Benzo[a]Pyrene Induced p53-Mediated Cell Cycle Arrest, DNA Repair, and Apoptosis Pathways in Chinese Rare Minnow (*Gobiocypris Rarus*)

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Abstract: The p53 pathways play an important role in carcinogenesis. In mammals, p53 and p53 target genes have been extensively studied, but little is known about their functions and regulation in fish. In this study, the cDNA fragments of p53 network genes, including *p53*, *p21*, *mdm2*, *gadd45 α* , *gadd45 β* , *igfbp-3*, and *bax*, were cloned from Chinese rare minnow (*Gobiocypris rarus*). These genes displayed high amino acid sequence identities with their zebrafish orthologs. The mRNA levels of p53 network genes and pathological changes in the liver were determined after adult rare minnow were exposed to 0.4, 2, and 10 $\mu\text{g/L}$ of benzo[a]pyrene (BaP) for 28 days. The results showed that *p53*, *p21*, *mdm2*, *gadd45 α* , and *bax* mRNA expressions in the livers from males and females were significantly upregulated compared with those of the controls ($p < 0.05$), but *gadd45 β* and *igfbp-3* expression was not significantly changed. Microphotographs revealed enlargement of the cell nuclei and cellular degeneration in males, while atrophy and vacuolization of hepatocytes were observed in females (10 $\mu\text{g/L}$). These results suggested that BaP induced liver DNA repair and apoptosis pathways and caused adverse pathological changes in rare minnow. The strongly responsive p53 network genes in the livers suggest that rare minnow is suitable as an experimental fish to screen environmental carcinogens. In addition, the p53 network genes in rare minnow could feasibly be used to identify the mechanism of environmental carcinogenesis. © 2016 Wiley Periodicals, Inc. *Environ Toxicol* 32: 979–988, 2017.

Keywords: benzo[a]pyrene; rare minnow (*Gobiocypris rarus*); p53 network genes; gene expression; histopathology

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INTRODUCTION

More than 50% of all human cancers have been shown to contain mutations in the *p53* gene (Olivier et al., 2010). *p53* is a tumor suppressor protein that has a key role in regulating the cell cycle, apoptosis, and DNA repair (Ananiev et al., 2011). In addition, recent studies have revealed that *p53* has a role in a wide range of biological processes, including aging and longevity, metabolic processes (Gottlieb and Vousden, 2010), autophagy (Vousden and Prives, 2009), reprogramming of stem cells (Zhao and Xu, 2010), and the insulin-like growth factor (IGF) and mTOR pathways (Feng et al., 2005). *p53* predominantly functions as a transcription factor that can bind to specific DNA sequences, activate downstream gene expression, and trigger cell cycle arrest, DNA repair, and apoptosis (Vousden and Prives, 2009). In mammals, a number of *p53* target genes were identified, and the functions of these genes have been studied, for example, *p21* (cyclin-dependent kinase inhibitor 1), *cdc25c*, and *gadd45* (growth arrest and DNA damage-inducible genes), which can initiate cell cycle arrest and DNA repair, and *bax* (BCL2-associated X protein), *puma*, *noxa*, and *apaf-1*, which promote apoptosis of cells (Shu et al., 2007; Beckerman and Prives, 2010). Moreover, recent studies have shown that *p53* activates transcription of microRNAs, which can trigger apoptosis and induce cellular senescence (Hermeking, 2007; Raver-Shapira et al., 2007).

