ORIGINAL RESEARCH PAPER

Characterization of the tilapia p53 gene and its role in chemical-induced apoptosis

Wei-jun Mai · Peng Liu · Weina Wang

Abstract Roles of p53 in mammals have been extensively studied but little is known about its functions in lower vertebrates. We have cloned and characterized the p53 gene from tilapia (*Oreochromis niloticus*), expressed it in *Escherichia coli*, generated a rabbit polyclonal antibody and examined the gene's expression patterns at both transcript and protein levels. The full-length p53 cDNA was 1288 bp with an ORF of 1,138 bp, encoding a polypeptide of 364 amino acids. The gene contains all four classical p53 family signature motifs but has low sequence similarity with other p53 genes. It was constitutively transcribed in all tissues examined, most abundantly in liver and blood cells. In addition, ATM, p53 and Bax proteins, associated with DNA damage responses and apoptosis, were all upregulated in tilapia liver after treatment with the stress inducers etoposide and $CdCl_2$. Tilapia p53 is thus both a constitutive and inducible acute-phase protein that is cooperatively activated via the ATM-p53 pathway in responses to stress and DNA-damaging signals.

Keywords Antibody · Apoptosis · Chemicals · p53 · Tilapia

W. Mai (✉) · P. Liu
Institute of Life Science, Jiangsu University,
Zhenjiang 212013, China
e-mail: mwj9876@yahoo.com.cn

W. Wang
College of Life Science, South China Normal University,
Guangzhou 510631, China

Introduction

p53 is a well-known tumor suppressor, which is also involved in organismal aging and developmental control processes. As a transcription factor, the p53 protein regulates the expression of target genes encoding proteins involved in cell growth control (Brady and Attardi 2010). It also participates in complex cellular responses to DNA damage-inducing stresses and agents, such as hypoxia, UVR, oxidative stress and mutagens (Kruse and Gu 2009). These responses involve DNA editing and repair followed by either normal cell division or apoptosis, resulting in cell survival and death, respectively (Devlin et al. 2008).

p53 has been identified in a number of fish species, including whitefish (Brzuzan et al. 2009), rainbow trout (Caron de Fromentel et al. 1992) and channel catfish (Luft et al. 1998). However, its expression, regulation and physiological significance in lower vertebrate species are not well understood at present. p53 expression may be induced producing a stable protein and thus accumulates in fish in response to DNA damage and other cellular perturbations

(Lee et al. 2008). However, in contrast, other researchers have detected little or no change in fish p53 protein levels in response to stress factors that normally upregulate mammalian p53. Instead, they have found that the p53 protein in some fish species is activated through phosphorylation, rather than either induction or stabilization (Brooks and Gu 2003; Rau Embry et al. 2006).

Here we show that tilapia p53 is upregulated by chemically induced DNA damage with concomitant apoptosis under control of the ATM p53-Bax signaling pathway, strongly supporting the view that stabilization of p53 protein is a crucial step in cellular responses to DNA damage in fish, or at least tilapia.

Materials and methods

Tilapia culture, chemical challenge, and tissue collection

Tilapias were collected from a commercial farm in Yangjiang, Guangdong Province, China. Individuals weighing 120 ± 20 g were cultured in filtered, aerated seawater at 20–23 °C for 2 weeks and then randomly divided into four treatment groups (blank, control, etoposide-stimulated and $CdCl_2$-stimulated) with three replicates per group. Etoposide and $CdCl_2$ stimulation was administered by injecting muscles of each tilapia with 10 or 100 μg/kg body wt of etoposide or $CdCl_2$ dissolved in 10 ml PBS, respectively. Controls were injected with 10 ml of PBS, and the blanks received no injection. At each of five time points (0, 4, 12, 24, and 48 h post-injection), five individuals per replicate were killed then their tissues were collected, pooled and used for DNA damage assessment, western blotting or immediately mixed with TRIzol reagent for RNA extraction, as described below.

Cloning full-length tilapia p53 cDNA

Primers (listed in Supplementary Table 1) were designed for a conserved region identified by comparing all p53 sequences compiled in the Genbank databases. Total RNA was extracted from blood cell samples using TRIzol reagent (Invitrogen), and reverse-transcribed into cDNA using Powerscript II reverse transcriptase with a CDS primer kit (Clontech). The PCR cycling conditions were as follows:

94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. PCR products were cloned into a pGEM-T vector (Promega) and sequenced using an ABI PRISM 377 DNA sequencer (Perkin-Elmer).

To recover the full-length cDNA sequence, 3′-RACE and 5′-RACE were performed using gene-specific and adaptor primers (Supplementary Table 1). The PCR cycling profile was 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 64 °C for 30 s and 72 °C for 1 min, with a final elongation step at 72 °C for 5 min.

