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MicroRNA regulation of DNA repair gene expression in 4-aminobiphenyl-treated HepG2 cells

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ABSTRACT

We examined the role of miRNAs in DNA damage response in HepG2 cells following exposure to 4-aminobiphenyl (4-ABP). The arylamine 4-ABP is a human carcinogen. Using the Comet assay, we showed that 4-ABP (18.75–300 μM) induces DNA damage in HepG2 cells after 24 h. DNA damage signaling pathway-based PCR arrays were used to investigate expression changes in genes involved in DNA damage response. Results showed down-regulation of 16 DNA repair-related genes in 4-ABP-treated cells. Among them, the expression of selected six genes (*UNG*, *LIG1*, *EXO1*, *XRCC2*, *PCNA*, and *FANCG)* from different DNA repair pathways was decreased with quantitative real-time PCR (qRT-PCR). In parallel, using the miRNA array, we reported that the expression of 27 miRNAs in 4-ABP-treated cells was at least 3-fold higher than that in the control group. Of these differential 27 miRNAs, the most significant expression of miRNA-513a-5p and miRNA-630 was further validated by qRT-PCR, and was predicted to be implicated in the deregulation of *FANCG* and *RAD18* genes, respectively, via bioinformatic analysis. Both FANCG and RAD18 proteins were found to be down-regulated in 4-ABP-treated cells. In addition, overexpression and knockdown of miRNA-513a-5p and miRNA-630 reduced and increased the expression of FANCG and RAD18 proteins, respectively.

Based on the above results, we indicated that miRNA-513a-5p and miRNA-630 could play a role in the suppression of DNA repair genes, and eventually lead to DNA damage.

1. Introduction

The arylamine 4-aminobiphenyl (4-ABP) is classified as human carcinogen (Nauwelaërs et al., 2013), and has been found as a contaminant in color additives, paints, food colors, leather and textile dyes, diesel-exhaust particles, and cooking oil fumes (Nauwelaers et al., 2011). A potential source of exposure to 4-ABP occurs through tobacco smoke and commercial hair dyes (Nauwelaers et al., 2011).

The liver is the major organ in the biotransformation of 4-ABP to genotoxic metabolites (Butler et al., 1989; Turesky et al., 1991). Human hepatocytes can also effectively metabolize a variety of chemical carcinogens (Guillouzo et al., 1997) such as aflatoxin B1 (Langouët et al., 1995), MeIQx (Langouët et al., 2001), PhIP (Langouët et al., 2002) to reactive metabolites capable of damaging DNA. DNA damage caused by 4-ABP is thought to be mediated by the formation of DNA adducts. In liver, this compound is oxidized to its *N*-hydroxy derivative [4-ABP(NHOH)] by the cytochrome P450 1A2 isozyme (Kadlubar et al., 1977). The resulting *N*-hydroxylamine can be further bioactivated by *O*-acetyltransferase to the *N*-acetoxy derivatives or it can be glucuronidated (Murata et al., 2001). Subsequently, the *N*-acetoxy derivative and *N*-glucuronide are converted to arylnitrenium, which is able to react with DNA to form DNA adducts (Kadlubar et al., 1977). We also previously showed that 4-ABP induces DNA damage in human hepatoma (HepG2) cells, and suggested the involvement of reactive oxygen species (ROS) in DNA damage (Wang et al., 2006). Epidemiological studies have suggested that cigarette smoking is a risk factor for the development of hepatocellular carcinoma (HCC) (Wang et al., 1998). Exposure to 4-ABP, which is primarily a result of cigarette smoking, plays a role

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in the development of HCC in humans (Wang et al., 1998). It was reported that the DNA adduct of 4-ABP was formed in human hepatocytes (Nauwelaers et al., 2011; Nauwelaërs et al., 2013). However, the underlying mechanisms of 4-ABP-induced DNA damage still are unclear.