In mammals, *p53* and *p53* target genes have been extensively studied, but little is known about their functions and regulation in lower vertebrates, including fish (Liu et al., 2011; Mai et al., 2012). *p53* homologs have been identified and studied in a number of fish species, such as tilapia (*Oreochromis niloticus*; Mai et al., 2012), whitefish (*Coregonus lavaretus*; Brzuzan et al., 2009), zebrafish (*Danio rerio*; Lee et al., 2007), medaka (*Oryzias latipes*; Krause et al., 1997), rainbow trout (*Oncorhynchus mykiss*; Liu et al., 2011), and channel catfish (*Ictalurus punctatus*; Luft et al., 1998). Fish *p53* is highly similar to mammalian *p53* in both structure and function (Krumschnabel and Podrabsky, 2009; Storer and Zon, 2010), and many proteins involved in the regulation of *p53* levels and stability as well as transcriptional targets of *p53* are well-conserved between mammals and fish (Krumschnabel and Podrabsky, 2009). Similar to mammals, *p53* is also required for transcriptional activation of a number of *p53* target genes in fish, including *p21*, *bax*, and *mdm2* (mouse double minute 2; Langheinrich et al., 2002; Lee et al., 2007). Nonetheless, functional analyses revealed both similarities and differences between the *p53* of mammals and that of several species of fish. For example, genotoxic agents and UV treatment enhanced the expression of *p53* in Gulf killifish (Blas-Machado et al., 2000) and Atlantic cod (*Gadus morhua*; Lesser et al., 2001), respectively. However, in several fish species, researchers have detected little or no change in *p53* levels in response to stress

factors that are known to upregulate mammalian *p53* (Chen et al., 2001; Embry et al., 2006). Moreover, *p53* deficiency or over-expression of *mdm2* led to a high rate of spontaneous cancers in mice (Donehower et al., 1992; Jones et al., 1998), but no spontaneous tumor formation occurred during early embryonic development of zebrafish (Langheinrich et al., 2002).

A number of studies have shown the effects of *p53* network genes in fish exposed to various environmental pollutants. For example, heavy fuel oil significantly upregulated *p53* and *gadd45 α* transcripts in turbot (Ruiz et al., 2012), BaP and microcystin-LR significantly induced the expression of *p53* and *p21* mRNA in whitefish (Brzuzan et al., 2005). Most previous studies examined the effects of *p53* network genes, including *p53*, *p21*, *gadd45 α* , and *mdm2*, in zebrafish (Soares et al., 2012) and turbot (Ruiz et al., 2012) exposed to environmental pollutants (e.g., PAHs). However, molecular characterization and the functions of *p53* network genes in rare minnow have not been investigated.

Benzo[a]pyrene (BaP) is one of the most studied environmentally relevant PAHs, and its carcinogenic and mutagenic properties have been extensively evaluated. Previous reports have shown that BaP induced/repressed large numbers of genes, including genes involved in the cell cycle, apoptosis, and DNA repair (Verhofstad et al., 2010; Hamouchene et al., 2011). BaP is most often linked to cancer because it is a well-established chemical mutagen (Thompson et al., 2010). Therefore, BaP is suitable as a reference chemical to study the responses of *p53* target genes in fish.

The rare minnow is distributed mostly in the upstream region of the Yangtze River and in the Sichuan province of China, and it is used as an experimental animal for the assessment of environmental pollutants due to its small size, ease of culture, short life cycle, and prolific egg production with high fertilization and hatching rates (Zha et al., 2007; Li et al., 2009). The aim of this study was to identify *p53*, *p21*, *mdm2*, *gadd45 α* , *gadd45 β* , *igfbp-3* (IGF-binding protein 3), and *bax* genes in rare minnow and to investigate the effects of BaP on *p53* network genes and histopathological changes in the livers of rare minnow. These data could contribute to our understanding of the mechanism of carcinogenesis induced by environmental pollutants (e.g., BaP).

MATERIALS AND METHODS

Chemicals and Reagents

BaP (>98% purity) and acetone were purchased from Sigma (USA). A stock solution of BaP was prepared by dilution in acetone. A vehicle treatment containing a combination of acetone served as a control. The ratio of vehicle to water was 1:10,000 (v/v).

