

Bisphenol A is a carcinogen that induces lipid accumulation, peroxisome proliferator-activated receptor- γ expression and liver disease

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Abstract. Bisphenol (BP) A is an exogenous endocrine disruptor that mimics hormones closely associated with health complications, e.g., obesity and cancers. The present study aimed to evaluate the effects of BPA on human liver cells and tissue. The peroxisome proliferator-activated receptor (PPAR)- γ expression profile across tumour samples and paired normal tissue was first analysed using GEPIA. Subsequently, BPA-treated liver THLE-2 cell viability was evaluated using an MTT assay. Clusterin, PPAR α and PPAR γ gene expression in BPA-treated THLE-2 cells was assessed using GEPIA before validating the gene expression using real-time PCR and analysing overall survival using TCGA data in GEPIA. Cytoplasmic lipid accumulation was examined in BPA-treated THLE-2 cells using Oil Red O staining, and liver tissue was examined using haematoxylin and eosin staining. Finally, cytochrome P450 (CYP) gene expression was assessed in BPA-treated THLE-2 cells using real-time PCR. PPAR γ is likely the primary nuclear receptor protein involved in lipid accumulation in THLE-2 cells following BPA treatment and is associated with liver disease. THLE-2 cells exposed to BPA showed a decrease in viability and lipid accumulation after 48 h treatment. Higher PPAR γ gene expression was significantly associated with survival of patients with liver cancer, with an average survival time of <80 months. Haematoxylin and eosin-stained sections

showed notable disruption of the liver architecture in tissue exposed to BPA. Downregulated CYP1A1 and CYP1B1 gene expression implied that BPA-treated THLE-2 cells decreased capacity for carcinogen metabolism, while upregulated CYP2S1 gene expression exerted minimal cytotoxicity. The present study revealed that BPA served as a carcinogen, enhanced tumorigenesis susceptibility and may induce other types of liver disease.

Introduction

The increasing rate of obesity is directly associated with specific factors, including a sedentary lifestyle, overeating and genetic disorders (1). Furthermore, environmental chemicals that disrupt metabolic function have been linked to obesity (2). Individuals are exposed to various chemicals through numerous sources (1,2). Currently, 2,2-bis(4-hydroxyphenyl) or bisphenol (BP) A is one of the most abundant environmental chemicals to which individuals are widely exposed worldwide (3-5). BPA is an exogenous endocrine disruptor associated with human health complications. BPA is a solid crystal, organic, colourless compound with a phenolic odour under ambient conditions, with chemical formula C₁₅H₁₆O₂ and molecular weight of 228.29 g/mol (3). BPA is highly soluble in lipids and weakly soluble in water (2). BPA is primarily used to synthesise plastic, epoxy resin, thermal paper, dental sealant, flame retardant, baby bottles and lacquer coatings for metal products (4). Numerous studies have indicated that BPA easily contaminates food and drinks under certain conditions, such as heating (6,7). During the fabrication, degradation and treatment of BPA-containing materials, BPA is released into the environment (7). It easily enters the human body through the respiratory and digestive tracts and is absorbed via the skin (6). BPA release from food products is increased by heating, contact with acid and alkaline substances, exposure to microwaves and repeated use of plastic containers containing BPA (8,9). BPA is also a xenobiotic endocrine disruptor chemical. In mammary glands, xenobiotic-metabolising enzymes metabolise BPA via two pathways:

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Glucuronidation and sulfation (8). Cytochrome P450 (CYP) enzymes are key enzymes in the liver that mediate the metabolism of BPA into BPA-o-quinone and BPA-semiquinone (10,11). Several studies have indicated that BPA also mimics hormones, such as oestrogens and androgens, and may promote health complications, such as hepatotoxicity, cardiotoxicity, type 2 diabetes, obesity and cancer (1,12).

The present study investigated the effects of BPA on human adult THLE-2 cells. Analysis of the peroxisome proliferator-activated receptor (PPAR)-gamma expression profile in tumour samples and paired normal tissue was performed using Gene Expression Profiling Interactive Analysis (GEPIA). Then, THLE-2 cell viability following BPA treatment was assessed and gene expression of clusterin of PPAR genes (lipid sensor) in BPA-treated THLE-2 cells was measured by reverse transcription-quantitative PCR (RT-qPCR). Subsequently, validation of gene expression data and overall survival analysis were performed using The Cancer Genome Atlas (TCGA) data in GEPIA. The present study assessed cytoplasmic lipid accumulation in BPA-treated THLE-2 cells and performed histopathological examination of liver tissue of rats fed normal or high-fat diet with or without BPA. Finally, CYP gene expression in BPA-treated THLE-2 cells was investigated by RT-qPCR. The present results are important to raise awareness among the public and policy-makers on general BPA use in the food and beverage industry and to provide a foundation for development of strategies to curb the high prevalence of obesity.

Materials and methods

Analysis of PPAR γ expression profile in tumour samples and paired normal tissue. The PPAR γ expression profile in tumour samples and paired normal tissue was investigated using GEPIA. This web server extracts data from TCGA data portal and Genotype-Tissue Expression (GTEx) database of normal tissue (gepia.cancer-pku.cn) (13). A total of 30 types of human tumour and paired normal tissue was used to investigate gene expression. These tumours included the following: Adrenocortical carcinoma, bladder urothelial carcinoma, breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangiocarcinoma, colon adenocarcinoma (COAD), lymphoid neoplasm diffuse large B cell lymphoma, oesophageal carcinoma, glioblastoma multiforme, head and neck squamous cell carcinoma (HNSC), kidney chromophobe, kidney renal clear cell carcinoma, kidney renal papillary cell carcinoma, acute myeloid leukaemia, brain lower grade glioma, liver hepatocellular carcinoma (LIHC), lung adenocarcinoma, lung squamous cell carcinoma (LUSC), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma, prostate adenocarcinoma, rectum adenocarcinoma (READ), sarcoma, skin cutaneous melanoma (SKCM), stomach adenocarcinoma, testicular germ cell tumour, thyroid carcinoma (THCA), thymoma and uterine corpus endometrial carcinoma (UCEC). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for comparing differential gene expression in tumour vs. paired normal samples and log₂(transcripts per million+1) was used for log-scale plotting. The log₂ fold-change cut-off was set to 1 and Q-value cut-off of 0.01 was used for dot and box

plots. The overexpressed genes were marked in red; underexpressed genes were marked in green.

Evaluation of BPA-treated THLE-2 cell viability. Before evaluating PPAR γ expression in THLE-2 cell lines, the half maximal inhibitory concentration and the effect of the carcinogen BPA on cell viability were investigated using MTT assay, according to the manufacturer's instructions (Sigma-Aldrich; Merck KGaA). The human adult liver THLE-2 cell line was obtained from the American Type Culture Collection and BPA (>99% purity) was purchased from Merck KGaA. THLE-2 cells were cultured with bronchial epithelial growth medium (Thermo Fisher Scientific, Inc.) in T25 culture flasks at 37°C in a humidified atmosphere with 5% CO₂ for at least 2-3 days or until the cells reached 80% confluence before subjecting to experiments. The growth medium was supplemented with frozen additives, including 10% foetal bovine serum (Thermo Fisher Scientific, Inc.), 0.4 ml phosphoethanolamine (100 µg/ml stock; cat. no. P0503; Sigma-Aldrich; Merck KGaA) and 0.3 ml human recombinant epithelial growth factor (EGF; 10 µg/ml stock; cat. no. 354052; Corning, Inc.). Cells were cultured until ~80% confluence. Then, cells were trypsinized and seeded in three 96-well plates at a density of 5x10³ per well in growth medium. The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24 h. Following incubation, the medium in each well was removed and 100 µl fresh medium containing different concentrations of BPA (7.81, 15.63, 31.25, 62.50, 125.00, 250.00, 500.00, 1,000.00 µg/ml) was added to each well. The 100 mg/ml BPA stock solution was prepared by dissolving 500 mg BPA in 1 ml DMSO. Then, the stock solution was aliquoted and stored at -20°C. The growth medium was used to adjust BPA solutions to the concentrations needed for cell treatment. Treated cells were incubated at 37°C for 24, 48 and 72 h. After each incubation period, 24 µl 2.5 mg/ml MTT reagent was added to each well. The plate was incubated at 37°C in 5% CO₂ for 4 h. Finally, 100 µl DMSO was used to dissolve the purple formazan crystals. A TECAN Sunrise™ microplate reader (Tecan Group, Ltd.) was used to measure the absorbance of the samples at 570 nm. The percentage of cytotoxicity was calculated as described previously (14). Each experiment was performed in triplicate and ≥3 independent experiments were performed.