Several studies have used toxicogenomic approaches to evaluate the global response of miRNAs in rodent livers following chemical insults. For example, exposure by Fisher of 344 rats to tamoxifen, a potent hepatocarcinogen, caused statistically significant differential expression of 33 miRNAs in the liver after chronic dietary exposure (Pogribny et al., 2007). Wang et al. (2009) proved that Balb/C mice fed with acetaminophen induced a large change in miRNA profiles in liver and plasma, indicating that specific circulating miRNAs may provide sensitive and informative biomarkers for drug-induced liver injury. However, the role of miRNA in response to toxicant insult is not well established, although miRNAs were suggested to be important in cellular responses to xenobiotics (Taylor and Gant, 2008). As such, several miRNAs have been shown to be involved in DNA damage response and repair (Hu and Gatti, 2011; Wan et al., 2011), and have regulatory roles in double-strand break repair (Lal et al., 2009; Crosby et al., 2009; Moskwa et al., 2011), nucleotide excision repair (NER) (Crosby et al., 2009), and mismatch repair (MMR) (Valeri et al., 2010; Yu et al., 2010). It was shown that miRNA-16, miRNA-34c, and miR-199a down-regulate the DNA repair enzyme uracil-DNA glycosylase (UNG) activity, UNG mRNA, and UNG protein levels (Hegre et al., 2013).

Here, using an miRNA array platform, pathway-based PCR array, and quantitative PCR analysis, we first reported that 4-ABP can suppress many genes involved in different DNA repair pathways such as double-strand break repair, NER, MMR, and base-excision repair, and induce many miRNAs. In this study, some miRNAs are predicted to inhibit the expression of some DNA repair genes via TargetScan algorithms analysis, suggesting that the role of miRNAs in inhibiting the DNA repair genes leading to DNA damage in 4-ABP-treated cells.

2. Materials and methods

2.1. Cell culture

HepG2 cells were grown in a DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were grown in 25 cm^2 flasks at 37 °C in a humidified atmosphere containing 5% CO_2. Fresh growth medium was added every 2 days until confluence was achieved.

2.2. Cell treatment

Cells were cultured in six-well plates until 70% confluence was reached. The medium was then replaced with a fresh serum-free medium containing 18.75–300 μM 4-ABP in DMSO. Negative controls were exposed to 0.5% DMSO, and the final concentration was used in 4-ABP-treated cultures. Cells were exposed for a total of 24 h or 48 h. Thereafter, cells were washed twice with Hank's buffered salt solution, harvested, transferred to a-1.5 ml RNase-free centrifuge tube, and spun at 300 × *g* for 5 min. An aliquot of each cell suspension was retained before centrifugation for use in the cytotoxicity and Comet assays. After centrifugation, the supernatant was removed, and total RNA was isolated for the pathway-specific real-time PCR array using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol (see below).

2.3. Cytotoxicity assays

HepG2 cells were seeded onto 96-well plates at a density of 10^4 cells/well and incubated for 24 h at 37 °C. The medium was then replaced with fresh complete medium containing 4-aBP at the indicated doses and incubated for 24 h and 48 h, respectively. 3-(4,5-Cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (0.5 mg/ml) (Steinheim, Germany) was added to the medium, and then the plates were incubated for additional 3 h. At the end of the MTT incubation, the medium was removed and the formazan crystals were dissolved with DMSO. The optical density (OD) was measured at 570 nm (reference filter 690 nm) using a microplate spectroflurometer. Viability was determined by comparing the OD of the wells containing 4-ABP-treated cells with those of untreated cells. The results are expressed as the mean of at least three independent experiments.

2.4. Comet assay

The Comet assay was performed under alkaline conditions using our previously described methods (Wang et al., 2006; Wu et al., 2009; Chen et al., 2011). At least 300 images were randomly selected from each sample and analyzed for DNA damage with the Comet Assay IV software. The tail moment comet parameter (mean ± SD) was used as an indicator of DNA damage (Wu et al., 2011a,b; Chen et al., 2011).