Fish Care and Exposure

The brood stock of rare minnow was raised in a flow-through system with dechlorinated tap water (pH 7.2–7.6; hardness 44.0–61.0 mg CaCO₃/L; and temperature 25 ± 1°C) and a photoperiod of 16:8 h (light:dark) that has been used for testing chemicals in our laboratory for more than 10 years (Zha et al., 2007; Li et al., 2009). Fish were fed a commercial diet (Trea, Germany) at a rate of 0.1% body weight per day and newly hatched brine shrimp (*Artemia nauplii*) two times daily.

Healthy five-month-old adult rare minnow ($n = 150$) and the offspring from the same pair of brood stock were randomly divided into five groups. The body weights and lengths were 0.7 ± 0.21 g and 48.21 ± 3.7 mm, respectively. Fish were kept in glass containers (3 L) in a water bath at 25°C and were exposed after two weeks of acclimation. Fish were exposed to BaP concentrations of 0.4, 2, and 10 µg/L, a control group received water, and a solvent control group received 0.01% acetone in water. The fish were randomly distributed into five experimental groups. Each group contained three replicate aquaria, and each replicate aquarium included 10 fish. The test solution was renewed each day during a 28 day exposure period. During the experiment, the water temperature was maintained at 25 ± 1°C and the pH at 7.0 ± 0.2. Fish were fed twice a day with brine shrimps. After 28 days of exposure, the fish were sacrificed, and the tissues were excised, immediately frozen into liquid nitrogen, and stored at –80°C.

Cloning cDNA Fragments of p53 Pathway Genes

Total RNA was isolated from the livers of rare minnow using the SV Total RNA Isolation System following the manufacturer's protocol (Promega, USA). Then, RNA samples were dissolved in ribonuclease-free water and stored at –80°C for RT-PCR analysis. The quantity and purity of the RNA samples were determined with a spectrophotometer (Thermo Fisher). The OD_{260/280} ratio ranged from 1.8 to 2, and the integrity of the RNA was assessed by visualization of the 28S and 18S ribosomal RNA bands.

The RT-PCR analysis was carried out as previously described (Li et al., 2009). The reverse transcription reaction mixtures containing 10 µL of total RNA, 2 µL (0.05 µg/µL) of oligo(dT)₁₅ (Promega), and diethylpyrocarbonate-treated water (a total volume of 12 µL) were heated to 70°C for 5 min and quickly chilled on ice. After cooling, 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂, 2 mM deoxynucleotide triphosphates (10 mM each), 40 units of RNasin (RNase inhibitor; Promega), and 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) were added for a total volume of 25 µL and incubated for 1 h at 37°C. Then, the reaction mixture

was heated to 70°C for 10 min to stop the reverse transcription.

To obtain cDNA fragments of the p53 pathway genes (*p53*, *p21*, *mdm2*, *gadd45α*, *gadd45β*, *igfbp-3*, and *bax*), the primer pairs were designed based on the highly conserved regions of known sequences from other fish species available in GenBank (Table I). The detailed information of the PCR experiment is as follows: each 50 µL DNA amplification reaction contained polymerase chain reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂), 200 µmol dNTPs (Promega), 20 pmol of each gene-specific primer, 1 µL of a 20-fold dilution of total cDNA, and 2.5 U Taq DNA polymerase (Invitrogen). The thermocycler program included a denaturation step at 94°C (8 min); 35 cycles of 94°C (30 s), 55°C (30 s), and 72°C (30 s); and a final elongation step at 72°C (10 min). The PCR amplification products were analyzed by 1.5% agarose gel electrophoresis. The bands with the expected sizes were excised and purified using an Agarose Gel DNA Purification Kit Version 2.0 (TaKaRa, Japan). The isolated fragments were cloned into the pMD 19-T vector (TaKaRa, Japan) and transformed into *Escherichia coli* (DH 5α). The partial cDNAs were sequenced and compared using BLAST to the sequences available in GenBank, and sequence alignment and phylogenetic analyses were performed with known sequences from other fish species and mammals to confirm the identity of the clones.

