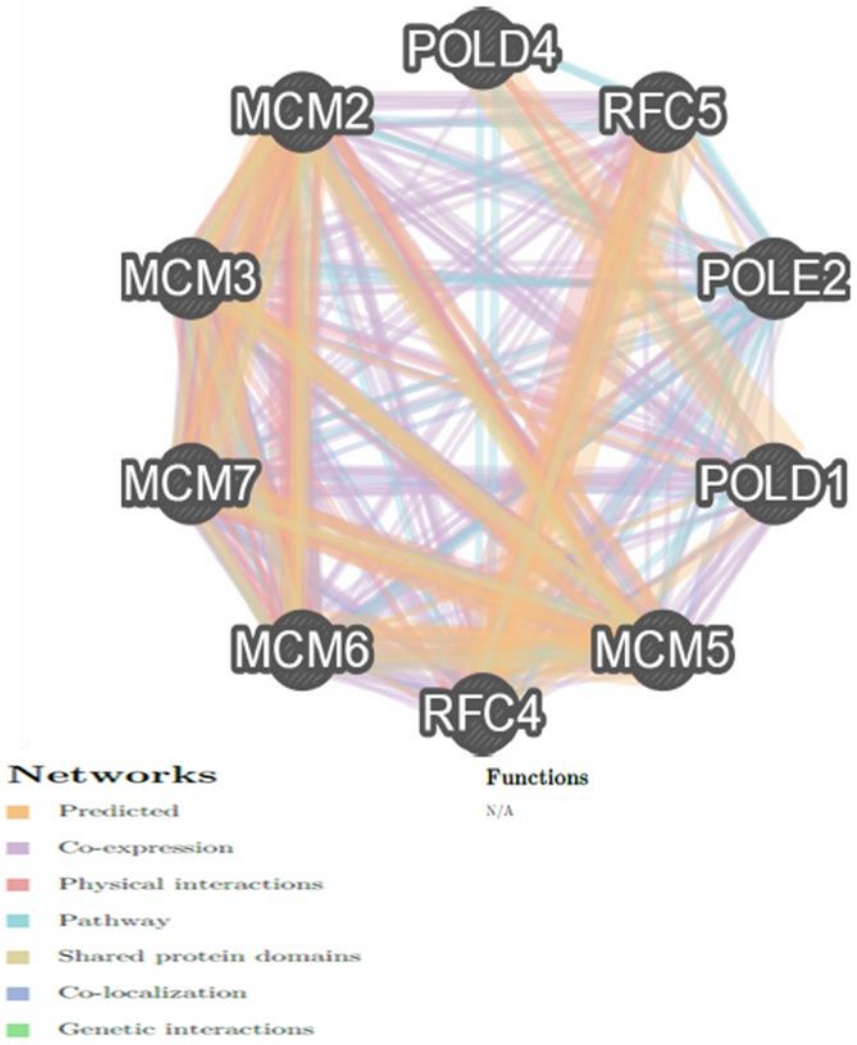


Figure 17. Biological network of significant genes in DNA replication pathway

Finally, **Figure 18** showed significantly expressed genes in steroid biosynthesis which had a biological network. The results indicated that, these genes could interact with each other also through co-localization, co-expression or shared protein domains.

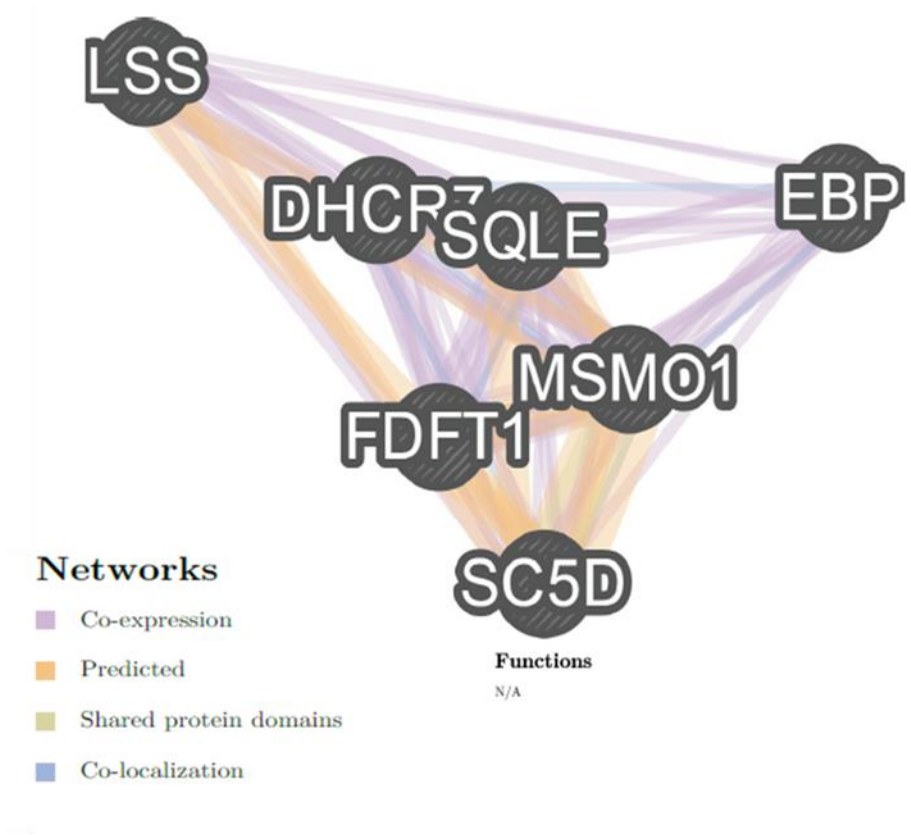


Figure 18. Biological network of significant genes in steroid biosynthesis pathway.

In order to understand the relation between these pathways further research is required. The relation between cell cycle and DNA replication pathways are actually confirming our previous genotoxicity results. After validation of microarray results with by PCR and western blot experiments, functional studies on individual pathways such as steroid pathway including the

cholesterol synthesis should be investigated. Besides the roles of steroid hormones in the progression of certain type of cancers, the results may provide a totally different role of boric acid on lipid metabolism especially in cholesterol synthesis.