Assessment of clusterin, PPAR and CYP gene expression in BPA-treated THLE-2 cells. The study performed assessment of clusterin, PPAR and CYP gene expression in BPA-treated THLE-2 cells by culturing THLE-2 cells as aforementioned. Cells were then trypsinised and sub-cultured with fresh growth medium in 6-well plates at a density of 2x10⁵ cells/well and subjected to 35 µl/ml BPA treatment at 37°C for 24, 48 and 72 h. Total RNA was extracted from BPA-treated THLE-2 cells using TRIzol® Total RNA Isolation Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The extracted total RNA concentration was determined using a NanoDrop™ (Thermo Fisher Scientific, Inc.) and the high purity of the extracted RNA was preserved by storage at -80°C until further use. The extracted RNA integrity was checked using a 1% (w/v) agarose gel with an electrophoresis system (Bio-Rad Laboratories, Inc.). The extracted total RNA was reverse-transcribed into cDNA using a Tetro cDNA Synthesis

kit (Bioline; Meridian Bioscience) at room temperature. The entire process took approximately one hour to accomplish, according to the manufacturer's instructions. The following primer pairs were used for qPCR: β -actin forward, 5'-CAT TGCCGACAGGATGCA-3' and reverse, 5'-CCGATCCAC ACGGAGTACTTG-3'; clusterin forward, 5'-TCCACCGCC GGTATATGA-3' and reverse, 5'-GCCCAGCTATGG TTCAGACTAAAA-3'; PPAR α forward, 5'-GCCAGTATT GTCGATTTTCAAG-3' and reverse, 5'-CTCCTTGTT CTGGATGCCATT-3'; PPAR γ forward, 5'-CTTTATGGA GCCAAGTTTGAG-3' and reverse, 5'-GCTTCACATTCA GCAAACCTG-3'; cytochrome P450 family 1 subfamily A member 1 (CYP1A1) forward, 5'-TCAGGAGAAGCAGCT GGATGA-3' and reverse, 5'-GAGGTCCAAGACGATGTT AATGATC-3'; CYP1 subfamily B member 1 (CYP1B1) forward, 5'-ATCAGGTGAGGTGTGCTCCAT-3' and reverse, 5'-TCTCCAGAAGCTCCTGCATA-3'; and CYP family 2 subfamily S member 1 (CYP2S1) forward, 5'-GACAGGGTT AATGTCTCCAGAGTGT-3' and reverse, 5'-GGACAGACT CCGGAAAACAAC-3'. All primer pairs were designed using Primer Express Software v.3.0.1 (Thermo Fisher Scientific, Inc.). All oligonucleotide primers were obtained from Integrated DNA Technologies, Inc. The primer stock was packed in desalted lyophilised form and dissolved in RNase-free water (Sigma-Aldrich; Merck KGaA) to generate a final 100 μ M stock of each primer solution. The primer stock and working solutions were stored at -20°C until further use. RT-qPCR was performed using an Agilent AriaMx Real-Time PCR System (Agilent Technologies, Inc.). The PCR cocktail was prepared by adding 10.0 μ l Applied Biosystems PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, Inc.), 0.8 μ l 10.0 μ M forward and reverse primers solutions for the gene of interest (GOI), as well as 50.0 ng cDNA diluted in nuclease-free water to a total volume of 20.0 μ l in each well. All solutions were prepared in 0.1 ml/8-tube quantitative PCR strips (AITbiotech Pte, Ltd.). The thermocycling conditions were as follows: Hot start at 95°C for 30 sec followed by 40 cycles of amplification at 95°C for 15 sec as the denaturation step and 60°C for 1 min as the annealing and extension steps. The reactions were continued for one cycle of 95°C for 15 sec followed by 60°C for 1 min, 95°C for 30 sec and 60°C for 15 sec to generate a melting curve to ensure that the specific target region of GOI was amplified. The expression of GOI was normalised to β -actin. The fold-change used to determine the association between the normalised GOI in treated and untreated (control) samples was calculated using the $2^{-\Delta\Delta C_q}$ method (15). The fold-change value for the vehicle control was set to 1. Expression change >1 represented upregulation and <1 represented downregulation.

Gene expression validation and overall survival analysis using TCGA data in GEPIA. The gene expression validation presented in the box plot based on GEPIA dynamically shows the expression profiles of the following genes: Clusterin, PPAR γ and PPAR α . The colour density of each block represents the median expression value of a gene in LIHC normalised by the maximum median expression value across all blocks. The plot modification parameters, such as width, jitter size and group colours provided by GEPIA, can be accessed in do-it-yourself expression sub-features. Additionally, GEPIA performed an

overall survival analysis based on the identified gene expression levels in LIHC for the overall or disease-free survival analysis. GEPIA uses a log-rank test for hypothesis evaluation, also known as the Mantel-Cox test (13). The study also included the Cox proportional hazard ratio and the survival plot 95% confidence interval (CI). In the present study, plots for high and low gene expression levels were analysed using the two-stage hazard rate comparison method for Kaplan-Meier plots in the case of crossing curves (16). The null (h_0) and alternative (h_1) hypotheses were set as the survival hazard ratio of subjects over high and low expression, respectively. P-value ranked the gene expression of overall survival analysis in LIHC as significant when the plots run parallel patterns (h_0). P-value ranked the gene expression of overall survival analysis in LIHC as not significant when the plots crossed (h_1).

Examination of cytoplasmic lipid accumulation in BPA-treated THLE-2 cells. Oil Red O staining was performed to examine cytoplasmic lipid accumulation in BPA-treated THLE-2 cells. THLE-2 cells were trypsinised and sub-cultured at 37°C for 24 h with fresh growth medium in 24-well plates at a density of 2×10^5 cells/well. Subsequently, THLE-2 cells were treated with 35 μ g/ml BPA at 37°C for 24, 48 and 72 h. The BPA-treated cells were fixed with cold 4% paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature before being stained with Oil Red O solution (Sigma Aldrich; Merck KGaA) at 37°C for 2 h. The stained cells were counterstained with Mayer haematoxylin solution at room temperature for 5 min (Sigma Aldrich; Merck KGaA) and mounted with an aqueous mounting medium. The morphology of the stained cells was examined using an Eclipse TS100 Inverted Light Microscope (Nikon Corporation) at 200x magnification and images were captured with a Nikon COOLPIX digital camera (Nikon Corporation).

Histopathological examination. For histological examination, liver tissue of 96 Sprague Dawley rats (age, 5-6 weeks) weighing 150-180 g were obtained from Dr Yong Yoke Keong at Animal Research Centre of Universiti Putra Malaysia (UPM), Serdang, Malaysia. Following the guidelines, the study was conducted and approved by the Institution of Animal Care and Use Committee at UPM (17). Briefly, the rats were housed in plastic cages distilled water and standard pellets ad libitum under housing condition 22°C, with 12 h light/dark cycles. Rats (n=18/group) were then fed normal diet + vehicle (olive oil), normal diet + BPA (50 mg/kg/day), high-fat diet without vehicle, high-fat diet + vehicle or high-fat diet + BPA (50 mg/kg/day). After 8 weeks, all rats were fasted for 12 h and administered with a lethal dose of sodium thiopental (150 mg/kg) intraperitoneally, which caused a quick and painless death by acting on the central nervous system to induce a cardiopulmonary arrest (18). The rats were confirmed dead when lacking a heartbeat, respiration or corneal reflex. The rats were dissected, and liver tissue was collected for histopathological examination. The tissue samples were fixed in 10% neutral formalin for 12 h at 25°C. The tissue pieces were dehydrated using ascending concentrations of ethyl alcohol and embedded in paraffin at room temperature for 2 h to form blocks. The blocks were trimmed and cut into 6 μ m thick sections. The sections were dewaxed in an oven at 60°C overnight. The section was dipped in xylene twice for 1-2 min. Next, the sections were gone through a series of washing and

staining steps, starting with dipping the sections with absolute alcohol twice for 1-2 min, 95% alcohol twice for 1-2 min, haematoxylin for 2 min, 1% acid alcohol for 1 min, then washing with running tap water for 2 min. The sections were then incubated with 1% ammonia for 1 min, washed with running tap water for 2 min, 95% alcohol for 1-2 min, eosin for 3 min, 95% alcohol for 1-2 min, followed by absolute alcohol twice. Lastly, immersed the sections in xylene twice before mounting. All the procedures were performed at room temperature. The stained tissue sections were examined under a light microscope to assess the histological changes. Qualitative analysis of the sections was performed using ToupView digital imaging software (BX41; Olympus Corporation) at 100x magnification and ImageJ version 1.46r (National Institutes of Health).