Hepatosomatic Indices (HSI) and Histological Analysis

The gender-specific body weight, liver weight, and body length of the experimental fish were measured. To determine the gender, the fish was dissected, and the gender was identified by observing the gonads of each fish. The HSI were calculated as follows: $HSI = [\text{Liver weight (g)}/\text{Fish weight (g)}] \times 100$.

Liver samples fixed in Bouin's solution were transferred to 70% ethanol and processed according to standard histological methods and embedded in paraffin wax as described by Wolf et al. (2004). The sections were cut at 3–4 µm, stained with hematoxylin and eosin, and observed on a Axioskop 2 mot plus optical microscope (Zeiss, Germany) and digitized with a AxioCam digital camera (Zeiss, Germany) using the Application Suite software AxioVision Rel. 4.5 (Zeiss, Germany).

Real-Time PCR

Real-time PCR was performed to determine the expression levels of *p53*, *p21*, *mdm2*, *gadd45α*, *gadd45β*, *igfbp-3*, and *bax*. Real-time PCR was performed using a MX3005P real-time quantitative PCR system (Stratagene) with a total volume of 25 µL, consisting of the Brilliant II SYBR Green QPCR master mix (Promega), 300 nM forward primer and

TABLE I. Primer sequences used for cloning of *bax*, *gadd45*, *igfbp-3*, *mdm2*, *p21*, and *p53*, and quantification of *p53* pathway genes expression by real-time PCR in rare minnow

Gene	Sequence (5'→3')	Product Size (bp)	Genbank Accession No.
Cloning			
<i>Bax</i>	F: CTGGGGAAGAGTTGTGGC R: GCGAGGAAAACCTCCGACT	228	KC477758
<i>gadd45β</i>	F: AAAAGCCAGGTCCATAAAG R: GAAACCCCTTTGGTCACAG	204	KC477759
<i>igfbp-3</i>	F: AAAAGCAAAGCGGCAGTC R: GAACGATCCAACCGGAAA	454	KC477760
<i>mdm2</i>	F: AGCCTGTCTTGGTGCG R: CTCGGTTATCTCAGTATCTTCA	298	KC477761
<i>p21</i>	F: ATCTGGTTGGCGATGAGC R: GCCGAAATCAGTGAGTTTGA	280	KC477762
<i>p53</i>	F: GCAGGCCCATCCTCACAA R: AAGAAGATTCTTTCACCAAA	240	KC477763
<i>gadd45α</i>	F: TGTGGAGATAACGCAACG R: GGTGAAGTGAATCTGGAG	232	KC477764
Real-time PCR			
<i>β-actin</i>	F: CAGGGCGTGATGGTGGGGAT R: GGTGGCTTTGGGGTTGAG	226	
<i>Bax</i>	F: ATTTCAACCAGGGTTCTT R: GTCCCATCCACCCTGTTC	110	
<i>gadd45β</i>	F: CTGCGTTGTGCTGTGCAT R: GAAACCCCTTTGGTCACAG	127	
<i>igfbp-3</i>	F: GAAACCCCTTTGGTCACAG R: TCCAGGTATGCGAGGTTG	106	
<i>mdm2</i>	F: GTCTTCGGACGCACGCAG R: ATGGACTCCACCTCAAACCT	114	
<i>p21</i>	F: TAAGGTACTATGAAGGATGCC R: AGAGGGATTATGGGACAGA	92	
<i>p53</i>	F: CCATCCTCACAATCATCAC R: CTCCTCAGTTTTCTGTCTC	74	
<i>gadd45α</i>	F: TGTCGTCGGGAGATGGAAT R: GTTGTTCACCTCGCAAGAT	139	

300 nM reverse primer. The thermal cycle parameters were 10 min at 95°C, 40 cycles of 30 s at 95°C, 1 min at 57°C, and 30 s at 72°C. All samples were analyzed in triplicate, and the mean value of these triplicate measurements was used to calculate the mRNA expression. The results were analyzed according to the delta–delta Ct method (Schmittgen and Livak, 2008), and *β-actin* was used as the internal control gene. Dissociation curve analysis was performed for each gene to evaluate the amplification of untargeted fragments. The efficiency of the primer sets was assessed with a standard curve via a dilution series, the linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was >0.99 (ranging from 0.991 to 0.998) in each case, and the efficiencies ranged from 95.1 to 104.8%.