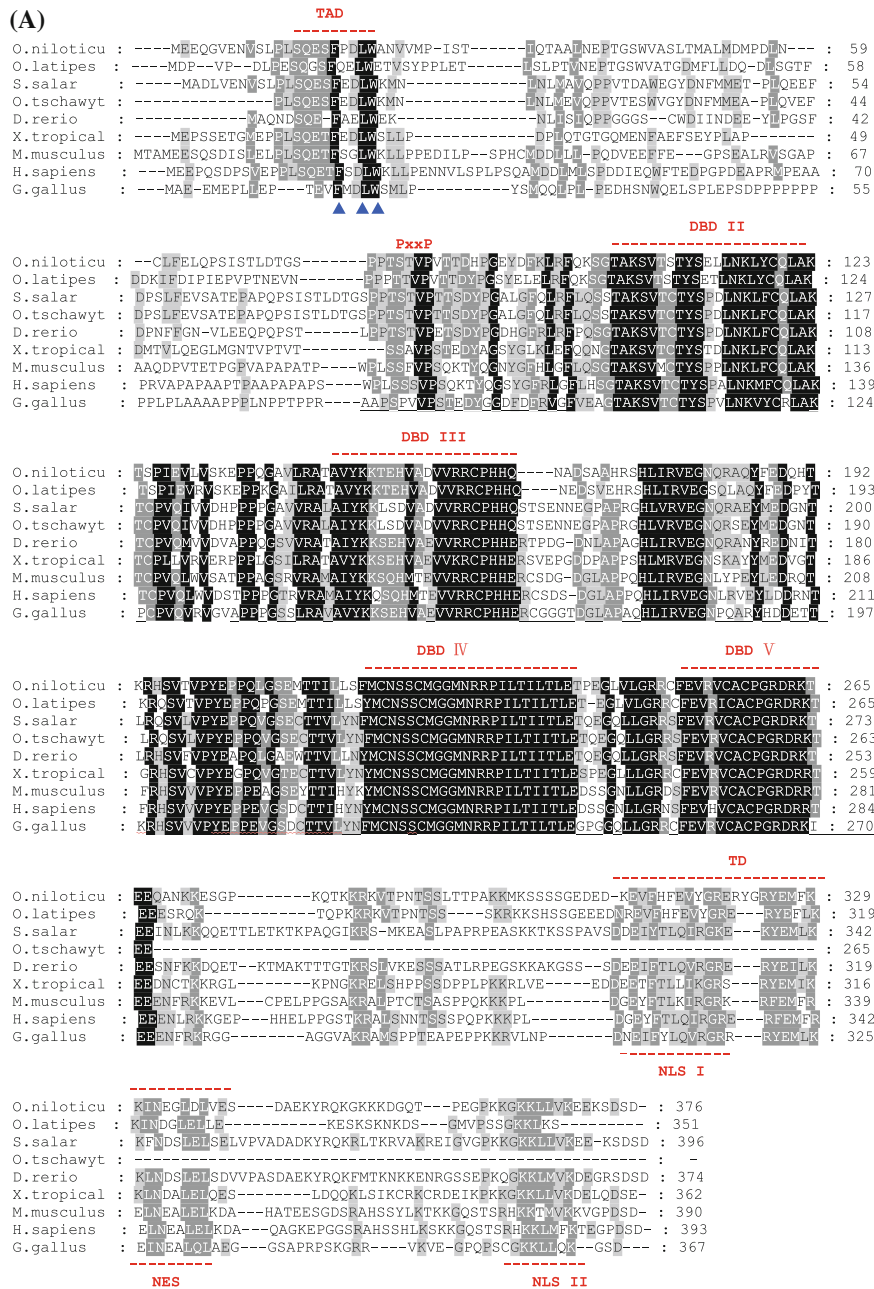### Sequence analysis of tilapia p53

The tilapia p53 amino acid sequence was deduced using DNAstar software, and compared (using BLAST) to previously reported p53 amino acid sequences compiled in the GenBank, EMBL, and DDBJ databases. It was then aligned with homologous sequences by GENE-DOC, version 2.6.001, and a phylogenetic tree based on the alignment was constructed using MEGA version 4.1 (with 1,000 bootstrap replications).

Production of recombinant tilapia p53 in E. coli

A cDNA sequence encoding full-length tilapia p53 was amplified by RT-PCR. The amplified product was inserted into the BamHI-HindIII cloning sites of the vector pET-32a (Novagen). Then, following Novagen's recommended protocols, the construct was used to transform Escherichia coli BL21 and expression of histidine (His)-tagged tilapia p53 protein (His-p53) was induced by IPTG. The recombinant protein was purified in its native conformation using Ni–NTA His Bind Resin (Novagen), its purity was assessed using 12 % SDS-PAGE followed by staining. Its concentration was determined using the Bradford method.

Immunizations

To raise a polyclonal antibody against tilapia p53, four New Zealand white rabbits were each immunized with 1 mg purified His-p53 emulsified in complete Freund's adjuvant (Sigma). Subsequent booster immunizations with 1 mg purified His-p53 in incomplete Freund's adjuvant (Sigma) were administered to each rabbit 14, 28, and 42 days thereafter, then the rabbits were bled 7 days after the final immunization

(A)

                                                  TAD
O.niloticu : ----MEEQGVENVSLPLSQESFPDLWANVVMP-IST------IQTAALNEPTGSWVASLTMALMDMPDLN--- : 59
O.latipes : ----MDP--VP--DLPESQGSFQELWETVSYPPLET------LSLPTVNEPTGSWVATGDMFLLDQ-DLSGTF : 58
S.salar   : -----MADLVENVSLPLSQESFEDLWKMN------------LNLVAVQPPVTDAWEGYDNFMMET-PLQEEF : 54
O.tschawyt : -----------PLSQESFEDLWKMN------------LNLMEVQPPVTESWVGYDNFMMEA-PLQVEF : 44
D.rerio   : -----------MAQNDSQE-FABLWEK--------------NLISLQPPGGGS--CWDIINDEE-YLPGSF : 42
X.tropical : ----MEPSSETGVEPPLSQETFEDLWSLLP-----------DPLQTGTGQMENFAEFSEYPLAP------- : 49
M.musculus : MTAMEESQSDISLELPLSQETFSGLWKLLPPEDILP--SPHCMDDLLL-PQDVEEFFE---GPSEALRVSGAP : 67
H.sapiens : ---MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLMLSPDDIEQWFTEDPGPDEAPRMPEAA : 70
G.gallus   : ---MAE-EMEPLLEP---TEVEMDLWSMLP----------YSMQQLIPL-PEDHSNWQELSPLEPSDPPPPPPP : 55
                    ▲ ▲▲