In the present study, our microarray results suggested that boric acid treatment at IC50 concentration affect mainly cell cycle, DNA replication and steroid biosynthesis pathways. According to our cell cycle and DNA replication pathway analysis, boric acid treatment caused cell cycle arrest and improper DNA replication in HepG2 cells. Cytotoxicity and genotoxicity test results also showed the inhibition of cell growth. So at this concentration of boric acid, proliferation of hepatocellular carcinoma cell line, HepG2, was inhibited which is also shown at the level of gene expressions.

In literature there are studies about boron compounds and boron neutron capture therapy (BNCT) treatment which could alter gene expressions of cancer cells and normal cell line. In BNCT, boron related products are used for cancer treatment. There are several studies in order to show the effect of BNCT on gene expressions. In one study, BNCT treatment was found to induce cell cycle arrest and apoptosis [61]. Interestingly, BNCT treatment could change gene expression levels of mitochondrial pathway so it triggered apoptosis.

Also, it was reported that boron treatment led to inhibition of protein synthesis in yeast via phosphorylation of eIF2a in a Gcn2 kinase [62]. Furthermore, in one study, it was detected that boron treatment reduced the expression of adipocyte related genes and proteins. In adipocyte cells, boron treatment couldn’t induce apoptosis but it led to arrest cell cycle genes [63].

In another study, the apoptotic effect of boron compounds was tested in rat livers and after gene expression analysis, it was reported that, though NF-κB gene expression was down-regulated, caspase-3 gene expression was up-regulated suggesting that boron compounds could trigger apoptotic pathways [64].

In our study, 24 mM boric acid concentration showed no effect on apoptotic pathway genes.

Capati and coworkers [59], treated osteoplastic cells with 0.1 mM of boron compound in order to detect the effect on Ca^{2+} metabolism. According to the microarray experiments, 5 genes which are part of the Ca^{2+} metabolism, were up regulated compared to control groups.

In another study, it was reported that DU-145 prostate cancer cell line when treated with 10 μM boric acid, it could inhibit cell proliferation without apoptosis and activate EIF2α/ATF4 and ATF6 Pathways [60]. These pathways are important for tumor suppression and cell differentiation, and in this way boric acid led to suppress oncogenes and tumor growth.

In conclusion, our present study showed that, boric acid treatment at IC50 concentration did not induce apoptotic pathways directly but it could trigger mainly cell cycle arrest and inhibit DNA replication pathways. Therefore, boric acid exposure could inhibit the proliferation of the HepG2, hepatocarcinoma cells. An interesting result which is obtained from microarray studies is that, boric acid treatment could reduce steroid biosynthesis by down regulating the gene of an important enzyme in the cholesterol synthesis so it could be new treatment approach for lipid metabolism disorders. In order to test this effect of boric acid, further studies with normal liver cell lines using PCR and Western blot experiments should be done.

CHAPTER 4

CONCLUSION

Boron compounds such as boric acid and borax are essential elements for human, animal and plant health. They are involved in cell membrane function, hormonal metabolism and enzyme reactions. Some studies reported that, boron compounds can play important roles in inflammatory pathways as well as cell cycle showing anti-proliferative effect mainly on carcinomas. Still, in literature there are no detailed studies on both cytotoxic and genotoxic effects of boron compounds on carcinoma and normal cells. Also, regulation of cell cycle, apoptosis and effected pathways upon boron treatment remains mystery.

In our study, we aimed to understand the cytotoxic and genotoxic effects of boric acid on human hepatocarcinoma cells (HepG2) and then determine the affected pathways by this treatment. As a result,

- In order to find the inhibitory effect of boric acid on HepG2 cell line, XTT cell proliferation and trypan blue exclusion assays were carried out. IC50 value was found as 24 mM. Cell death initiated at 12 mM boric acid concentration for 24-hour incubation.
- In order to study genotoxicity of boric acid on HepG2 cell line, single cell gel electrophoresis (SCGE) and cytokines block micronucleus (CBMN) assays were performed. Boric acid treatment at IC50 concentration induced DNA damage and increased MN frequency which is an indicator of chromosomal damage also.
- Microarray and pathway analysis results revealed that, 828 genes were differentially expressed in IC50 concentration boric acid treated HepG2

cells compared to 0.1% DMSO control. According to microarray results, 361 genes up-regulated and 467 genes down-regulated upon boric acid exposure for 24 hours. After visualizing the data, differentially expressed genes were uploaded into DAVID database for pathway analysis. Pathways analysis results suggested that boric acid at IC50 concentration could influence cell cycle and DNA repair and replication mechanism as well as steroid biosynthesis pathways. In conclusion, boric acid treatment induced cell cycle arrest at mitotic phase to inhibit cell viability.

- In our present study, we suggest that boric acid treatment can be a new approach for cancer therapy and also lipid metabolic disorders. Boron toxicity observed at relatively high concentrations of boric acid with IC50 value of 24 mM. In that aspect boron nanoparticles can be used for targeted therapies. Also though the concentration is high but at these high concentrations it can be used in order to inhibit cell proliferation. But most interesting outcome of this study is the effect on steroid biosynthesis mainly on cholesterol metabolism. Further studies are required in order to reveal this role.

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APPENDICES

APPENDIX A

LIST OF DIFFERENTIALLY EXPRESSED GENE

Table A.1. Differentially expressed gene list between control group and treatment group