2.5. Total RNA isolation

Briefly, following cells treated cells for 24 h were washed with cold 1 × phosphorous buffer solution (PBS). After washing, 1 ml Trizol reagent and 200 μl of chloroform were added. After mixing vigorously, the solution was centrifugated at 13,000 × *g* for 20 min. The supernatant was isolated, mixed with an equal volume of isopropanol, and incubated for 10 min. This mixture was then centrifuged at 13,000 × *g* for 10 min. The supernatant was discarded, and the pellet was treated with 70% alcohol and 1 ml of diethylpyrocarbonate (DEPC). After drying, the RNA was dissolved in DEPC. RNA quantity and purity were measured spectrophotometrically (BioPhotometer, Eppendorf). Samples were considered suitable for further processing if the A_{260}/A_{280} ratios were between 1.8 and 2.0. RNA integrity was determined with a 1.8% agarose electrophoresis gel.

2.6. Pathway-specific real-time PCR assay

We followed the procedures described by Wu et al. (2011a,b) and Chen et al. (2011), and used the human DNA damage signaling RT^2 profile PCR array (SuperArray Bioscience) to determine the effect of 4-ABP on the expression of 84 genes related to DNA damage responses. Synthesis of complementary DNA, real-time PCR, and statistical analyses was performed according to the manufacturer's instructions. The data shown represent the average of three replicates. Cycle thresholds (C_t) with no more than 35 cycles were determined for each gene product. In addition to 84 tested genes, there were additional five housekeeping genes (*Rpl13a*, *B2m*, *Gapdh*, *Hprt1*, and *Actb*) included as RNA content controls in this RT^2 profile PCR array. Among these five genes, only three genes (*Rpl13a*, *B2m*, and *Gapdh*) were constitutively expressed independent of 4-ABP addition were therefore used for the analysis. The C_t value of each target gene was compared to the average C_t value of these three housekeeping genes. In addition, a genomic DNA control primer set was included in each PCR run to detect the possible DNA contamination. Gene expression differences in the PCR expression array were determined using the $-2\Delta\Delta C_t$ method. Independent experiments were performed in duplicate and repeated at least three times. Statistical significance between treated groups and controls was determined by two-tailed Student's *t*-test, and $P < 0.05$ was considered significant.

2.7. RNA Labeling and Hybridization

A concentration at 0.1 μg of total RNAs were dephosphorylated and labeled with pCp-Cy3 by Agilent miRNA Complete Labeling and Hyb Kit (Agilent Technologies, USA, microRNA Spike-In Apply). A 2X hybridization buffer (Agilent Technologies, USA) was added to the labeled mixture to a final volume of 45 μl. The mixture was heated for 5 min at 100 °C and immediately cooled to 0 °C. Each 45 μl sample was hybridized onto an Agilent human miRNA Microarray R16 (Agilent Technologies, USA) at 55 °C for 20 h. After hybridization, slides were washed for 5 min in Gene Expression Wash Buffer 1 at room temperature, then for 5 min in Gene Expression Wash Buffer 2 at 37 °C. Microarrays were scanned with an Agilent microarray scanner (Agilent Technologies, model G2505C) at 535 nm for Cy3. Feature Extraction (Agilent Technologies) software version 10.7.3.1 was used for image analysis.

2.8. miRNA forced expression and inhibition

To force and inhibit the expression of miRs, pre-miRs and anti-miRs were purchased as PG/miR/eGFP/Blasticidin constructs (GenDiscovery Biotechnology) separately. The sequences for hsa-miR-513-5p mimic and its inhibitor as well as hsa-miR-630 mimic and its inhibitor were listed as below.

The sequences of miRNA-513-5p mimic (product ID, B0199) were

5′-AATTCGTTCACAGGGAGGTGTCATGTTTTGGCCACTGACTGACATGACACCCCT-GTGAACA-3′ (sense) 5′-CCGGTGTTCACAGGGGTGTCATGTCAGTCAGTGGCCAAAAC-ATGACACCTCCCTGTGAACG-3′ (antisense), while the sequences of hsa-miR-513-5p inhibitor (product ID, B0133) were

5′-AATTCGATGACACCTCCCTGTGAAGTTTTGGCCACTGACTTCACAGGGGTGT-CATCA-3′ (sense)

5′-CCGGTGATGACACCTCCCTGTGAAGTCAGTCAGTGGCCAAAACTTCACAGGGA-GGTGTCATCG-3′ (antisense).

The sequences of miRNA-630 mimic (product ID, B0197) were