                                PxxP   DBD II
O.niloticu : --CLFELQPSISTLDTGS-------FPTSTVPVTTLHPGEYDFKLRFQKSGTAKSVITSYSELLNKLYCQLAK : 123
O.latipes : DDKIFDIPIEPVPTNEVN-------PPPTIVPVTTDYPGSYELBLRFQKSGTAKSVTSTYSETLNKLYCQLAK : 124
S.salar   : DPSLFEVSATEPAPQPSISTLDTGSPPTSTVPTTSDYPGALGFQLRFLQSSTAKSVTCTYSPDLNKLFCQLAK : 127
O.tschawyt : DPSLFEVSATEPAPQPSISTLDTGSPPTSTVPTTSDYPGALGFQLRFLQSSTAKSVTCTYSPDLNKLFCQLAK : 117
D.rerio   : DPNFFGN-VLEEQPQPST-------LPPTSTVPESDYPGDHGFRLRFPQSGTAKSVTCTYSPDLNKLFCQLAK : 108
X.tropical : DMTVLQEGLMGNTVPTVT--------SSAVPSTEDYACSYGLKLEFQQNCTAKSVTCTYSTDLNKLFCQLAK : 113
M.musculus : AAQDPVTETPGPVAPAPATP----WPLSSFVPSQKTYQCNYGFHLGFLQSGTAKSVMCTYSPPLNKLFCQLAK : 136
H.sapiens : PRVAPAPAAPTPAAPAPAPS----WPLSSSVPSQKTYQGSYGFRLGFLHSGTAKSVTCTYSPALNKMFCQLAK : 139
G.gallus   : PPLPLAAAAPPPLNPPTPPR----AAPSPVVPSTEDYGGDFDERVGFVEAGTAKSVTCTYSPVLNKVYCRLAK : 124

                                DBD III
O.niloticu : TSPIEVLVSKEPPQGAVLRATAVYKKTEHVADVVRRCPHHQ----NALSAAHRSLLIRVEGNQRACYFEDQHT : 192
O.latipes : TSPIEVRVSKEPPKGAILRATAVYKKTEHVADVVRRCPHHQ----NELSVEHRSHLIRVEGSQLACYFEDPYT : 193
S.salar   : TCPVQLIVDHPPPGAVVRALAIYKKLSDVADVVRRCPHHQSTSENNEGPAPRGHLVRVEGNQRABYMEDGNT : 200
O.tschawyt : TCPVQLIVDHPPPGAVVRALAIYKKLSDVADVVRRCPHHQSTSENNEGPAPRGHLVRVEGNQRSBYMEDGNT : 190
D.rerio   : TCPVQMWVDVAPPGCSVVRAAAIYKKSEHVABVVRRCPHHERTPDG-ENLAPAGHLIRVEGNQRANYREDNIT : 180
X.tropical : TCPLLVRWERPPPLGSILRAPAVYKKSEHVAEVVRRCPHHERSVEPGDPAPPSHLMRVEGSNSKAYYMEDVQT : 186
M.musculus : TCPVQLWVSATPPAGSRVRAMAIYKKSQHMTEVVRRCPHHERCSDG-DGLAPPQHLIRVEGNLYPEYLEDRQT : 208
H.sapiens : TCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPHHERCSDS-DGLAPPQHLIRVEGNLRVEYLDDRNT : 211
G.gallus   : TCPVQVRVGVAPPPGSSLRAWAVYKKSEHVAEVVRRCPHHERCGGGTDGLAPAQHLIRVEGNPQARYHDDETT : 197

                        DBD IV   DBD V
O.niloticu : KRHSVTVPYEPPQLGSEMTTILLSFMCNSSCMGGMNRRPILTILTLETPPGLVLGRRCFEVRVCACPGRDRKT : 265
O.latipes : KRQSVTVPYEPPQPGSEMTTILLSYMCNSSCMGGMNRRPILTILTLET-PGLVLGRRCFEVRICACPGRDRKT : 265
S.salar   : LRHSVLVPYEPPQVGSECTTVLYNFMCNSSCMGGMNRRPILTIITLETQEGQLLGRRSFEVRVCACPGRDRKT : 273
O.tschawyt : LRQSVLVPYEPPQVGSECTTVLYNFMCNSSCMGGMNRRPILTIITLETQEGQLLGRRSFEVRVCACPGRDRKT : 263
D.rerio   : LRHSVHVPYEPAPQLGAEWTTVLLNYMCNSSCMGGMNRRPILTIITLETQEGQLLGRRSFEVRVCACPGRDRKT : 253
X.tropical : GRHSVCVPYEGPQVGTECTTVLYNYMCNSSCMGGMNRRPILTIITLESPEGLLLGRRCFEVRVCACPGRDRRT : 259
M.musculus : PRHSVVVPYEPPEAGSEYTTIHYKYMCNSSCMGGMNRRPILTIITLEDSSGNLLGRDSFEVRVCACPGRDRRT : 281
H.sapiens : FRHSVVVPYEPPEVGSDCTTIHYNYMCNSSCMGGMNRRPILTIITLEDSSGNLLGRNSFEVHVCACPGRDRRT : 284
G.gallus   : KRHSVVVPYEPPEVGSDCTTVLYNFMCNSSCMGGMNRRPILTILTLEGPCGQLLGRRCFEVRVCACPGRDRKT : 270