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 9.4.0 (GraphPad Software, Inc.). The data are expressed as the mean \pm standard deviation. Data were compared using one-way ANOVA with Tukey's post hoc test or two-way ANOVA with Bonferroni's post hoc test. One-way ANOVA was used for lipid accumulation analysis whereas two-way ANOVA was used for gene mRNA expression analyses. Each sample was measured in ≥ 3 replications and ≥ 3 independent experiments were performed. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Increased PPAR γ expression profile in tumour samples associated with digestive system. The present analysis compared PPAR γ expression levels between tumour and normal tissue. Data extracted from TCGA database revealed that PPAR γ expression was notably different in only 11 types of tumours compared with matched normal tissue using RNA-sequencing data from GTEx. Of these, only three showed increased PPAR γ expression and eight showed decreased expression (Fig. 1A). The number of samples for these three tumours was as follows: COAD, PAAD and READ. Number of samples for the eight tumours that showed decreased expression was as follows: BRCA, CESC, HNSC, LUSC, OV, SKCM, THCA and UCEC (Fig. 1B). PPAR γ showed increased expression in cancers associated with the digestive system (COAD, PAAD and READ), compared with normal tissue using GEPIA. Therefore, carcinogens that are associated with colorectal carcinogenesis or digestive system, including the liver, may also be associated with PPAR γ expression.

BPA induced chemosensitivity of THLE-2 cells. MTT assay analysis showed that treating THLE-2 cells with BPA for 24 h did not produce an ideal dose-response (Fig. 2A). This phenomenon was also observed in THLE-2 cells following BPA treatment for 48 h (Fig. 2B). The curves of the descending hyperbola represent a growing drug resistance in the test cells. The hyperbolic curve shows that BPA did not exhibit ideal dose-response cell death at all concentrations. By contrast, BPA was toxic to cells. The ideal dose-response curve (logistic or sigmoidal shape) of BPA was observed exclusively in THLE-2 cells following treatment with BPA for 72 h (Fig. 2C). The best-fit value of the hillslope at the curves following 72 h treatment was -0.568, indicating that the effect of BPA increased

with treatment duration. The maximal response following BPA treatment of THLE-2 cells for 72 h was $\leq 25\%$. The EC_{50} value of BPA in THLE-2 cells following 72 h treatment was $\sim 33.70 \mu\text{g/ml}$, indicating that the BPA distribution of chemosensitivity occurred at lower concentrations and increased with treatment duration. Therefore, subsequent experiments used a concentration of $35 \mu\text{g/ml}$ BPA.

Increased gene clusterin and PPAR mRNA expression in BPA-treated THLE-2 cells. RT-qPCR analysis showed an inhibitory effect on clusterin mRNA expression in THLE-2 cells following 24 h $35 \mu\text{g/ml}$ BPA treatment (24.58% vs. control; $P < 0.05$; Fig. 3A). However, mRNA clusterin expression was significantly increased in THLE-2 cells following 48 h BPA treatment (156.54% vs. control; $P < 0.05$; Fig. 3A). Clusterin mRNA expression was decreased in THLE-2 cells following 72 h BPA treatment. RT-qPCR analysis also indicated that treatment of THLE-2 cells with $35 \mu\text{g/ml}$ BPA for 48 h significantly increased PPAR γ mRNA expression (377.11% vs. control; $P < 0.001$; Fig. 3B). On the other hand, PPAR α mRNA expression was significantly decreased in THLE-2 cells treated with $35 \mu\text{g/ml}$ BPA for 24 h (22.18%-fold change; $P < 0.05$; Fig. 3C) compared with the control. However, the greatest PPAR α mRNA expression was observed following 48 h treatment (167.42% vs. control; $P < 0.05$; Fig. 3C). However, these levels were lower than those of PPAR γ . The semi-ideal bell-shaped curve was observed for clusterin and PPAR α mRNA expression and an ideal bell-shaped curve was observed across the treatment time points for PPAR γ mRNA expression. The results revealed the greatest PPAR γ mRNA expression at 48 h treatment. In addition, clusterin and PPAR α mRNA expression may also serve an important role in cell signaling.

Association of PPAR γ expression with poorer overall survival in LIHC. Gene expression validation of the box plots for clusterin, PPAR γ and PPAR α in LIHC was performed using TCGA data in GEPIA. Clusterin (Fig 4A-a) and PPAR α (Fig. 4A-c) did not significantly differ between tumour and paired normal tissue, while only PPAR γ showed significant differences between tumour and paired normal tissue ($P < 0.05$; Fig. 4A-b). As tumour progression affects prognosis, the analysis also validated the identified genes by investigating their role in LIHC overall survival time (19). Higher clusterin expression possessed a significantly longer overall survival time compared with lower clusterin expression (log-rank $P < 0.05$; Fig. 4B-a). However, a shorter overall survival time was associated with higher PPAR γ expression of LIHC, for a mean survival time of < 80 months (log-rank $P < 0.05$; Fig. 4B-b), which demonstrated higher PPAR γ expression was associated with poorer overall survival in LIHC.

Increased lipid accumulation in BPA-treated THLE-2 cells. Oil Red O staining showed high cytoplasmic lipid accumulation in THLE-2 cells treated with $35 \mu\text{g/ml}$ BPA for 72 h (Fig. 5A). Statistical analysis revealed that lipid accumulation in THLE-2 cells treated with BPA for 48 h was 156.20% of that in control ($P < 0.01$; Fig. 5B). Following 72 h treatment, lipid accumulation remained in the BPA-treated THLE-2 cells