p-value	FDR	F.C	Name	Symbol
< 1e-07	2.99 E-05	4.5	PYCR 1	pyrroline-5-carboxylate reductase 1
< 1e-07	2.99 E-05	4.37	TME M97	transmembrane protein 97
< 1e-07	2.99	3.71	S100A	S100 calcium binding protein A3
< 1e-07	2.99	0.23	DHFR	dihydrofolate reductase
1.00E-07	2.99	2.9	FAM1	family with sequence similarity 172,
2.00E-07	2.99	0.08	CLIC1	chloride intracellular channel 1
2.00E-07	2.99	0.38	KYN	kynureninase
3.00E-07	2.99	0.24	BEND	BEN domain containing 7
3.00E-07	2.99	0.39	ZNF1	zinc finger protein 146
3.00E-07	2.99	0.38	AKR1	aldo-keto reductase family 1, member D1
3.00E-07	2.99	4.55	HMG	3-hydroxy-3-methylglutaryl-CoA synthase 1
3.00E-07	2.99	3.68	ORC1	origin recognition complex, subunit 1
3.00E-07	2.99	3.12	C16orf	chromosome 16 open reading frame 72
3.00E-07	2.99	0.35	ERCC	excision repair cross- complementation
3.00E-07	2.99	2.57	PNM	paraneoplastic Ma antigen 1
4.00E-07	2.99	0.36	TRIM	tripartite motif containing 37
4.00E-07	2.99	3.5	SREB	sterol regulatory element binding
4.00E-07	2.99	2.79	CLIC1	chloride intracellular channel 1
4.00E-07	2.99	0.38	NCAP	non-SMC condensin II complex, subunit D3
4.00E-07	2.99	2.57	DHFR	dihydrofolate reductase
4.00E-07	2.99	3.04	ZWIN	ZW10 interacting kinetochore protein
4.00E-07	2.99 E-05	3.19	KPNA 4	karyopherin alpha 4 (importin alpha 3)
5.00E-07	2.99	0.28	GPR3	G protein-coupled receptor 39
5.00E-07	2.99	0.27	COX1	
5.00E-07	2.99	0.42	RPS19	ribosomal protein S19 binding protein 1
5.00E-07	2.99 E-05	0.45	CLIC1	chloride intracellular channel 1
5.00E-07	2.99	0.4	CDCA	cell division cycle associated 7-like
5.00E-07	3.14	5.09	CDC4	CDC42 effector protein (Rho GTPase
6.00E-07	3.14	0.4	ZNF2	zinc finger protein 260
6.00E-07	3.14	0.34	SETD	SET domain containing 5
6.00E-07	3.14	2.24	PTPN	protein tyrosine phosphatase, non- receptor
6.00E-07	3.27	3.32	RNF1	ring finger protein 14
7.00E-07	3.27	0.4	ALG1	ALG12, alpha-1,6- mannosyltransferase
7.00E-07	3.27	2.59	SSC4	scavenger receptor cysteine rich family, 4
8.00E-07	3.27	4.02	MAG	melanoma antigen family D1
8.00E-07	3.27	2.4	CDC6	cell division cycle 6

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
8.00E-07	3.27	3.18	WDH	WD repeat and HMG-box DNA binding
8.00E-07	3.27	2.44	USP21	ubiquitin specific peptidase 21
8.00E-07	3.50	2.88	SRPX	sushi-repeat containing protein, X- linked 2
9.00E-07	3.72	0.24	MCM	minichromosome maintenance complex
1.00E-06	3.72	2.34	NASP	nuclear autoantigenic sperm protein
1.00E-06	3.91	2.74	MND1	meiotic nuclear divisions 1
1.20E-06	3.91	3.32	DHCR	7-dehydrocholesterol reductase
1.30E-06	3.91	2.82	ZNF7	zinc finger protein 770
1.30E-06	3.91	0.47	TRAP	trafficking protein particle complex 6B
1.30E-06	3.91	0.4	MCM	minichromosome maintenance complex
1.30E-06	3.91	3.73	EXO1	exonuclease 1
1.30E-06	3.91	4.87	AAR2	AAR2 splicing factor homolog
1.30E-06	3.91	0.44	CSGA	chondroitin sulfate N-
1.40E-06	3.91	0.19	MSM	methylsterol monooxygenase 1
1.40E-06	3.91	3.62	CGNL	cingulin-like 1
1.40E-06	3.91	2.83	MET	MET proto-oncogene, receptor tyrosine
1.40E-06	3.91	0.46	CASC	cancer susceptibility candidate 5
1.40E-06	3.99 E-05	2.13	STK17A	serine/threonine kinase 17a
1.50E-06	3.99	0.45	TK1	thymidine kinase 1, soluble
1.50E-06	3.99	2.73	DUSP	dual specificity phosphatase 10
1.50E-06	4.45	0.28	UBR5	ubiquitin protein ligase E3 component n-
1.80E-06	4.50	0.42	SFXN	sideroflexin 2
1.80E-06	4.50	3.11	GAS2	growth arrest-specific 2 like 3
1.80E-06	4.54	3.33	DHRS	dehydrogenase/reductase (SDR family)
1.90E-06	4.54	2.4	PLOD	procollagen-lysine, 2-oxoglutarate 5-
1.90E-06	4.54	5.94	MCM	minichromosome maintenance complex
1.90E-06	4.65	2.45	FAM6	family with sequence similarity 64, member
2.00E-06	4.65	3.07	ZFAN	zinc finger, AN1-type domain 2A
2.00E-06	4.69	0.42	NANS	N-acetylneuraminic acid synthase
2.10E-06	4.69	2.16	STOM	stomatin
2.10E-06	4.69	0.3	ITPRI	inositol 1,4,5-trisphosphate receptor
2.20E-06	4.76	0.26	ZNF4	zinc finger protein 426
2.30E-06	4.76	0.32	FANC	Fanconi anemia, complementation group I
2.30E-06	4.76	2.77	MBN	muscleblind-like splicing regulator 1
2.40E-06	4.76 E-05	0.49	ZNF623	zinc finger protein 623
2.40E-06	4.76	0.42	GINS1	GINS complex subunit 1 (Psf1 homolog)
2.50E-06	4.76	2.24	MSH6	mutS homolog 6
2.50E-06	4.76	2.3	DTL	denticleless E3 ubiquitin protein ligase