                                                                    TD
O.niloticu : EEQANKKESGP--------KQTKKRKVTPNTSSLTTPAKKMKSSSSGEDED-KVPHFEVYGRERYGRYEMFK : 329
O.latipes : EEESROK-----------TQPKKFKVTPNTSS----SKRKKSHSSGEEEDNREVFHFEVYGRE---RYEFLK : 319
S.salar   : EEINLKKQQETTLETKTKPAQGIKRS-MKEASLPAPRPEASKKTKSSPAVSDDEIYTLQIRGKE---KYEMLK : 342
O.tschawyt : EE--- : 265
D.rerio   : EESNFKKDQET--KTMAKTTTGTKRSLVKESSSATLRPEGSKKAKGSS--SDEFIFTLQVRGRE---RYEILK : 319
X.tropical : EEDNCTKKRGL--------KPNGKRELSHPPSSDPPLPKKRLVE-----EDDEBTFTLLIKGRS---RYEMTK : 316
M.musculus : EEENFRKKEVL---CPELPPGSAKRALPTCTSASPPQKKKPL---------DGSYFTLKIRGRK---RFEMFR : 339
H.sapiens : EEENLRKKGEP---HHELPPGSTKRALSNNTSSSPQPKKKPL---------LGEYFTLQIRGRE---RFEMFR : 342
G.gallus   : EEENFRKRGG--------AGGVAKRAMSPPTEAPEPPKKRVLNP-------DNEIFYLQVRGRR---RYEMLK : 325

                                                                NLS I
O.niloticu : KINEGLDLVES----DAEKYRQKGKKKDGQT---PEGPKKGKKLLVKEEKSDSD- : 376
O.latipes : KINDGLEILLE-----------KESKSKNKDS---GMVPSSGKKLKS--------- : 351
S.salar   : KFNDSLELSELVPVADADKYRQKRLTKRVAKREIGVGPKKGKKLLVKEE-KSDSD : 396
O.tschawyt : -- : -
D.rerio   : KLNDSLELSDVVPASDAEKYRQKFMTKNKKENRGSSEPKQGKKLLMVKDEGRSDSD : 374
X.tropical : KLNDALELQES--------LDQQKLSIKCRKCRDEIKPKKGKKLLVKDELQDSE- : 362
M.musculus : ELNEALELKDA---HATEESGDSRAHSSYLKTKKGQSTSRHKKTMVKKVGPDSD- : 390
H.sapiens : ELNEALELKDA---QAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD- : 393
G.gallus   : EINEALQIAEG---GSAPRPSKGRR----VKVE-GPQPSCGKKLLQK--GSD--- : 367
             ----------   ----------
                  NES   NLS II

Fig. 1 a Multiple alignment of the deduced amino acid sequence of tilapia p53 with p53 of other vertebrates obtained using Clustal X. Residues in *black* and *grey* indicate 100 % identity and conserved substitutions, respectively. The characteristic domains of the p53 family are highlighted in *red* above the tilapia sequence. *TAD* Transcriptional activation domain (or DNA binding domain I), *Colons* (:) conserved hydrophobic residues involved in MDM2 interactions in human, *DBD* DNA-binding domain, *PxxP* Proline-rich domain, *TD* Tetramerization domain. The Nuclear export signal (NES) and Nuclear localization domain (NLS), in the *C*-terminus, are indicated by the *red dashed lines* under the sequences. **b** Majority-rule consensus phylogenetic tree constructed by the bootstrap method with heuristic search using MEGA 4.0 (bootstrap value 1,000). Zebrafish, *Danio rerio* p73 (NM_183340) was used as an out-group. Bootstrap values are shown above the branches. p53 sequences for alignment were retrieved from GenBank, as described in the text. **c** Expression and purification of recombinant tilapia p53 in *E. coli* cells. Collected cell lysates and purified proteins were separated on 12 % SDS gels. Lane 1, protein molecular standard; Lane 2, pET-32a without insert; Lane 3, pET-32a lysozyme following IPTG induction

(B)