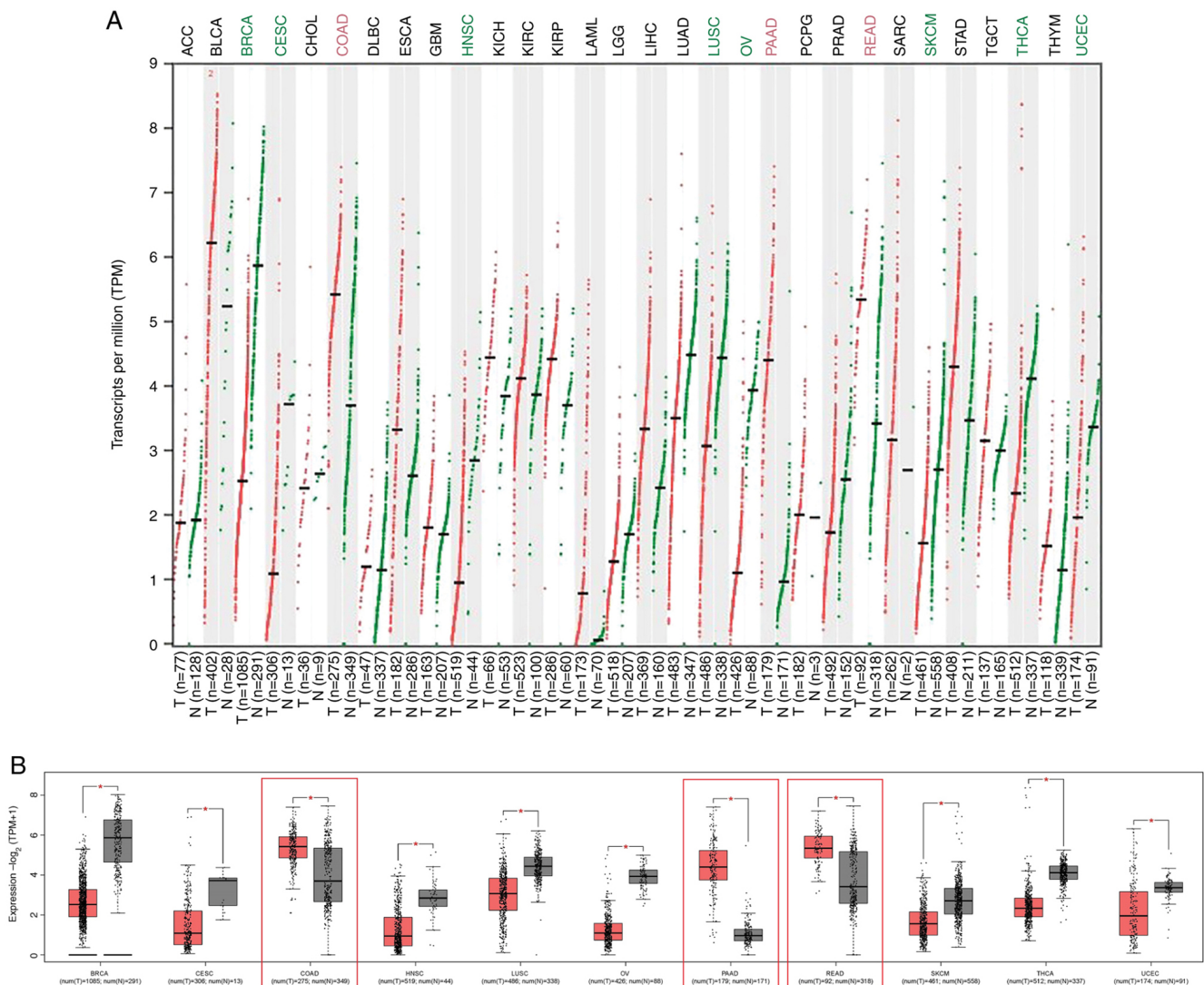


Figure 1. PPAR γ expression in cancer. PPAR γ expression levels in 30 TCGA T (tumour; red) and matched N (normal; green) samples with GTEx data using Gene Expression Profiling Interactive Analysis webserver. (A) Dot and (B) box plots showing upregulation of PPAR γ in three types of cancer. * $P < 0.05$. TPM, transcripts per million; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B cell lymphoma; ESCA, oesophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; AML, acute myeloid leukaemia; LGG, lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumour; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression; T, tumour; N, normal; PPAR, peroxisome proliferator-activated receptor.

was 166.24% of that in control ($P < 0.001$; Fig. 5B). This finding indicates that THLE-2 cells exposed to BPA showed differentiation due to increased lipid accumulation.

Increased lipid accumulation and inflammatory features in rat liver tissue. Haematoxylin and eosin staining showed that the rats fed with a normal diet (and vehicle; olive oil) displayed normal liver architecture had a classic arrangement with central nuclei, sharp nuclear membranes and red-stained cytoplasm (Fig. 6A). The liver tissue of rats fed normal diet + BPA showed deformed hepatocytes, potentially due to inflammation (Fig. 6B). The histopathology also revealed ballooning degeneration of hepatocytes with minimal congestion. On the other hand, liver tissue of rats fed high-fat diet with and without

vehicle showed grade 1 microvesicular steatosis, indicating small lipid droplets in the tissue (Fig. 6C and D). The analysis also found clusters (aggregates) of inflammatory cells in the hepatocytes. The liver tissue of rats fed high-fat diet + BPA showed periportal inflammation, which is a sign of sepsis (Fig. 6E). The tissue also showed lipid droplets, ballooning degeneration of hepatocytes and fibrous tissue proliferation around hepatic lobules. The results revealed that BPA exposure induced abnormal liver architecture, lipid accumulation and inflammation.

Changed CYP mRNA expression in BPA-treated THLE-2 cells. RT-qPCR analysis revealed altered mRNA expression of CYP1A1, CYP1B1 and CYP2S1, which are involved in

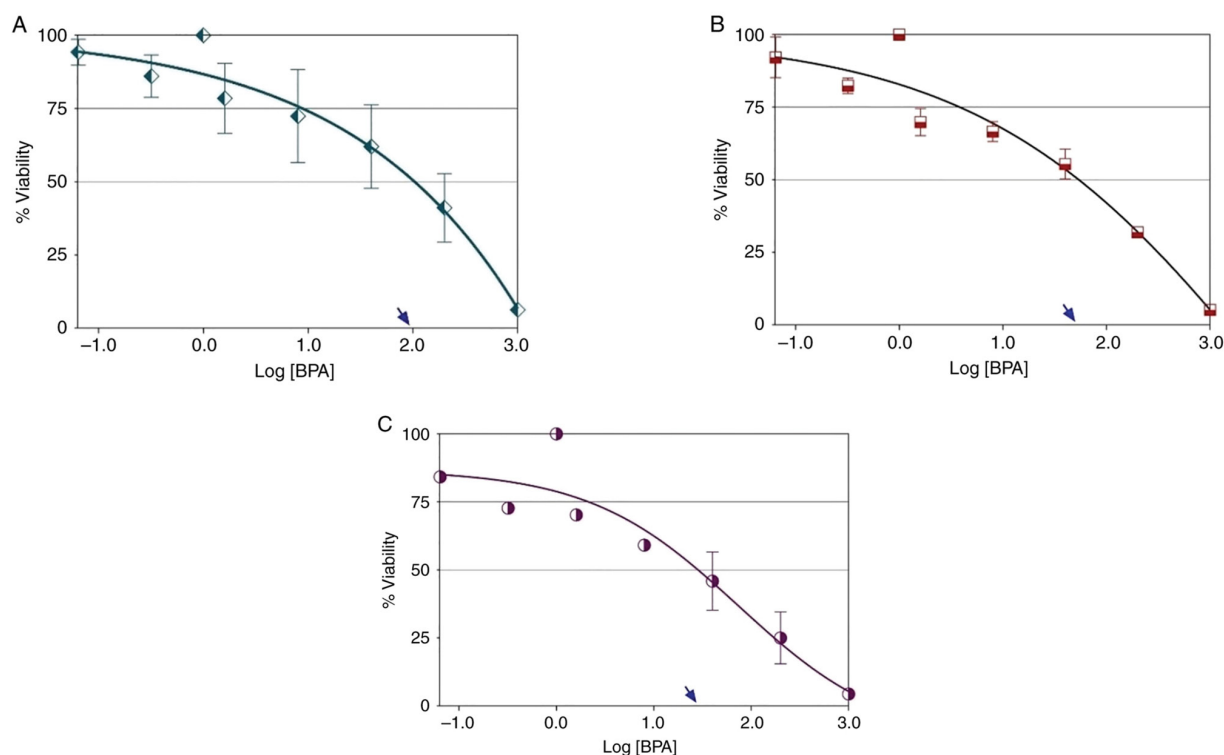


Figure 2. Growth curve of BPA-treated liver cells. EC₅₀ values (arrows) of BPA determined from growth curve following (A) 24, (B) 48 and (C) 72 h treatment were 119.40, 56.70 and 33.70 μg/ml, respectively. The data are expressed as the mean ± standard deviation (n=3). BPA, bisphenol A.