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
2.50E-06	4.76	2.69	DTNA	dystrobrevin, alpha
2.60E-06	4.76	0.37	POLR	polymerase (RNA) I polypeptide C, 30kDa
2.60E-06	4.76	0.46	MISP	mitotic spindle positioning
2.70E-06	4.76	0.33	SMCR	Smith-Magenis syndrome chromosome
2.70E-06	4.76	0.37	PLK2	polo-like kinase 2
2.70E-06	4.76	0.19	SLC41	solute carrier family 41 (magnesium
2.70E-06	4.76	0.42	BLM	Bloom syndrome, RecQ helicase- like
2.70E-06	4.76 E-05	2.58	HMG CR	3-hydroxy-3-methylglutaryl-CoA reductase
2.70E-06	4.83	3.02	DPEP	dipeptidase 1 (renal)
2.80E-06	4.83	2.2	STAR	StAR-related lipid transfer (START)
2.80E-06	4.90 E-05	2.14	TME M99	transmembrane protein 99
2.90E-06	4.90	0.3	ERAP	endoplasmic reticulum aminopeptidase 2
3.00E-06	4.90	3.83	OTUB	OTU deubiquitinase, ubiquitin aldehyde
3.00E-06	4.90	0.44	AMM	AMMECR1-like
3.00E-06	4.90	0.47	IRS1	insulin receptor substrate 1
3.10E-06	4.90	0.33	SLC1	solute carrier family 1 (glial high affinity
3.10E-06	4.90	0.46	LSS	lanosterol synthase (2,3- oxidosqualene-
3.10E-06	4.90 E-05	2.57	RDH1 0	retinol dehydrogenase 10 (all-trans)
3.10E-06	4.96	0.46	TIGA	TP53 induced glycolysis regulatory
3.20E-06	4.96	0.45	LURA	leucine rich adaptor protein 1-like
3.20E-06	4.98	0.32	VRK1	vaccinia related kinase 1
3.30E-06	4.98	2.37	CMTR	cap methyltransferase 2
3.30E-06	4.98	0.46	MCM	minichromosome maintenance 10
3.30E-06	5.23	3.8	SMAR	SWI/SNF related, matrix associated, actin
3.50E-06	5.25	2.24	MCM	minichromosome maintenance complex
3.60E-06	5.25 E-05	2.26	EBP	emopamil binding protein (sterol isomerase)
3.60E-06	5.25	3.22	TTLL	tubulin tyrosine ligase-like family member 4
3.70E-06	5.25	3.74	ZXDB	zinc finger, X-linked, duplicated B
3.70E-06	5.25	0.42	HSD1	hydroxysteroid (17-beta) dehydrogenase 7
3.90E-06	5.58	2.47	HIST1	histone cluster 1, H3b
4.00E-06	5.67	2.96	CLTB	clathrin, light chain B
4.10E-06	6.04	0.36	CDT1	chromatin licensing and DNA replication
4.40E-06	6.12	4.2	PHGD	phosphoglycerate dehydrogenase
4.50E-06	6.48	4.26	CLSP	claspin
4.80E-06	6.64	2.19	MPH	M-phase phosphoprotein 9
5.00E-06	6.64	2.7	H1F0	H1 histone family, member 0
5.00E-06	6.85	0.43	BIRC5	baculoviral IAP repeat containing 5

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
5.20E-06	6.93	2.46	RETS	retinol saturase (all-trans-retinol 13,14-
5.30E-06	6.95	0.41	TICR	TOPBP1-interacting checkpoint
5.40E-06	6.95	3.57	HMG	high mobility group box 2
5.40E-06	7.03	2.27	PPP2C	protein phosphatase 2, catalytic subunit, beta
5.50E-06	7.05	0.45	OTUD	OTU deubiquitinase 7B
5.60E-06	7.05	0.36	TATD	TatD DNase domain containing 1
5.60E-06	7.12	0.48	S100A	S100 calcium binding protein A16
5.70E-06	7.32 E-05	0.29	AZI2	5-azacytidine induced 2
5.90E-06	7.65	3.37	NUCB	nucleobindin 2
6.30E-06	7.65	2.5	SRXN	sulfiredoxin 1
6.30E-06	7.65	0.43	SQLE	squalene epoxidase
6.40E-06	7.65	2.52	MOSP	motile sperm domain containing 1
6.40E-06	7.65	0.17	LRP3	low density lipoprotein receptor- related
6.40E-06	7.95	2.21	ATP6	ATPase, H+ transporting, lysosomal
6.70E-06	8.31	0.23	C21orf	chromosome 21 open reading frame 59
7.10E-06	8.31	0.48	STK1	serine/threonine kinase 17b
7.10E-06	8.37	0.36	DUSP	dual specificity phosphatase 18
7.20E-06	8.43	0.32	TRMT	tRNA methyltransferase 1
7.30E-06	8.60	0.48	FDFT	farnesyl-diphosphate farnesyltransferase 1
7.60E-06	8.60	3.42	ASRG	asparaginase like 1
7.60E-06	8.65	2.86	DROS	drosha, ribonuclease type III
7.70E-06	8.70	2.12	BHM	betaine--homocysteine S- methyltransferase
7.80E-06	8.80	3.21	CORO	coronin, actin binding protein, 1A
8.00E-06	8.80	2.09	IL18	interleukin 18
8.00E-06	8.80	0.06	METT	methyltransferase like 9
8.10E-06	8.80	2.37	TAGL	transgelin 2
8.10E-06	8.85	0.39	HMG	3-hydroxymethyl-3-methylglutaryl- CoA
8.30E-06	8.85	0.44	SOCS	suppressor of cytokine signaling 4
8.30E-06	8.85	0.41	CDC4	CDC42 small effector 1
8.30E-06	8.89	0.45	NUA	NUAK family, SNF1-like kinase, 1
8.40E-06	8.89	0.38	SMO	spermine oxidase
8.50E-06	8.89	0.2	SC5D	sterol-C5-desaturase
8.50E-06	8.93	2.3	HEXI	hexamethylene bis-acetamide inducible 1
8.60E-06	8.93	0.4	DAGL	diacylglycerol lipase, beta
8.70E-06	8.98	0.42	CDCA	cell division cycle associated 7
8.80E-06	9.03	4.04	ZBTB	zinc finger and BTB domain containing 41
8.90E-06	9.08	0.41	CSNK	casein kinase 1, alpha 1
9.00E-06	9.42	0.49	LPXN	leupaxin
9.40E-06	9.52	0.43	ZNF8	zinc finger protein 860