(C)
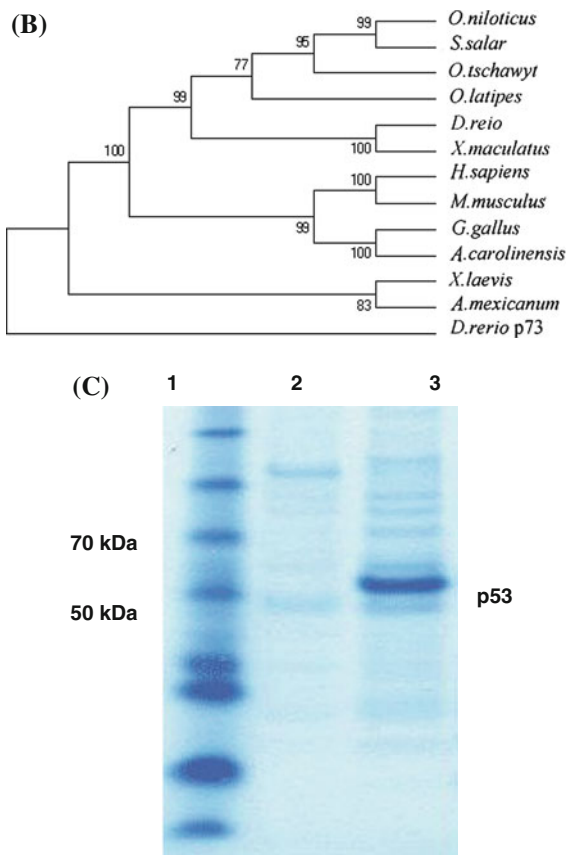
Fig. 1 continued

following procedures specified in the Guidelines for Animal Experimentation of the Shanghai Veterinary Research Institute. The antibody titer in blood serum was measured using an enzyme-linked immunosorbent assay using ELISA kit (BD science).

Single-cell gel electrophoresis (Comet assay)

Cells in liver tissues from four tilapias of each replicate sampled 0, 4, 12, 24, and 48 h after treatment were separated by incubation with 0.1 % trypsin at 37 °C for 2 h, then washed by centrifugation twice at $8,000\times g$ and resuspension in PBS (pH 7.4). The final cell density (as determined spectrophotometerically) was adjusted to 2×10^5 cells/ml with PBS, then the Comet assay was applied, as described by Singh et al. (2004). This assay indicates the extent to which DNA strands in individual cells have been broken, by measuring the migration of fragmented and relaxed DNA molecules away from nuclei immobilized in agarose gels.

TUNEL assay

The degree of DNA fragmentation induced in the tilapia's liver cells by the etoposide and cadmium chloride injections at each of the sampling time points (see above) was also assessed as follows. Liver cells extracted from each group of fish were fixed in 4 % (v/v) paraformaldehyde for 30 min, washed with PBS and permeabilized by incubation with 20 µg proteinase K/ml for 5 min at room temperature. The degree of DNA fragmentation in the cells was then quantified using a commercially available TUNEL assay kit (Pharmingen) according to the manufacturer's protocol.

Quantitative real-time PCR assay of tilapia p53 gene expression

To assess the p53 gene transcription profile in healthy tilapia, total RNA was extracted from gills, heart, spleen, liver, muscle and blood cells of three healthy fish randomly selected just before the experiment started. In addition, to assess changes in its expression following challenge with DNA-damaging agents, total RNA was extracted from the same samples of fish stimulated with either etoposide or $CdCl_2$ at 10 or 100 µg/ml and collected at each of the time point. We then quantified the p53 transcripts (normalized to levels of beta actin transcripts) by quantitative real-time PCR (qRT-PCR) using primers based on published tilapia mRNA sequences designed by Primer Premier V5 (Premier Biosoft International) The primer sequences are shown in Supplementary Table 1. qRT-PCR amplifications were carried out using a SYBR PrimeScript miRNA RT-PCR Kit (Takara) and an ABI7300 real-time PCR instrument (Applied Biosystems), with a profile consisting of 94 °C for 10 s, followed by 40 cycles of 94 °C for 5 s and 58 °C for 30 s. Products obtained from the PCR reactions were subjected to melting curve analysis to confirm that expected PCR products had been amplified and detected.

Western blotting

Liver cells of tilapias sampled 0, 4, 12, 24, 36, and 48 h after etoposide and $CdCl_2$ administration were extracted, resuspended in 160 µl lysis buffer and incubated on ice for 45 min. Lysates were centrifuged at $20,000\times g$ for 20 min at 4 °C, and the resulting supernatant fractions were stored at -70 °C, prior to

(A)

(B)

(C)
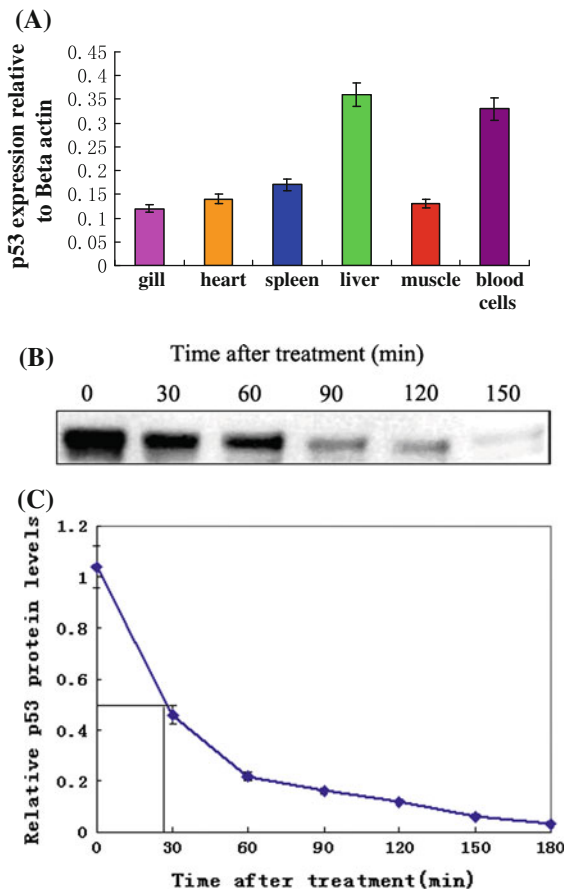
Fig. 2 **a** Tissue profile of p53 expression: mRNA levels relative to beta-actin transcripts, according to quantitative RT-PCR in gill, heart, spleen, liver, muscle, and blood cells of adult tilapia *Vertical bars* represent mean values ± S.E. (N = 3). **b** Western blot showing time course of changes in p53 protein levels in tilapia liver cells following treatment with etoposide or CdCl$_2$ at either 10 or 100 μg/kg body weight. **c** quantified data obtained by scanning the autoradigrams with a densitometer and normalizing p53 levels with respect to levels at time zero, results from three independent experiments

electrophoresis. Protein concentrations were determined with the Bradford assay. Finally, changes in the abundance of ATM, ATR, p53, p21 and Bax proteins were evaluated by western blotting using antibodies as previously described by Mai et al. (2010), and half-lives of the p53 protein were evaluated following the procedure of Chernov et al. (1998).