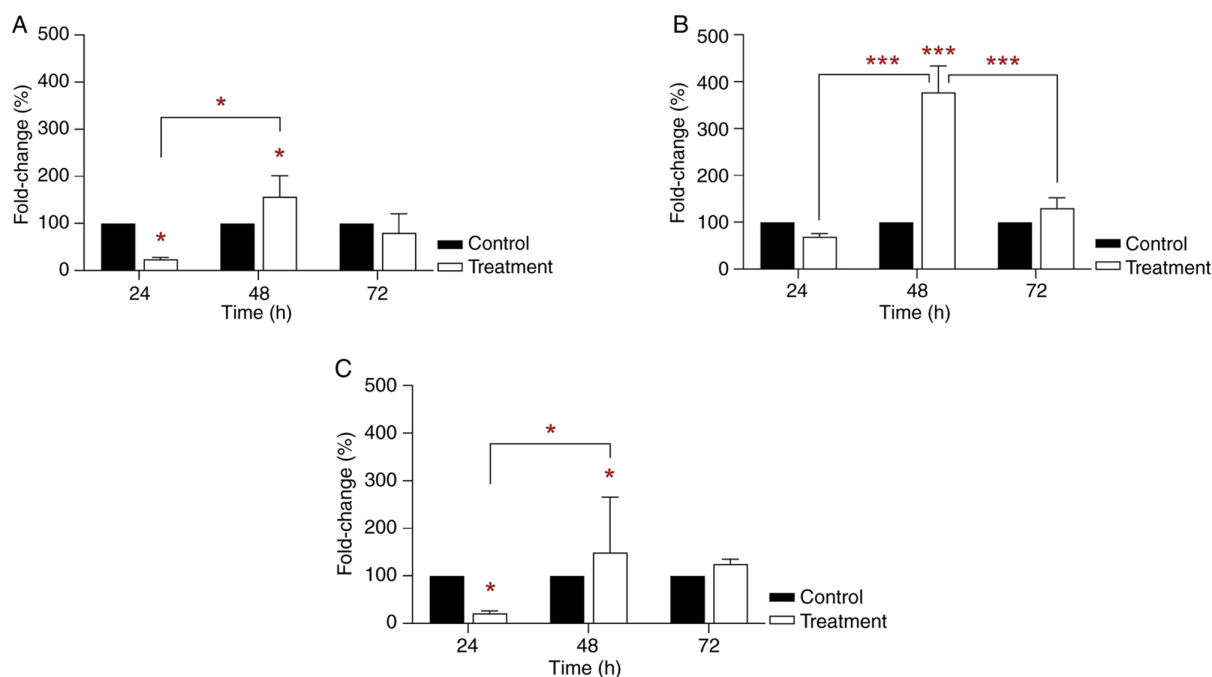


Figure 3. Gene clusterin and PPAR mRNA expression in BPA-treated THLE-2 cells. Reverse transcription-quantitative PCR analysis indicated changes in mRNA expression of (A) clusterin, (B) PPARγ and (C) PPARα in the cytoplasm following BPA treatment in THLE-2 cells for 24, 48 and 72 h. The data are expressed as the mean ± standard deviation (n=3) and were compared using two-way ANOVA followed by Bonferroni's post hoc test. The treatment group at each time point was compared with the respective control. *P<0.05 and ***P<0.001. BPA, bisphenol A; PPAR, peroxisome proliferator-activated receptor.

cell metabolism (20), in THLE-2 cells following treatment with 35 μg/ml BPA for 72 h. Compared with the control, 35 μg/ml BPA significantly decreased CYP1A1 mRNA expression to 76.24 (P<0.01) and 41.39% (P<0.001; Fig. 7A)

following 48 and 72 h treatment, respectively. Compared with control, 35 μg/ml BPA significantly inhibited CYP1B1 expression levels to 55.82 (P<0.01; Fig. 7B) and 45.67% (P<0.01; Fig. 7B) following 48 and 72 h treatment,

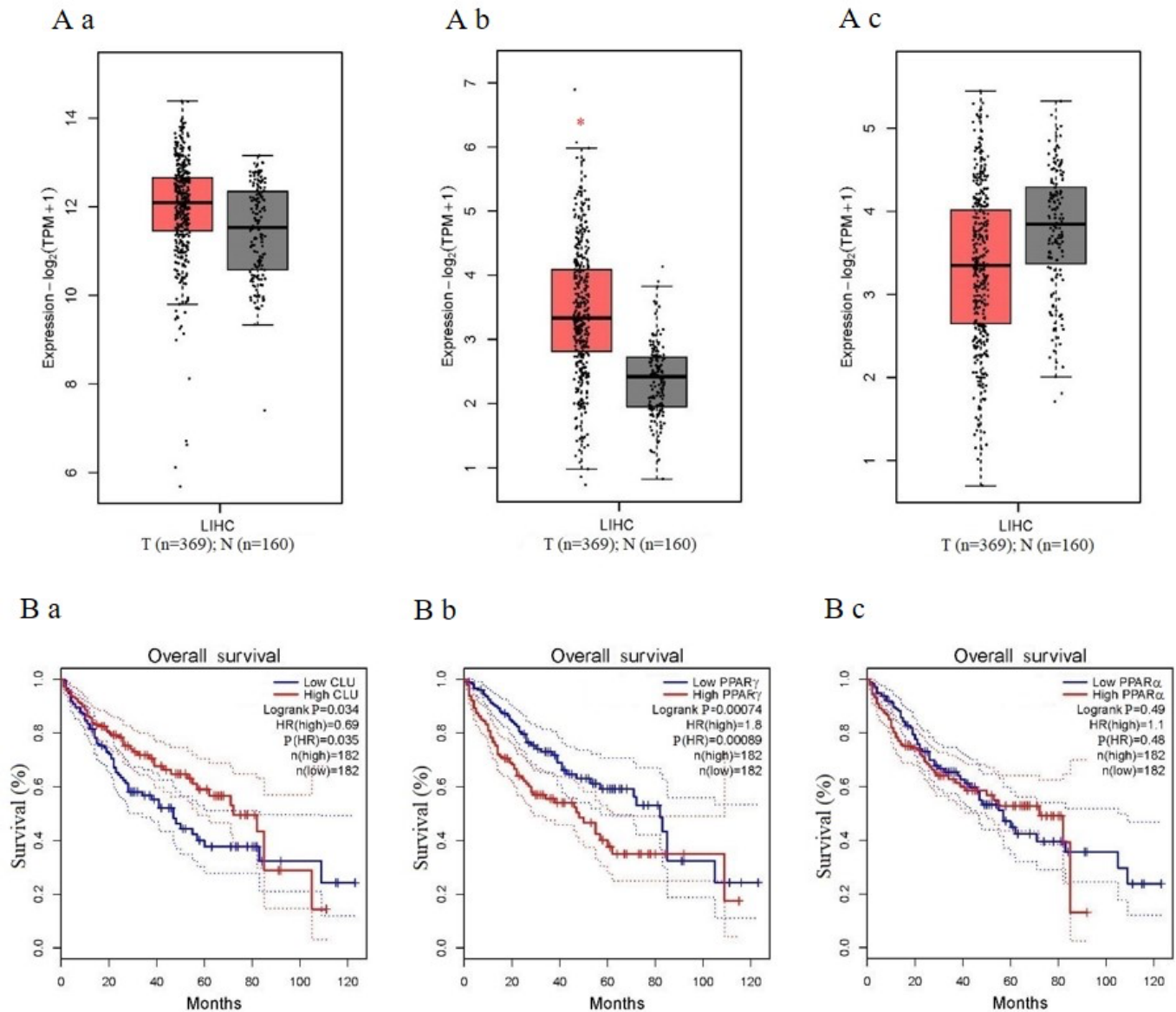


Figure 4. Gene expression validation and overall survival analysis. (A) Validation of (a) CLU, (b) PPAR γ and (c) PPAR α gene expression based on TCGA data in GEPIA. (B) Overall survival analysis of identified (a) CLU, (b) PPAR γ and (c) PPAR α in LIHC based on TCGA data in GEPIA. The significance log-rank P-value is only valid before the plots cross. *P<0.05. HR, hazard ratio; TCGA, The Cancer Genome Atlas; GEPIA, Gene Expression Profiling Interactive Analysis; LIHC, liver hepatocellular carcinoma; PPAR, peroxisome proliferator-activated receptor; TPM, transcripts per million; CLU, clusterin.

respectively. On the other hand, CYP2S1 mRNA expression was significantly decreased to 56.16% after 24 h treatment compared with the control (P<0.05; Fig. 7C). However, CYP2S1 mRNA expression was significantly increased to 146.36% following 48 h treatment compared with the control (P<0.05; Fig. 7C). Semi-ideal bell-shaped curve for CYP2S1 mRNA expression was observed across the treatment time points. This suggested that BPA decreased capacity for carcinogen metabolism, while exerting minimal cytotoxicity in THLE-2 cells.