Statistics

Statistical analyses were performed with SPSS (version 13.0) and OriginPro (version 8.0). The experimental data

were assessed for homogeneity of variance across treatments using Levene's test. All quantitative data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis of the data was performed using an ANOVA, followed by Duncan's multiple range test. A probability of $p < 0.05$ was considered statistically significant.

RESULTS

Nucleotide and Predicted Amino Acid Sequences Analysis

The partial cDNA fragments of *p53*, *p21*, *mdm2*, *gadd45α*, *gadd45β*, *igfbp-3*, and *bax* were isolated and cloned from rare minnow. Cloned cDNAs were sequenced and compared to sequences in GenBank using BLAST analysis. Phylogenetic analysis revealed that the putative proteins of *p53*, *p21*, *mdm2*, *gadd45α*, *gadd45β*, and *igfbp-3* in rare minnow had high amino acid similarities to those of other fish and

TABLE II. Growth and the HSI of adult fish exposed to BaP for 28 d

Concen. ($\mu\text{g/L}$)	Body weight (g)		Body length (mm)		HSI (%)	
	Males	Females	Males	Females	Males	Females
Control	0.86 ± 0.13	1.35 ± 0.56	44.81 ± 4.01	52.32 ± 3.51	2.18 ± 0.38	2.18 ± 0.38
0.4	0.87 ± 0.22	1.24 ± 0.37	45.11 ± 1.61	51.51 ± 1.11	2.01 ± 0.39	1.88 ± 0.45
2	0.89 ± 0.24	1.11 ± 0.31	47.51 ± 4.11	53.11 ± 2.61	2.14 ± 0.54	2.07 ± 0.44
10	0.84 ± 0.19	1.12 ± 0.23	42.01 ± 4.31	52.71 ± 2.91	1.95 ± 0.37	1.47 ± 0.42^a

Data expressed as mean $\pm$ SEM of each treatment (males, $n = 15$; females, $n = 15$).

^aStatistically significant difference from the control ($p < 0.05$).

mammalian species, confirming their identities (Supporting Information Fig. S1). The partial cDNA sequences for rare minnow had high similarity with the same regions in zebrafish for *p53* (88%), *p21* (85%), *mdm2* (99%), *gadd45 α* (99%), *gadd45 β* (92%), *bax* (91%), and *igfbp-3* (90%). The cloned sequences have been deposited in GenBank, and the accession numbers of *p53*, *p21*, *mdm2*, *gadd45 α* , *gadd45 β* , *bax*, and *igfbp-3* are KC477763, KC477762, KC477761, KC477764, KC477759, KC477758, and KC477760, respectively.

Growth, HSI, and Histopathology

The body lengths and body weights of adult rare minnow exposed to BaP for 28 days were not significantly different compared with those of the controls (Table II). Because no significant difference was observed between the acetone control and water control for the body lengths, body weights and HSI,

as well as the gene expression, the water control was used as the control group for subsequent experiments. Compared with the control, BaP caused significant decreases in HSI in females exposed to BaP at 10 $\mu\text{g/L}$ ($p < 0.05$, Table II).

Normal hepatic tissue exhibited a regular hepatocyte structure with evident, well-defined nuclei [Fig. 1(A,C)]. No significant histological changes were found in the liver of males and females exposed to low concentrations of BaP. However, microphotographs revealed enlargement of the cell nuclei and cellular degeneration in male rare minnow treated with high concentrations of BaP (10 $\mu\text{g/L}$) [Fig. 1(B)]. Atrophy and vacuolization of the hepatocytes were observed in females at high concentrations (10 $\mu\text{g/L}$) [Fig. 1(D)].