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
9.60E-06	9.52	0.17	CBS	cystathionine-beta-synthase
9.70E-06	9.52	2.12	MNS1	meiosis-specific nuclear structural 1
9.90E-06	9.52	2.59	ARRD	arrestin domain containing 4
9.90E-06	9.52	0.14	INPP1	inositol polyphosphate-1- phosphatase
9.90E-06	9.52	0.36	CNOT	CCR4-NOT transcription complex, subunit
9.90E-06	9.57	2.5	RFX5	regulatory factor X, 5 (influences HLA class
1.00E-05	9.80	3.49	RFC5	replication factor C (activator 1) 5, 36.5kDa
1.03E-05	1.00 E-04	2.47	FAM117A	family with sequence similarity 117, member A
1.06E-05	1.00 E-04	2.27	ACAT2	acetyl-CoA acetyltransferase 2
1.07E-05	1.00 E-04	2.9	CDCA8	cell division cycle associated 8
1.07E-05	0.00 0101	2.34	LRRCC1	leucine rich repeat and coiled-coil centrosomal protein 1
1.09E-05	0.00 0102	3.51	SESN3	sestrin 3
1.10E-05	0.00 0102	2.29	BTBD10	BTB (POZ) domain containing 10
1.11E-05	0.00 0102	0.4	UBL3	ubiquitin-like 3
1.12E-05	0.00 0107	2.37	RAVER2	ribonucleoprotein, PTB-binding 2
1.19E-05	0.00 0107	2.47	GAREM	GRB2 associated, regulator of MAPK1
1.20E-05	0.00 0107	0.25	TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A
1.20E-05	0.00 0107	0.34	EMP3	epithelial membrane protein 3
1.21E-05	0.00 0108	2.25	BARD1	BRCA1 associated RING domain 1
1.23E-05	0.00 0109	2.53	ALKBH8	alkB homolog 8, tRNA methyltransferase
1.26E-05	0.00 0109	0.33	CTH	cystathionine gamma-lyase
1.29E-05	0.00 0113	0.46	KIAA0101	KIAA0101
1.33E-05	0.00 0113	3.52	PGP	phosphoglycolate phosphatase
1.35E-05	0.00 0114	0.37	TJP3	tight junction protein 3
1.36E-05	0.00 0116	2.04	BRCA1	breast cancer 1, early onset
1.39E-05	0.00 0116	3.68	ATP11B	ATPase, class VI, type 11B

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
1.40E-05	0.000116	0.46	ANKRD50	ankyrin repeat domain 50
1.41E-05	0.000116	0.48	TBL1X	transducin (beta)-like 1X-linked
1.41E-05	0.000117	2.95	SCD	stearoyl-CoA desaturase (delta-9-desaturase)
1.44E-05	0.000117	2.43	KIF14	kinesin family member 14
1.44E-05	0.000117	2.39	TFE3	transcription factor binding to IGHM enhancer 3
1.45E-05	0.000123	0.36	USP38	ubiquitin specific peptidase 38
1.56E-05	0.000124	0.38	FAM200A	family with sequence similarity 200, member A
1.56E-05	0.000126	0.43	GINS2	GINS complex subunit 2 (Psf2 homolog)
1.61E-05	0.000126	3.7	ANXA9	annexin A9
1.61E-05	0.000126	3.08	FAM114A1	family with sequence similarity 114, member A1
1.62E-05	0.000126	4.42	KLRC3	killer cell lectin-like receptor subfamily C, member 3
1.63E-05	0.000129	0.33	ATAD2	ATPase family, AAA domain containing 2
1.68E-05	0.000129	2.38	BCAT1	branched chain amino-acid transaminase 1, cytosolic
1.70E-05	0.000129	2.48	SKA3	spindle and kinetochore associated complex subunit 3
1.70E-05	0.000131	2.18	CENPI	centromere protein I
1.73E-05	0.000132	2.72	TMEM143	transmembrane protein 143
1.75E-05	0.000132	2.24	MPV17L	MPV17 mitochondrial membrane protein-like
1.76E-05	0.000134	4.06	ZNF134	zinc finger protein 134
1.79E-05	0.000136	0.36	IBA57	IBA57 homolog, iron-sulfur cluster assembly
1.83E-05	0.000139	0.43	ANXA3	annexin A3
1.88E-05	0.00014	0.25	CBX5	chromobox homolog 5
1.90E-05	0.000141	2.3	IDI1	isopentenyl-diphosphate delta isomerase 1
1.92E-05	0.000143	2.59	PXK	PX domain containing serine/threonine kinase

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
1.95E-05	0.000144	2.11	SNRNP25	small nuclear ribonucleoprotein 25kDa (U11/U12)
1.98E-05	0.000144	2.39	ARHGEF39	Rho guanine nucleotide exchange factor (GEF) 39
1.99E-05	0.000144	2.21	NEXN	nexilin (F actin binding protein)
2.02E-05	0.000144	0.25	SPC24	SPC24, NDC80 kinetochore complex component
2.03E-05	0.000144	2.17	NCAPH	non-SMC condensin I complex, subunit H
2.03E-05	0.000144	2	ABCG8	ATP-binding cassette, sub-family G (WHITE), member 8
2.03E-05	0.000146	2.2	AQP3	aquaporin 3 (Gill blood group)
2.07E-05	0.000146	0.086	C4orf46	chromosome 4 open reading frame 46
2.08E-05	0.000147	2.14	RAB3B	RAB3B, member RAS oncogene family
2.10E-05	0.000157	0.24	FAM50A	family with sequence similarity 50, member A
2.26E-05	0.000157	0.47	TRAF3	TNF receptor-associated factor 3
2.28E-05	0.000157	2.02	CCNI	cyclin I
2.28E-05	0.000161	3.6	SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1
2.35E-05	0.000161	0.46	MBTPS1	membrane-bound transcription factor peptidase, site 1
2.37E-05	0.000161	3.14	ZNF512	zinc finger protein 512
2.37E-05	0.000161	2.24	L1CAM	L1 cell adhesion molecule
2.40E-05	0.000162	0.43	MYO5C	myosin VC
2.41E-05	0.000163	2.23	UBASH3B	ubiquitin associated and SH3 domain containing B
2.44E-05	0.000168	0.39	BRIP1	BRCA1 interacting protein C- terminal helicase 1
2.54E-05	0.000168	2.89	IDS	iduronate 2-sulfatase
2.54E-05	0.000168	0.29	FAS	Fas cell surface death receptor
2.55E-05	0.000168	0.46	FILIP1L	filamin A interacting protein 1-like
2.55E-05	0.000168	0.38	ELF1	E74-like factor 1 (ets domain transcription factor)