Statistical analysis

Quantitative data are expressed as mean ± SD (standard deviation). Treatment effects were evaluated by two-way ANOVA, followed by pairwise comparisons of means using Tukey's multiple range test, with the significance level set at $P < 0.05$. All statistical analyses were performed using SPSS v13.0 software (SPSS).

Results and discussion

Cloning and characterization of the tilapia p53 gene

Using PCR-based techniques we isolated a full-length cDNA clone of the tilapia p53 gene, which was 1,288 bp long in total, with an ORF of 1,138 bp encoding a polypeptide of 364 amino acids with an estimated molecular mass of 53.11 kDa and theoretical isoelectric point of 6.83. Multiple alignment indicated that its amino acid sequence diverges substantially from other p53 sequences (sharing 74.1 and 63.3 % identity with rainbow trout and zebrafish p53, respectively, but less than 50 % similarity with other p53 isoforms). Nevertheless, tilapia p53 shares the main features found in all p53 proteins (Brady and Attardi 2010), including: (1) four highly conserved amino acid clusters that act as sequence-specific DNA-binding sites, (2) an *N*-terminal transactivation domain, (3) a hydrophilic, charged *C*-terminus involved in regulation of specific DNA-binding activity, and (4) a shorter proline-rich region (Fig. 1a). The apparent divergence of the tilapia p53 protein from those of other taxonomic groups is illustrated in Fig. 1b.

Production of a polyclonal antibody against tilapia p53 protein

To generate an antigen for raising a polyclonal anti-tilapia p53 antibody, tilapia p53 cDNA was amplified using RT-PCR then heterologously expressed with a histidine tag in *E. coli*. As shown in Fig. 1c, IPTG induction resulted in very strong expression of a protein with a molecular mass of 53 kDa, corresponding to that of His-p53. The expressed protein was purified using Ni^{2+} affinity chromatography. Peak A$_{280}$ fractions that eluted from the column were pooled and analyzed by SDS-PAGE, which revealed a predominant single His-p53 band (lane 3). The purified His-p53 was concentrated and injected into
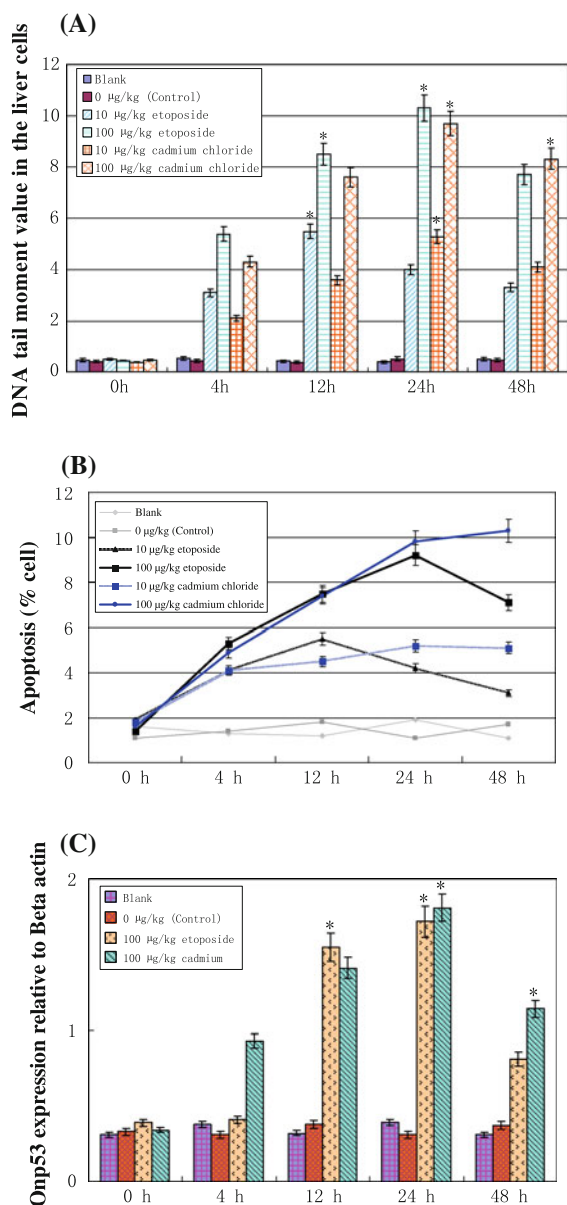
Fig. 3 Comet assay, TUNEL analysis and p53 mRNA expression analysis in tilapia after treatment with chemical agents. Level of DNA damage determined with the Comet assay (a) and quantitation of apoptotic cells using TUNEL analysis (b) in control and treated tilapia livers after challenge etoposide or CdCl$_2$ either for 0, 4, 12, 24, and 48 h. Results are mean values ± SD for triplicate samples. *Asterisks* indicate significant differences from control values ($P < 0.05$). c Tilapia p53 mRNA expression in etoposide or CdCl$_2$-treated samples relative to that in the relevant blank and control (normalized with respect to beta-actin transcript levels, for all samples). *Vertical bars* represent mean ± S.E (N = 3). Significant differences are indicated by *asterisks* ($P < 0.05$)

four rabbits for antibody production. The antiserum with the highest ELISA titer (1:1,000,000,000; data not shown), which is capable of immunoprecipitating p53 protein from tilapia cells (see Figs. 3b, 4a, b), was used for subsequent experiments.