Quantitation of p53 Pathway Genes

The mRNA expressions of *p53*, *p21*, *mdm2*, *gadd45 α* , *gadd45 β* , *igfbp-3*, and *bax* in the livers of males and females

Fig. 1. Microphotographs of the liver from rare minnow (*Grobicypris rarus*), stained with hematoxylin and eosin: (A) Liver from male control, (B) Liver from male fish exposed to 10 $\mu\text{g/L}$ BaP, (C) Liver from female control, (D) Liver from female fish exposed to 10 $\mu\text{g/L}$ BaP. The black arrows indicated enlargement of the cell nuclei in B, and vacuolization and atrophy of hepatocytes in C. The images shown are representative of three replicate aquaria per treatment, bar = 20 μm .

Fig. 2. Expression levels of *p53*, *p21*, *mdm2*, and *bax* mRNA in the livers of adult rare minnow (males ■ and females □) exposed to BaP for 28 d. Values are presented as the mean $\pm$ SEM. A significant difference between the groups of $p < 0.05$ ($n = 3-5$, ANOVA) is indicated by differences in the letters above the bars.

were determined after 28 days of exposure to BaP. The liver *p53* mRNA expression was significantly upregulated compared with that of the controls at BaP concentrations above 0.4 µg/L ($p < 0.05$, Fig. 2). For males, *p21* mRNA levels in all treatments and *mdm2*, *gadd45 α* , *bax* mRNA levels at 2 and 10 µg/L BaP were significantly induced compared with

those of the controls ($p < 0.05$, Figs. 2 and 3). For females, mRNA levels of *p21*, *mdm2*, *gadd45 α* , and *bax* were significantly induced in all treatments compared with those of the controls ($p < 0.05$, Figs. 2 and 3). In addition, expressions of *gadd45 β* and *igfbp-3* in the livers of males and females in all treatment groups were not significantly changed (Fig. 3).

Fig. 3. Expression levels of *gadd45 α* , *gadd45 β* , and *igfbp-3* mRNA in the livers of after adult rare minnow (males ■ and females □) exposed to BaP for 28 d. Values are presented as the mean $\pm$ SEM. A significant difference between the groups of $p < 0.05$ ($n = 3-5$, ANOVA) is indicated by differences in the letters above the bars.

DISCUSSION

Identification of New p53 Pathway Genes in Rare Minnow

In our study, seven previously unidentified p53 network genes in rare minnow were cloned and identified. The partial fragments of these p53 network genes were sequenced and submitted to GenBank. Based on the sequence and phylogenetic analysis of their predicted amino acid sequences, the new transcripts were classified as *p53*, *p21*, *mdm2*, *gadd45 α* , *gadd45 β* , *igfbp-3*, and *bax*. In general, the predicted amino acid sequences of the genes exhibited high homology with the same regions in zebrafish (Soares et al., 2012).

Histopathology

Histopathological alterations have been assessed in various tissues of fish for decades to determine the effects of environmental contaminants both in field and laboratory settings. In addition, the liver has been the focus of histopathological biomarkers because of its key role in processes such as metabolism of xenobiotics and synthesis of vitellogenin (Dabrowska et al., 2012). There have been numerous reports demonstrating the histopathological alterations in the livers of fish exposed to various environmental pollutions (Au, 2004).