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
2.60E-05	0.00017	2.15	UHRF1	ubiquitin-like with PHD and ring finger domains 1
2.61E-05	0.00176	3.05	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2
2.75E-05	0.00176	2.33	MICALL1	MICAL-like 1
2.75E-05	0.00176	0.5	TTLL5	tubulin tyrosine ligase-like family member 5
2.75E-05	0.00019	2.09	ZNF562	zinc finger protein 562
3.01E-05	0.00019	0.5	ATF5	activating transcription factor 5
3.01E-05	0.00191	0.44	RAB9A	RAB9A, member RAS oncogene family
3.07E-05	0.00193	0.49	GPAM	glycerol-3-phosphate acyltransferase, mitochondrial
3.09E-05	0.00195	4.51	PRUNE	prune exopolyphosphatase
3.13E-05	0.00203	2.63	NKD1	naked cuticle homolog 1 (Drosophila)
3.28E-05	0.00203	2.39	UNG	uracil DNA glycosylase
3.28E-05	0.00215	2.15	CABLES2	Cdk5 and Abl enzyme substrate 2
3.51E-05	0.00217	2.51	LDLRAD1	low density lipoprotein receptor class A domain containing 1
3.61E-05	0.00221	2.21	SPSB1	splA/ryanodine receptor domain and SOCS box containing 1
3.64E-05	0.00221	0.28	ACD	adrenocortical dysplasia homolog (mouse)
3.66E-05	0.00221	2.2	CEP152	centrosomal protein 152kDa
3.67E-05	0.00221	2.04	SH3BP1	SH3-domain binding protein 1
3.76E-05	0.00224	2.33	UTP14A	UTP14, U3 small nucleolar ribonucleoprotein, homolog A (yeast)
3.78E-05	0.00227	2.47	POLE2	polymerase (DNA directed), epsilon 2, accessory subunit
3.86E-05	0.00227	2.05	NCF2	neutrophil cytosolic factor 2
3.86E-05	0.00228	0.39	P2RY8	purinergic receptor P2Y, G-protein coupled, 8
3.91E-05	0.00228	2.05	P2RY8	purinergic receptor P2Y, G-protein coupled, 8

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
3.93E-05	0.000228	2.05	MAP1B	microtubule-associated protein 1B
3.93E-05	0.000228	0.42	RBP1	retinol binding protein 1, cellular
3.94E-05	0.000232	0.47	FOXRED2	FAD-dependent oxidoreductase domain containing 2
4.04E-05	0.000232	2.15	ASNS	asparagine synthetase (glutamine-hydrolyzing)
4.05E-05	0.000239	2.59	GNAI1	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1
4.18E-05	0.000239	0.43	DZIP1	DAZ interacting zinc finger protein 1
4.19E-05	0.000243	2.74	ZC3H12C	zinc finger CCCH-type containing 12C
4.29E-05	0.000243	0.43	HELLS	helicase, lymphoid-specific
4.29E-05	0.000243	2.24	ZNF804A	zinc finger protein 804A
4.31E-05	0.000245	0.33	C20orf96	chromosome 20 open reading frame 96
4.36E-05	0.000245	2.67	SLC13A3	solute carrier family 13 (sodium- dependent dicarboxylate transporter), member 3
4.37E-05	0.000245	3.57	SACS	sacsin molecular chaperone
4.39E-05	0.000245	0.46	SPR	sepiapterin reductase (7,8-dihydrobiopterin:NADP+ oxidoreductase)
4.40E-05	0.000247	2.31	ZNF528	zinc finger protein 528
4.47E-05	0.000247	0.43	BTG1	B-cell translocation gene 1, anti-proliferative
4.47E-05	0.000247	0.34	KDELR3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3
4.48E-05	0.000248	3.01	SOX4	SRY (sex determining region Y)- box 4
4.53E-05	0.000248	0.47	GMCL1	germ cell-less, spermatogenesis associated 1
4.54E-05	0.000253	2.77	AMT	aminomethyltransferase
4.64E-05	0.000253	2.56	KIAA0101	KIAA0101
4.66E-05	0.000256	3.82	DQX1	DEAQ box RNA-dependent ATPase 1
4.75E-05	0.000256	2.79	SOCS2	suppressor of cytokine signaling 2

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
4.75E-05	0.000258	0.4	CHD2	chromodomain helicase DNA binding protein 2
4.84E-05	0.000259	2.19	BRCA2	breast cancer 2, early onset
4.85E-05	0.000261	2.1	SSUH2	ssu-2 homolog (C. elegans)
4.92E-05	0.000261	0.48	BUB1B	BUB1 mitotic checkpoint serine/threonine kinase B
4.94E-05	0.000261	2.19	LMNB1	lamin B1
4.95E-05	0.000267	2.16	MMS22L	MMS22-like, DNA repair protein
5.08E-05	0.000267	2.41	TYMS	thymidylate synthetase
5.09E-05	0.000267	2.24	GNG5	guanine nucleotide binding protein (G protein), gamma 5
5.11E-05	0.000274	0.5	CCR6	chemokine (C-C motif) receptor 6
5.28E-05	0.000275	0.39	WBP1L	WW domain binding protein 1-like
5.30E-05	0.000283	0.36	PKLR	pyruvate kinase, liver and RBC
5.49E-05	0.000283	2.12	SLC23A1	solute carrier family 23 (ascorbic acid transporter), member 1
5.52E-05	0.000283	2.84	ESAM	endothelial cell adhesion molecule
5.54E-05	0.000283	0.16	RHOF	ras homolog family member F (in filopodia)
5.55E-05	0.000283	0.33	MEGF8	multiple EGF-like-domains 8
5.56E-05	0.000283	2.04	HARBI1	harbinger transposase derived 1
5.58E-05	0.000283	0.5	MMAB	methylmalonic aciduria (cobalamin deficiency) cblB type
5.61E-05	0.000283	2.3	TCF19	transcription factor 19
5.62E-05	0.000286	2.17	LY6E	lymphocyte antigen 6 complex, locus E
5.68E-05	0.000286	5.62	TAF13	TAF13 RNA polymerase II, TATA box binding protein (TBP)- associated factor, 18kDa
5.71E-05	0.000292	0.37	RPL22L1	ribosomal protein L22-like 1
5.85E-05	0.000293	0.45	GINS4	GINS complex subunit 4 (Sld5 homolog)