As p53 is a key component of the checkpoint pathway, mutations in its four highly conserved domains can lead to tumorigenesis and cancer. Indeed, p53 mutations have been detected in over 50 % of human tumors (Olivier et al. 2010). Some fish species living in polluted water also develop tumors, although the mutation rate of fish p53 is lower than that of mammals (Berghmans et al. 2005). Populations of fish inhabiting highly polluted environments provide information on the etiology of pollutant-mediated cancer. Thus, fish provide useful models for studying environmental carcinogenesis, and our newly generated tilapia p53 antibody may be an effective tool for analyzing p53 pathways in fish models.

Tilapia p53 protein stability

Since increases in p53 protein levels in cells have previously been attributed to increases in protein stability (Chernov et al. 1998), we investigated the half-life of tilapia p53 protein in tilapia liver cells, following the procedure of the cited authors, and found it to be ~28 min (Fig. 2b, c). This is ca. twice as long as that of p53 in mammalian cells (15 ~ 18 min) reported by Chernov et al. (1998), indicating that different mechanisms may govern p53 protein levels in fish and mammals.

Tilapia p53 mRNA tissue expression profile

Quantitative RT-PCR analysis of tissue-specific levels of tilapia p53 mRNA, normalized to beta-actin transcript levels, indicated that p53 mRNA is constitutively expressed in all tissues we examined (gill, heart, spleen, liver, muscle, and blood cells). However, at least under our growth conditions, it appears to be expressed more strongly in blood cells and liver than in muscle and heart (Fig. 2a). In contrast, p53 is reportedly expressed most strongly in brain, gill and heart in rainbow trout, and only weakly expressed in spleen and liver (Caron de Fromentel et al. 1992), while in flounder p53 transcript is present, at low

Fig. 4 Western blots showing levels of ATM, ATR, p53, Bax and p21 proteins at indicated time-points following challenge with 100 µg/kg body weight etoposide (**a**) and CdCl$_2$ (**b**)
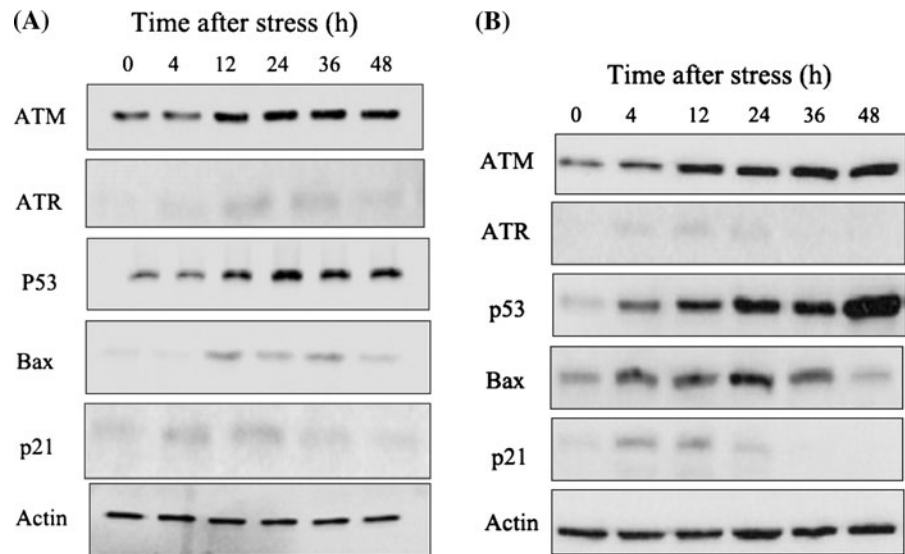
levels, in all tissues examined by Cachot et al. (1998), including ovary, testicle, liver, intestine and muscle. The results indicate that the p53 gene is constitutively expressed in diverse tissues, generally at very low steady-state levels, and that expression patterns differ among animal species.

Chemical agents induce DNA damage, apoptosis and p53 expression in tilapia

p53 plays a major role in the induction of apoptosis, and numerous chemical agents trigger DNA damage and apoptosis via both p53-dependent and -independent mechanisms (Kruse and Gu 2009). In mammalian systems, an established mechanism of p53 activation is protein stabilization, leading to increases in the normally low steady-state levels of endogenous p53. In mammalian systems, protein stabilization that allows normally low p53 level increase is the well-understood mechanism by which p53 is activated (Warmerdam and Kanaar 2010). Genotoxic stress can also induce p53 activation through a variety of various post-translational modifications, including phosphorylation, dephosphorylation and acetylation (Tedeschi and Di Giovanni 2009). However, to date, only a few studies have focused on the specific roles of p53 in fish. Therefore, to explore possible biological functions of tilapia p53 we examined its mRNA tissue profiles, and correlations between temporal changes in its transcript levels and both DNA damage and apoptosis in liver cells following challenges with

chemical agents (etoposide and CdCl$_2$) that are known to activate p53 and induce DNA damage and apoptosis in mammalian systems (Yu et al. 2011). DNA damage, expressed as Olive Tail Moment values obtained from Comet assays, was evident in tilapia liver cells of injected with either etoposide or cadmium chloride at doses of 10 or 100 µg/kg body weight, and apoptosis occurred within 48 h of treatment (Fig. 3a).