Discussion

TCGA database revealed that PPAR γ showed increasing levels in cancers associated with the digestive system. PPAR γ mRNA was expressed in THLE-2 cells exposed to BPA with an EC₅₀

value of 35 μ g/ml. In addition, 48 h treatment may have been the optimal time point to signal cellular differentiation in treated THLE-2 cells. BPA is a colorectal cancer carcinogen that caused increased expression of lipid sensors, such as PPAR γ , and decreased metabolism in cytoplasm of THLE-2 cells (12,21). Higher PPAR γ expression was significantly associated with ~80 months overall survival of LIHC in the TCGA database. PPAR γ may have been the primary signaling molecule that induces lipid accumulation in THLE-2 cells following BPA treatment, which was observed in the present study. The liver tissue of rats fed high-fat diet + BPA showed signs of periportal inflammation, lipid droplets, ballooning degeneration of hepatocytes and fibrous tissue proliferation around hepatic lobules. In THLE-2 cells, BPA treatment also decreased the capacity for carcinogen metabolism and increased cytotoxicity. This indicated that PPAR γ expression

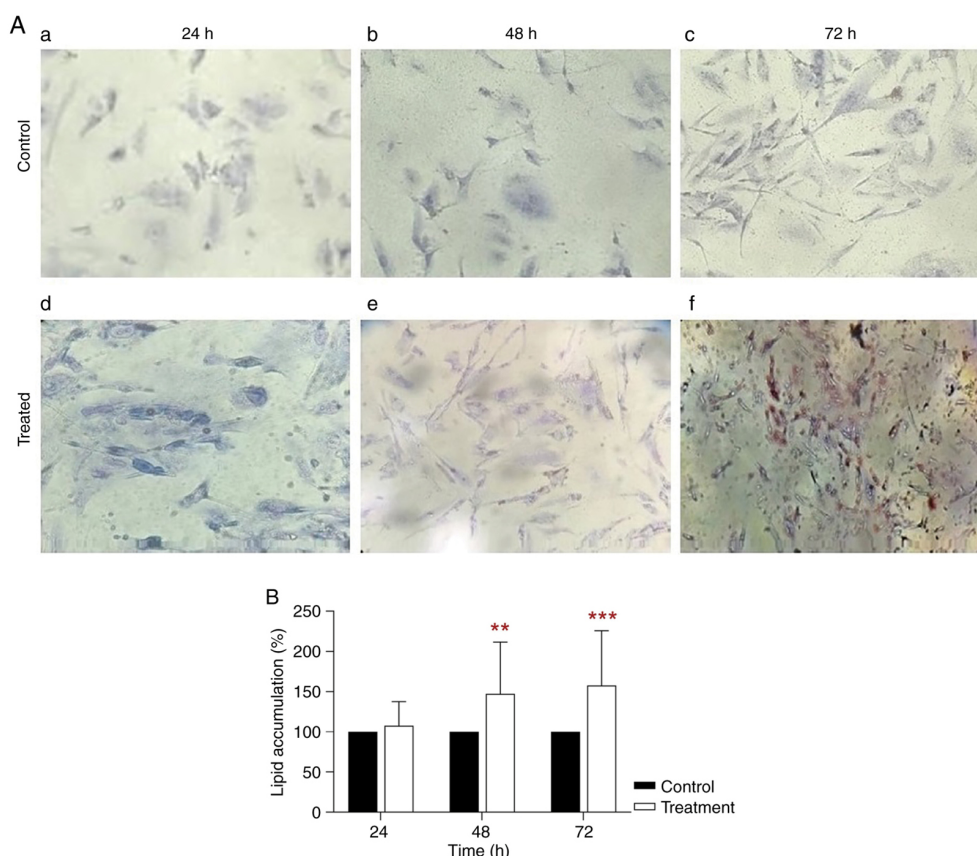


Figure 5. Lipid accumulation in BPA-treated THLE-2 cells. (A) Oil Red O staining demonstrated high cytoplasmic lipid accumulation in THLE-2 cells treated with 35 $\mu\text{g/ml}$ BPA. THLE-2 cells were not treated with BPA (control) for (a) 24 h, (b) 48 h and (c) 72 h, and THLE-2 cells were treated with BPA for (d) 24 h, (e) 48 h and (f) 72 h. All images were captured at 200x magnification. (B) Statistical analysis of lipid accumulation in THLE-2 cells treated with BPA for 72 h. The data are expressed as the mean \pm standard deviation ($n=3$) and were compared using one-way ANOVA followed by Tukey's post hoc test. The treatment group at each time point was compared with the respective control. ** $P<0.01$ vs Control for 48 h and *** $P<0.001$ vs Control for 72 h. BPA, bisphenol A.

and BPA exposure were associated with colorectal cancer and may induce liver disease.

PPAR γ is a master regulator of adipogenesis and fat storage (22). PPAR γ regulates adipocyte differentiation and insulin sensitivity in adipose tissue (22). Several reports have highlighted the anti-proliferative and pro-differentiative actions of PPAR γ ligands in cancer cell lines and animal models of human neoplastic disease (22,23). Previous studies illustrating the tumour-promoting effects of PPAR γ in colon and breast cancer models raise concerns about the practicability and safety of PPAR γ ligands as anticancer agents (22,24,25). Consistently, increased PPAR γ expression was found in cancer associated with the digestive system, including urothelial bladder carcinoma and colon, pancreatic and rectum adenocarcinoma. A potential role for PPAR γ as an inducer of differentiation of cancer stem cells has been explored. PPAR γ protein levels in tumour specimens serve as a key prognostic marker (26). The PPAR γ signalling pathway may explain discrepancies regarding the dual role of PPAR γ , whether it acts as an anti-proliferative or a tumour-promoting action in gastrointestinal cancer (22,27). PPAR γ acts as an anti-proliferative agent, but PPAR γ also acts as a tumour-promoting molecule (22). PPAR γ mRNA expression was also increased in liver cells exposed to BPA.

BPA is a ubiquitous ingredient in epoxy resin and polycarbonate plastic commonly used in manufacturing water bottles

and food containers (28). BPA is also a synthetic oestrogen associated with increased risk of type 2 diabetes mellitus and obesity (29). Despite an official ban against the use of BPA in baby bottles in Malaysia since 2012, BPA remains present in other food containers and beverage packaging (30). Additionally, BPA leaches into foods from these containers when placed in microwaves for extended periods or exposed to vegetable oil and sodium chloride solution (1). The present study investigated the potential role of BPA in cancers. Several studies have suggested that the degree of BPA toxicity to human and animal health depends on the dose, duration of exposure, age of the exposed individual and frequency of exposure (3,31,32). Liver and kidney are the organs with the highest bioaccumulation of BPA (33); therefore, these organs should be checked for BPA accumulation. Several studies have reported that BPA interacts with nuclear receptors, including oestrogen receptors, PPARs, retinoid and thyroid receptors (34). BPA induces lipid accumulation by stimulating these liver receptors (3,35). BPA has been shown to activate PPAR γ , thereby inducing expression of PPAR γ and its target genes in a mouse RAW264.7 macrophage cell line (36,37). These results indicated that BPA disrupts lipid metabolism by regulating PPAR γ (38). To the best of our knowledge, only one study has used the human liver HL-7702 cells as an *in vitro* model to study the effect of BPA on PPARs (39). The study used human liver HL-7702 cells as an *in vitro* model to study