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
5.90E-05	0.000293	2.23	HIST1H4D	histone cluster 1, H4d
5.90E-05	0.000293	2.25	SHOC2	SHOC2 leucine-rich repeat scaffold protein
6.47E-05	0.000309	2.32	ABCA12	ATP-binding cassette, sub-family A (ABC1), member 12
6.47E-05	0.000313	0.45	PLCD3	phospholipase C, delta 3
6.56E-05	0.000313	0.46	GPSM2	G-protein signaling modulator 2
6.58E-05	0.000313	2.3	RRP12	ribosomal RNA processing 12 homolog
6.59E-05	0.000315	0.34	ZMAT3	zinc finger, matrin-type 3
6.67E-05	0.000327	0.48	DCUN1D3	DCN1, defective in cullin neddylation 1, domain containing 3
6.95E-05	0.000332	0.44	UBR4	ubiquitin protein ligase E3 component n-recognin 4
7.15E-05	0.000337	2.05	BTN3A2	butyrophilin, subfamily 3, member A2
7.23E-05	0.000337	3.48	KLHL28	kelch-like family member 28
7.25E-05	0.000338	0.47	VNN1	vanin 1
7.28E-05	0.000338	0.3	RAD51	RAD51 recombinase
7.32E-05	0.000344	2.18	MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase
7.45E-05	0.000361	2.14	EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
7.88E-05	0.000368	2.09	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)
8.07E-05	0.00	2.1	IL11	interleukin 11
8.08E-05	0.00	0.27	HAM	hepcidin antimicrobial peptide
8.09E-05	0.00	6.42	SPC25	SPC25, NDC80 kinetochore complex
8.15E-05	0.00	2.73	EPHA	EPH receptor A2
8.32E-05	0.00	0.37	MIXL	Mix paired-like homeobox
8.35E-05	0.00	4.69	WDR7	WD repeat domain 76
8.38E-05	0.00	2.61	KCNA	potassium channel, voltage gated subfamily
8.43E-05	0.00	2.57		
8.46E-05	0.000378	2.68	C14orf1	chromosome 14 open reading frame 1
8.47E-05	0.00	2.38	ZFYV	zinc finger, FYVE domain containing 1

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
8.56E-05	0.00038	0.47	POLQ	polymerase (DNA directed), theta
8.64E-05	0.00	2.69	GYLT	glycosyltransferase-like 1B
8.65E-05	0.00	2.43	DIO1	deiodinase, iodothyronine, type I
8.66E-05	0.00	3.82	PMAI	phorbol-12-myristate-13-acetate- induced
8.66E-05	0.00	0.49	METT	methyltransferase like 6
8.71E-05	0.00	0.49	TTPA	tocopherol (alpha) transfer protein- like
8.88E-05	0.00	0.45	KLF6	Kruppel-like factor 6
8.92E-05	0.00	0.43	CANT	calcium activated nucleotidase 1
9.03E-05	0.00	2.17	FRG1	FSHD region gene 1 family member B,
9.10E-05	0.00	2.55	HAVC	hepatitis A virus cellular receptor 1
9.26E-05	0.00	0.14	HIST1	histone cluster 1, H2ab
9.29E-05	0.00	2.29	E2F8	E2F transcription factor 8
9.39E-05	0.00	3	LPCA	lysophosphatidylcholine acyltransferase 2
9.40E-05	0.00	2.09	SH3R	SH3 domain containing ring finger 1
9.63E-05	0.00	0.35	ATAD	ATPase family, AAA domain containing 5
9.67E-05	0.00	2.04	TXNI	thioredoxin interacting protein
9.85E-05	0.00	0.18	CYP2	cytochrome P450, family 2, subfamily W,
9.86E-05	0.00	2.12	PTGR	prostaglandin reductase 1
9.86E-05	0.00	0.45	MYO5	myosin VA
9.90E-05	0.00	0.43	ARHG	ARHGAP5 antisense RNA 1 (head to head)
9.91E-05	0.00	0.42	PBK	PDZ binding kinase
0.0001011	0.00	2.57	CD10	CD109 molecule
0.0001023	0.00	0.37	GSTM	glutathione S-transferase mu 1
0.0001035	0.00	2.06	ARHG	Rho/Rac guanine nucleotide exchange factor
0.0001039	0.00	0.39	PTPN	protein tyrosine phosphatase, non- receptor
0.000104	0.00	0.42	ORC6	origin recognition complex, subunit 6
0.0001042	0.00	2.39	C1orf4	chromosome 1 open reading frame 43
0.0001043	0.00	2.21	AREG	amphiregulin
0.0001053	0.00	0.34	TNFR	tumor necrosis factor receptor superfamily,
0.0001056	0.00	0.45	MAL2	mal, T-cell differentiation protein 2
0.0001064	0.00	2.13	F2RL1	coagulation factor II (thrombin) receptor-
0.0001072	0.00	0.22	IFIH1	interferon induced with helicase C domain 1
0.0001079	0.00	2.23	FOSL	FOS-like antigen 2
0.0001082	0.00	0.35	ESCO	establishment of sister chromatid cohesion
0.0001082	0.00	2.38	CPEB	cytoplasmic polyadenylation element
0.0001122	0.00	0.48	BCKD	branched chain keto acid dehydrogenase E1,
0.0001125	0.00	2.13	NID1	nidogen 1
0.0001128	0.000454	2.92	TMEM63A	transmembrane protein 63A

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
0.000115	0.00	2.25	AHR	aryl hydrocarbon receptor
0.0001152	0.00	0.48	HIST1	histone cluster 1, H2ak
0.0001185	0.00	2.01	RAD5	RAD54-like (S. cerevisiae)
0.0001215	0.00	2.03	FAM1	family with sequence similarity 111,
0.0001222	0.00	6.52		
0.0001348	0.00	0.46	SDPR	serum deprivation response
0.0001356	0.00	0.37	TME	transmembrane protein 135
0.0001365	0.00	2.27	ZNFX	zinc finger, NFX1-type containing 1
0.000139	0.00	0.48	SIRT7	sirtuin 7
0.000145	0.00	0.41	SLC22	solute carrier family 22 (organic anion
0.0001462	0.00	3.59	SEMA	sema domain, immunoglobulin domain (Ig),
0.0001475	0.00	0.35	PRLR	prolactin receptor
0.0001484	0.00 0565	2.21	MKI6 7	marker of proliferation Ki-67
0.0001521	0.00	2.82	MYO	myoferlin
0.0001522	0.00	0.21	RIT1	Ras-like without CAAX 1
0.0001528	0.00	0.31	C1orf1	chromosome 1 open reading frame 106
0.0001537	0.00	0.49	PPAR	peroxisome proliferator-activated receptor
0.0001542	0.00	2.5	FADS	fatty acid desaturase 2
0.0001548	0.00 0587	2.99	OIP5	Opa interacting protein 5
0.000158	0.00	2.97	AHN	AHNAK nucleoprotein
0.0001585	0.00	0.34	MCM	minichromosome maintenance complex
0.0001604	0.00	3.36	GPRC	G protein-coupled receptor, class C, group
0.0001672	0.00	0.18	HIST1	histone cluster 1, H1b
0.0001674	0.00	3.53	TCF19	transcription factor 19
0.0001713	0.00	2.25	TCF19	transcription factor 19
0.0001715	0.00	2.25	DUSP	dual specificity phosphatase 14
0.0001715	0.00	0.48	FSTL3	follistatin-like 3 (secreted glycoprotein)
0.0001771	0.00	0.45	MIS18	MIS18 binding protein 1
0.0001772	0.00	2.05	CCDC	coiled-coil domain containing 51
0.0001792	0.00	0.41	MPP7	membrane protein, palmitoylated 7
0.000181	0.00 066	2.94	TNFR SF10D	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain
0.0001837	0.00	0.43	SOBP	sine oculis binding protein homolog
0.000188	0.00 067	2.15	SESN 2	sestrin 2
0.0001883	0.00	0.39	GPAT	glycerol-3-phosphate acyltransferase 3
0.0001885	0.00	0.28	DGK	diacylglycerol kinase, kappa