In this study, significant histopathological changes, including enlargement of the cell nuclei, atrophy and vacuolization of hepatocytes, and decreases in the diameter of hepatocytes, were observed in the livers of rare minnow exposed to BaP (10 μ g/L). Similarly, significantly more hepatic foci in Atlantic killifish exposed to 200 μ g/L BaP for 24 h were observed (Wills et al., 2010). Atrophy and vacuolization of the hepatocytes have been observed in several fish exposed to PAHs, and cytoplasmic vacuolation is usually produced by deposition of glycogen and lipids, which eventually leads to the displacement and deformation of the nucleus (Pal et al., 2011). In addition, hypertrophy of the hepatocytes in males and females was observed in rare minnow exposed to atrazine and 3-amino-1,2,4-triazole at 10 μ g/L (Li et al., 2009; Yang et al., 2010). These results suggest that histopathological alteration is a direct and valid way to identify the effects of environmental carcinogens.

Induction of p53 Pathway Genes

p53 is a tumor suppressor protein that has a key role in regulating the cell cycle, apoptosis, and DNA repair. The principal mechanisms regulating the activity of p53 at the protein level (Braithwaite et al., 2005) and the increased cellular p53 protein levels following exposure to various genotoxic agents are mainly due to an increase in the stability of the p53 protein rather than an increase in the levels of p53 mRNA (Park et al., 2006). However, several studies have suggested that PAH-stimulated p53 accumulation may also

be transcriptionally induced (Pei et al., 1999; Park et al., 2006). Moreover, a number of studies have indicated that genotoxic chemicals could modulate p53 mRNA expression, and it has been suggested as a biomarker (Brzuzan et al., 2005; Brzuzan et al., 2011). In this study, BaP significantly induced the expression of p53 mRNA in rare minnow livers. Similar results were obtained in human cells (Pei et al., 1999) and whitefish (Brzuzan et al., 2005) exposed to BaP, indicating that BaP-induced p53 expression is partly regulated at the transcriptional level (Pei et al., 1999). In contrast, other studies have shown that BaP and dioxin suppressed the mRNA expression of p53 in rainbow trout (Brzuzan et al., 2011) and human epidermal keratinocytes (Ray and Swanson, 2004), respectively. The authors indicated that the repression of p53 mRNA expression requires the involvement of AhR and is mediated by DNA methylation (Ray and Swanson, 2004). Nonetheless, p53 mRNA could be a potential biomarker to screen environmental carcinogens.

Because p53 has crucial roles in regulating cell survival and death, its expression is tightly regulated at multiple levels (Vilborg et al., 2010) and maintained at low levels under normal conditions. The turnover of p53 is regulated by the p53 target gene *mdm2*, which is the major negative regulator of p53 levels and activity in mammals (Momand et al., 1992). In this study, a significant increase in *mdm2* gene transcription in the males and females was observed. Similarly, statistically significant differences in *mdm2* expression levels were observed in laryngeal carcinoma samples (Hassumi-Fukasawa et al., 2012). Our finding that p53 and *mdm2* increased upon BaP exposure could be due to the auto-regulatory feedback loop described in mammals for this complex of proteins (Soares et al., 2012). However, molecular analysis of zebrafish *mdm2* revealed that it seems to lack some key amino acids for DNA binding found in other models (Neel et al., 2000), and the regulation of p53 activity in fish may not be dependent on *mdm2* in the same way or to the same extent as that in mammals (Krumnschnabel and Podrabsky, 2009).

The p53 pathway responds to various cellular stress signals by activating p53, which functions as a transcription factor. Figure 4 shows the program that p53 respond to BaP exposure. In the present study, to elucidate the effects of BaP on p53 signaling pathways, we analyzed the transcriptional levels of several p53 downstream genes (e.g., *p21*, *gadd45a*, and *bax*), which are key players in cell cycle regulation, DNA repair and apoptosis. The cyclin-dependent kinase inhibitor p21 (also known as p21^{WAF1/Cip1}) promotes cell cycle arrest in response to many stimuli (Abbas and Dutta, 2009). In this study, BaP significantly induced the *p21* transcripts in the livers of rare minnow (both males and females). Previous studies have indicated that the increase in *p21* mRNA expression was related to p53 functional status, and it was useful for the analysis of p53-mediated cell cycle checkpoints (Węglarz et al., 2006). Thus, the elevated

Fig. 4. The program that p53 responds to BaP perturbation. The figure was according to Shu et al. (2007), and there was minor revision for it. [Color figure can be viewed at wileyonlinelibrary.com]

expression of *p21* could indicate a possible role for the p53/p21 pathway in the cell cycle arrest in rare minnow livers.