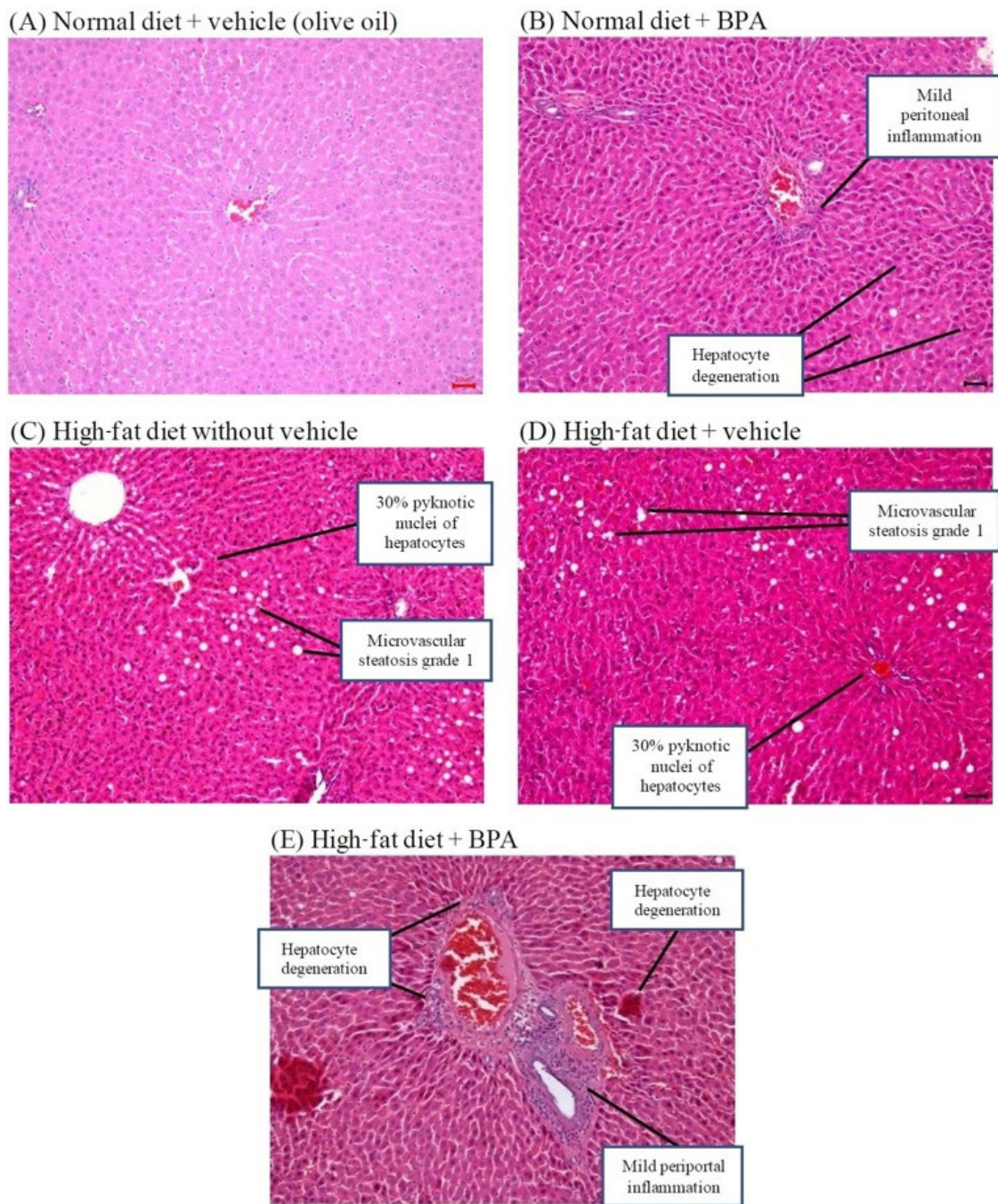


Figure 6. Haematoxylin and eosin staining of Sprague-Dawley rat liver tissue. (A) Normal hepatocytes were well arranged with central nuclei, sharp nuclear membranes and red-stained cytoplasm. (B) Liver tissue of rats fed normal diet containing BPA showed deformed hepatocytes due to inflammation. Tissue also showed ballooning degeneration of hepatocytes and fibrous tissue proliferation around hepatic lobules. Liver tissues of rats fed high-fat diet with (C) and without (D) vehicle showed microvesicular steatosis grade 1, indicating the presence of small lipid droplets on the tissues. The analysis also found clusters or aggregates of inflammatory cells white circles in hepatocytes. (E) Liver tissue of rats fed high-fat diet + BPA showed periportal inflammation. The tissue also showed ballooning degeneration of hepatocytes and fibrous tissue proliferation around hepatic lobules. Each analysis was repeated using ≥ 3 independent experiments ($n=3$). All photomicrographs were captured at 100x magnification. BPA, bisphenol A.

the effect of BPA on PPARs, whereas our study used the cells obtained from human adult hepatocytes, THLE-2. Because the cell sources are different, both studies imply two different mechanistic studies induced by BPA. In the present study, BPA induced toxic effect in THLE-2 cells during early treatment, such as reduced cell viability and increased cytoplasmic lipid accumulation after 48 h of treatment.

BPA exhibits obesogenic properties that alter PPAR γ mRNA expression in THLE-2 cells. The obesogenic

properties include adipogenesis, stimulating lipid accumulation in adipose tissue and liver, and perturbs obesity-related cytokines levels (40,41). The expression of this adipogenesis gene or lipid sensor indicates an increase in lipid accumulation and adipogenesis in the liver in response to BPA exposure. The liver is the key organ that controls lipid metabolism via numerous signalling pathways (42). The present study showed that cytoplasmic lipid accumulation in THLE-2 cells exposed to 35 $\mu\text{g/ml}$ BPA for 72 h was significantly increased due to

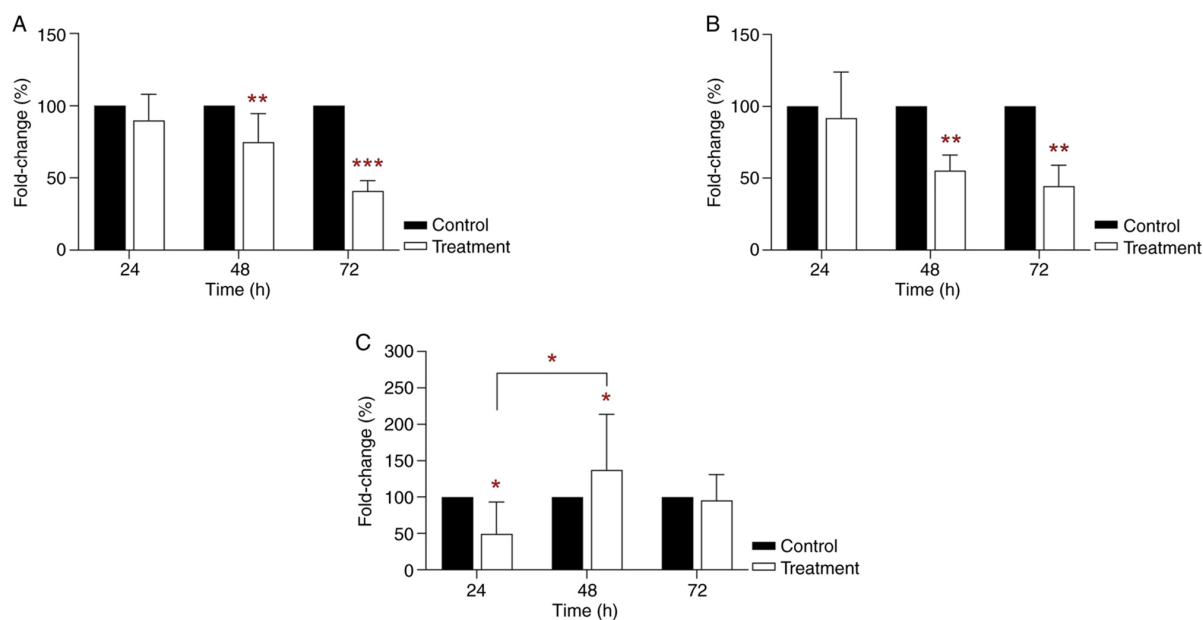


Figure 7. CYP mRNA expression in BPA-treated THLE-2 cells. Reverse transcription-quantitative PCR revealed changes in (A) CYP1A1, (B) CYP1B1 and (C) CYP2S1 mRNA expression in cytoplasm following BPA treatment in THLE-2 cells for 24, 48 and 72 h. The data are expressed as the mean \pm standard deviation and were compared using two-way ANOVA with Bonferroni's post hoc test. Treatment group was compared with control at each time point. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. The P value is compared to the control at a specific time point. CYP, cytochrome P450; BPA, bisphenol A.