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
0.0001886	0.00 067	6.98	DSN1	DSN1 homolog, MIS12 kinetochore complex component
0.0001893	0.00	2.13	FGFR	fibroblast growth factor receptor- like 1
0.0001894	0.00	2.12	STMN	stathmin 1
0.0001939	0.00	3.65	SQRD	sulfide quinone reductase-like (yeast)
0.0001952	0.00	0.35	RBL2	retinoblastoma-like 2
0.0001963	0.00	2.29	ENPP	ectonucleotide
0.0001989	0.00	5.04	DAB2	Dab, mitogen-responsive phosphoprotein,
0.0002017	0.00 071	0.47	NCEH 1	neutral cholesterol ester hydrolase 1
0.0002044	0.00 071	0.45	RRN3	RRN3 homolog, RNA polymerase I transcription factor
0.0002052	0.00	0.49	SLC25	solute carrier family 25, member 51
0.0002053	0.00	0.38	IDS	iduronate 2-sulfatase
0.0002097	0.00	0.35	ICK	intestinal cell (MAK-like) kinase
0.0002104	0.00	3.76	POLD	polymerase (DNA-directed), delta 4,
0.0002106	0.00	0.48	PQLC	PQ loop repeat containing 2
0.000217	0.00	0.47	SLC40	solute carrier family 40 (iron- regulated
0.0002259	0.00	0.2	PPIP5	diphosphoinositol pentakisphosphate kinase
0.0002259	0.00	2.03	USP30	ubiquitin specific peptidase 30
0.0002274	0.00	2.66	TOM	translocase of outer mitochondrial
0.0002355	0.00	2.04	STAR	StAR-related lipid transfer (START)
0.0002395	0.00	2.4	RFC4	replication factor C (activator 1) 4, 37kDa
0.0002401	0.00	2.05	DUSP	dual specificity phosphatase 1
0.0002406	0.00	0.3	MAN	mannosidase, endo-alpha
0.0002469	0.00	2.05	TRPV	transient receptor potential cation channel,
0.000247	0.00	0.37	DDC	dopa decarboxylase (aromatic L- amino acid
0.0002471	0.00	2.06	ERP27	endoplasmic reticulum protein 27
0.0002597	0.00	2.1	GRB1	growth factor receptor-bound protein 10
0.0002603	0.00	0.33	MTM	myotubularin related protein 11
0.0002693	0.00	0.42	CHIC	cysteine-rich hydrophobic domain 2
0.0002753	0.00	0.42	MSH2	mutS homolog 2
0.0002799	0.00	2.29	RAB1	RAB11 family interacting protein 4 (class
0.0002841	0.00	0.45	CPN2	carboxypeptidase N, polypeptide 2
0.00029	0.00	0.29	NEIL3	nei-like DNA glycosylase 3
0.0002934	0.00	2.48	STX3	syntaxin 3
0.0002961	0.00	0.49	LGAL	lectin, galactoside-binding, soluble, 1
0.0003006	0.00	0.21	LYVE	lymphatic vessel endothelial hyaluronan
0.0003081	0.00	2.45	ELF4	E74-like factor 4 (ets domain transcription
0.0003101	0.00	0.49	DGK	diacylglycerol kinase, alpha 80kDa
0.0003149	0.00	0.42	GIPC2	GIPC PDZ domain containing family,

Table A.1. (Cont`d)

p-value	FDR	F.C	Name	Symbol
0.0003155	0.00	2.87	KCNJ	potassium channel, inwardly rectifying
0.000326	0.00	0.3	TSR2	TSR2, 20S rRNA accumulation, homolog
0.000327	0.00	2.28	PXYL	2-phosphoxylose phosphatase 1
0.0003273	0.00	2.19	MPP1	membrane protein, palmitoylated 1, 55kDa
0.0003331	0.00	0.25	NFAT	nuclear factor of activated T-cells,
0.0003362	0.00	2.65	STXB	syntaxin binding protein 1
0.0003408	0.00 108	2.05	ETS1	v-ets avian erythroblastosis virus E26 oncogene homolog 1
0.0003523	0.00	0.17	TGFB	transforming growth factor, beta 2
0.0003613	0.00	0.24	JAG1	jagged 1
0.0003633	0.00	0.5	COM	COMM domain containing 9
0.0003672	0.00	2.14	SEMA	sema domain, transmembrane domain (TM),
0.0003689	0.00	2.69	SCN9	sodium channel, voltage gated, type IX
0.0003714	0.00 113	0.18	ZNF5 01	zinc finger protein 501
0.0003751	0.00	0.43	KIAA	KIAA2018
0.0003758	0.00	2	CYR6	cysteine-rich, angiogenic inducer, 61
0.000376	0.00	0.28	EPHX	epoxide hydrolase 2, cytoplasmic
0.0003802	0.00	2.45	GSTM	glutathione S-transferase mu 4
0.0003803	0.00	2.41	BAK1	BCL2-antagonist/killer 1
0.0003825	0.00	0.38	PIK3R	phosphoinositide-3-kinase, regulatory
0.0003891	0.00	2.41	CLCF	cardiotrophin-like cytokine factor 1
0.0003925	0.00	0.45	CDKN	cyclin-dependent kinase inhibitor 1A (p21,