The *gadd45* family genes have been implicated in stress signaling in response to various physiological or environmental stressors, which results in cell cycle arrest, DNA repair, cell survival and senescence, or apoptosis (Liebermann and Hoffman, 2008). Previous studies have shown that *gadd45* family members are rapidly induced by various genotoxic stress agents (Liebermann and Hoffman, 2007; Vairapandi et al., 2002). In this study, the expression of *gadd45α* but not *gadd45β* was induced by BaP in both males and females. Similarly, previous studies have shown that individual members of the *gadd45* family are differentially induced by various genotoxic and environmental stress agents (Shaulian and Karin, 1999; Zhang et al., 1999). Moreover, previous studies have indicated that only *gadd45α* is a p53 target gene (Liebermann and Hoffman, 2008). These results and our study on rare minnow suggested that *gadd45* members serve similar, but not identical, functions in different stress response pathways (Liebermann and Hoffman, 2008).

Another key gene involved in p53-mediated responses is *bax*, which is a p53 apoptosis-induced gene (Soares et al., 2012). *Bax* plays an essential role in the mitochondrial pathway of apoptosis (Donauer et al., 2012); after being released or activated, it undergoes a conformational change and forms an oligomeric pore in the outer mitochondrial membrane through which the apoptosis-stimulating factors (e.g., cytochrome c) are released (Krumschnabel and Podrabsky, 2009). In our study, BaP significantly induced the mRNA expression of *bax* in rare minnow liver. Similarly, elevated expression of *bax* was observed in human cell lines exposed to BaP (Jiang et al., 2011). In a previous study, following treatment with p53-activating agents, *bax* was transcriptionally upregulated together with cyclin G1, *mdm2* and *p21* in zebrafish embryos (Lee et al., 2007), which was consistent

with our results on rare minnow. Furthermore, the BaP-induced *bax* mRNA expression may indicate that apoptosis occurred in the liver of rare minnow, possibly through activation of the intrinsic pathway and related caspases.

IGF play an important role in the etiology of breast cancer (Ren et al., 2004). The IGF binding proteins regulate the action of IGFs, and >90% of circulating IGFs are bound to igfbp-3 (Al-Zahrani et al., 2006). Therefore, igfbp-3 functions as a carrier of IGFs in circulation and a mediator of growth suppression in cells. In our study, expression of liver *igfbp-3* was not significantly changed in all treatment groups (Fig. 3). Our findings indicated that liver cells did not become cancerous; this could be due to low concentrations of BaP and a short exposure period. Similarly, tumor cells were not found in light micrographs of hepatic tissues in male and female adult rare minnow (Fig. 1).

CONCLUSIONS

In summary, we identified and cloned the partial cDNAs of previously unreported p53 network genes, including *p53*, *p21*, *mdm2*, *gadd45α*, *gadd45β*, *igfbp-3*, and *bax* in rare minnow liver. BaP significantly upregulated these genes, including *p53*, *p21*, *mdm2*, *gadd45α*, and *bax*. Our findings suggest that rare minnow exposed to BaP could trigger p53/p21/*gadd45α*-mediated cell cycle arrest and DNA repair and p53/*bax*-mediated apoptosis pathways. Additionally, all these outcomes of the p53 transcriptional program can lead to the suppression of tumors in rare minnow liver. Further studies focusing on combining gene and protein expressions should be performed to identify the mechanism underlying the action of BaP in fish. Additionally, the p53 network genes in rare minnow could serve as potential molecular biomarkers of environmental carcinogens.

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