the effect of gene expression on hepatic lipid accumulation. A previous study showed that BPA concentrations of 10-100 ng/ml are representative of occupational exposure (43). Based on the magnitude value analysis, humans are exposed to 0.4-4.2 $\mu\text{g/kg}$ body weight/day BPA (44). These BPA concentrations are high, especially considering BPA levels associated with long-term environmental exposure (45,46). Therefore, for an *in vitro* study, treatment of THLE-2 cells with 35 $\mu\text{g/ml}$ BPA for 72 h and feeding experimental rats 50 mg/kg BPA was equivalent to the predicted concentrations, which is ~ 10 -fold higher than the actual situation. Numerous studies have also demonstrated that BPA has a specific role in hepatic lipid accumulation (47,48). For example, lipid accumulation increases in HepG2 cells treated with 10 μM BPA for 24 h (47). Furthermore, an *in vivo* study illustrated that long-term BPA exposure serves a crucial role in lipid accumulation in the liver of mice (48). In the present study, PPAR α , PPAR γ and clusterin mRNA expression levels were increased in the BPA-treated THLE-2 cells. PPAR γ gene exhibited the most significant increase in expression in THLE-2 cells treated with 35 $\mu\text{g/ml}$ BPA for 72 h. Consistently, BPA treatment enhances the expression of PPAR γ (3,47,49). A recent study demonstrated that HepG2 cells treated with 10 μM BPA also exhibit increased PPAR α expression (47). In addition, BPA upregulates PPAR α expression levels in the liver of offspring rats (50). However, the increase in PPAR α expression was not as high as that for PPAR γ following BPA treatment in the present study. Therefore, only PPAR γ expression is significantly upregulated in BPA-treated THLE-2 cells.

Haematoxylin and eosin staining showed that exposure to BPA caused notable disruption of liver architecture, resulting in cellular infiltration, large cytoplasmic vacuoles and hepatic sinusoids, and an increased number of Kupffer cells in liver tissue (51). The present analysis showed deformed

hepatocytes due to inflammation in the liver tissue of rats fed normal diet + BPA. Tissue histopathology also revealed hepatocyte balloon degeneration with minimal congestion lesions compared with rats fed normal diet. Liver tissue of rats fed high-fat diet with and without vehicle showed grade 1 microvesicular steatosis, which is a sign of small lipid droplets on the tissue. Further analysis found inflammatory cell aggregates in hepatocytes of rats fed high-fat diet + BPA. A previous study demonstrated that liver tissue exposed to BPA show vacuolar degeneration, sinusoidal dilatation, vascular congestion and glycogen depletion that increases with exposure (52). The hepatic lobular structure obtained from liver tissue of rats and pigs exposed to BPA are close to physiological levels observed in the human liver (52). BPA causes liver damage in developing children prone to the common neural, psychological and metabolic disorders (52). Children continuously exposed to BP compounds for several years may experience a significantly increased risk of liver damage and altered metabolism (52). BPA also causes hepatic oxidative stress and steatosis, impairing secretory function and liver integrity (7). Moreover, BPA increases lipid peroxidation and decreases antioxidant defence enzymes in rat liver (53). BPA reacts with oxygen radicals, decomposing the radicals to reactive metabolites with potent oxidant activity (54). These metabolites increase production of reactive oxygen species (ROS), inhibit activity of antioxidant enzymes and increase the reactivity of H_2O_2 and thiobarbituric acid in the liver (54). BPA-mediated increase in ROS content enhances peptide chain cleavage and amino acid cross-linking in enzymes, leading to change or loss of liver enzyme activity (55). Therefore, the mechanism of oxidative damage caused by BPA may involve inhibition of the antioxidant enzyme system. BPA also causes dyslipidaemia, increases accumulation of triglycerides (steatosis) and cholesterol,

and induces intracellular accumulation of fat droplets in a dose-dependent manner (56). Furthermore, BPA exposure results in upregulation of genes associated with *de novo* lipogenesis, such as ATP citrate lyase, and cholesterol synthesis, such as mevalonate (diphospho) decarboxylase (Mvd) (57). BPA induced hepatic lipid accumulation may arise from increased expression of transcription factor sterol regulatory element-binding protein-1, which increases the quantity and activity of the enzymes that catalyse lipogenesis, triggering hepatic lipid accumulation (56). Hepatic lipid accumulation and oxidative stress followed by liver injury and inflammation are pathogenic manifestations of steatohepatitis not caused by consumption of alcohol (58). Oxidative stress induced by BPA and lipid peroxidation enhances hepatic damage and disrupts cellular membrane integrity leading to leakage of cytoplasmic liver enzymes (59). Additionally, patients with liver disease exhibit higher levels of BPA than healthy individuals, suggesting a link between BPA exposure and liver health status (60).

The effect of BPA on THLE-2 cells may also be associated with CYP gene expression. The present study illustrated significant inhibition of CYP1A1 and CYP1B1 mRNA expression in THLE-2 cells treated with BPA for 72 h compared with the untreated group. Several studies have suggested that CYPs are strongly associated with the toxicity and metabolism of BPA in the mammalian body via enhancement or inhibition of activity (3,9). As observed in mice, *in utero* exposure to BPA disrupts CYP1A1 mRNA expression levels in the embryo (61). Other studies have shown downregulation of CYP1A1 mRNA expression in mouse Hepa-1c1c7 cells treated with 10 and 50 μ M BPA for 18 h (62). Various nuclear receptors, including PPARs and oestrogen-associated receptors, regulate sulphotransferase expression, such as CYP1B1, thereby altering xenobiotic disposition (20). Inhibition of CYP1B1 mRNA expression has been found in human foetal liver tissue treated with 35.4–56.1 ng/g BPA (63). Previous studies have demonstrated that BPA upregulates CYP1B1 expression, which induces oxidative stress (64,65). For example, exposure to 100 μ M BPA for 24 h increases CYP1B1 mRNA expression in human foetal lung fibroblasts (65). CYP1A1 and CYP1B1 enzymes serve a role in catalysing oxidation of procarcinogens to carcinogenic reactive metabolites (66). Therefore, downregulation of CYP1A1 and CYP1B1 expression inhibits the cells from catalysing oxidation of procarcinogens to carcinogenic reactive metabolites.

CYP2S1 mRNA was significantly overexpressed in the present study. The present study also indicated that 48 h was the optimum treatment time point for THLE-2 cells to metabolise BPA via CYP2S1 expression. Higher CYP2S1 indicates better BPA metabolism. CYPs are enzymes predominantly secreted by the liver and involved in the metabolism or detoxification of xenobiotics and endogenous compounds during phase one reactions (66). CYP enzymes cause molecules to be hydrophilic, less toxic and easily excreted from the body based on the presence of polar functional groups in their structure (20). Human CYP2S1 is a complex and most prominent family of CYP genes that is located at the end of a cluster on chromosome 19q of CYP2 family members (67). Previous studies have revealed that frequent xenobiotic exposure induces the highest CYP2S1 mRNA expression in epithelial mouse and human

tissues (68–73). Another study demonstrated that oxaliplatin induces CYP2S1 expression in HCT116 cells and subsequently inhibits cell proliferation. Moreover, increased survival was observed in HCT116 cells with CYP2S1 knockdown (66,74). This phenomenon implies that cell growth is inhibited if CYP2S1 is expressed at high levels.

In conclusion, PPAR γ expression is associated with digestive system cancer (18,75). THLE-2 cells showed decreased viability and cytoplasmic lipid accumulation following 48 h exposure to BPA. Liver-specific PPAR γ gene expression was significantly associated with overall survival. Haematoxylin and eosin staining revealed notable disruption of the liver architecture in rat liver tissue exposed to BPA. The observed downregulation of CYP1A1 and CYP1B1 mRNA expression indicated that BPA-treated THLE-2 cells did not catalyse the carcinogen to reactive metabolites. By contrast, CYP2S1 mRNA expression was associated with decreased proliferation of cells treated with BPA.

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Availability of data and materials

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

Authors' contributions

MA, TJ, HB, YK and KY conceived and designed the study. LI and AA performed experiments. KY and YK confirm the authenticity of all the raw data. LI and KY interpreted the data, drafted and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Experiments using rat liver tissue were approved by the Institution of Animal Care and Use Committee at University Putra Malaysia (approval no. UPM/IACUC/AUP-R068/2019).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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