TUNEL analysis confirmed that etoposide and CdCl$_2$ significantly, and both time- and dose-dependently, induced apoptosis in tilapia liver cells, resulting in percentages of apoptotic cells of up to 9.2 and 10.3 %, respectively (see Fig. 3b for details). These findings are similar to those obtained in experiments with mouse fibroblast cells (Yu et al. 2011). Following stimulation with 100 µg etoposide/kg or CdCl$_2$, p53 mRNA expression in liver was upregulated 4.7 and 4.3-fold, respectively, at 48 h (Fig. 3c). Regulation of p53 at both the mRNA and protein levels in response to various types of stress has been described in zebrafish (Lee et al. 2008). Moreover, Brzuzan et al. (2009) observed increases in whitefish p53 mRNA expression in liver 24 h after microcystin-LR stimulation. Our data collectively show that stress-promoting chemical agents induce p53 expression, associated with increasing DNA damage and apoptosis, in tilapia liver.

ATM, ATR, p53, Bax and p21 levels

In mammalian systems, DNA damage triggers activation of the protein kinases ATM and ATR, followed by

stabilization of p53 via disruption p53-Mdm2 interactions, leading to promotion of transactivation and/or promotion of nuclear localization of p53. Upon activation, p53 binds to the enhancer/promoter elements of downstream target genes and regulates their transcription, thereby initiating the cellular programs underlying its tumor suppressing activity (Hennequin et al. 2011; Kotipatruni et al. 2011). Similarly, our western blot analysis revealed clear time-dependent accumulation of the p53, ATM and Bax proteins in tilapia liver cells following etoposide and $CdCl_2$ challenge, relative to control levels, albeit with indications of a tail-off in Bax levels towards the end of the 48-hour monitoring period (Fig. 4a, b). In contrast, ATR and p21 expression levels were not significantly altered relative to controls. Hence, we propose that ATM, but not ATR, activates p53 expression in response to DNA-damaging signals in tilapia, leading to initiation of p53-mediated apoptosis via activation of the Bax apoptotic gene, similar but not identical to the phenomena observed in mammals.

Some genotoxic agents, such as UV and bleomycin, known to trigger DNA damage and upregulate p53 in mammalian cells, do not stimulate increases in p53 expression in some fish species and cell lines (Rau Embry et al. 2006; Liu et al. 2011). Additionally, *Drosophila melanogaster* p53 protein is activated via phosphorylation rather than increased expression upon exposure to cellular stress, and its function is independent of protein stabilization (Brooks and Gu 2003). Hence, the p53 activation mechanism and possible function in fish species differ from those of other vertebrates, possibly due to differences in phylogenesis and acclimatization strategies.

Conclusions

In summary, we have identified (and raised a polyclonal antibody against) a p53 homolog in tilapia on the basis of sequence homology, biochemical properties and conserved cellular function. Fish provide useful model systems for investigations of p53 roles, action mechanisms, regulatory networks and environmental carcinogenesis. Thus, the data presented here and the newly generated tilapia p53 antibody should facilitate both further studies on p53 signaling pathways in fish models and their exploitation in environmental analyses.

References

Berghmans S, Murphey RD, Wienholds E, Neuberg D, Kutok JL, Fletcher CD, Morris JP, Liu TX, Schulte-Merker S, Kanki JP, Plasterk R, Zon LI, Look AT (2005) Tp53 mutant zebrafish develop malignant peripheral nerve sheath tumors. Proc Natl Acad Sci USA 102:407–412

Brady CA, Attardi LD (2010) P53 at a glance. J Cell Sci 123:2527–2532

Brooks CL, Gu W (2003) Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin Cell Biol 15:164–171

Brzuzan P, Wozny M, Ciesielski S, Luczynski MK, Gora M, Kuzminski H, Dobosz S (2009) Microcystin-LR induced apoptosis and mRNA expression of p53 and cdkn1a in liver of whitefish (*Coregonus lavaretus L.*). Toxicon 54:170–183

Cachot J, Galgani F, Vincent F (1998) cDNA cloning and expression analysis of flounder p53 tumour suppressor gene. Comp Biochem Physiol B Biochem Mol Biol 121:235–242

Caron de Fromentel C, Pakdel F, Chapus A, Baney C, May P, Soussi T (1992) Rainbow trout p53: cDNA cloning and biochemical characterization. Gene 112:241–245

Chernov MV, Ramana CV, Adler VV, Stark GR (1998) Stabilization and activation of p53 are regulated independently by different phosphorylation events. Proc Natl Acad Sci USA 95:2284–2289

Devlin HL, Mack PC, Burich RA, Gumerlock PH, Kung HJ, Mudryj M, White RWD (2008) Impairment of the DNA repair and growth arrest pathways by p53R2 silencing enhances DNA damage-induced apoptosis in a p53-dependent manner in prostate cancer cells. Mol Cancer Res 6:808–818

Hennequin C, Quero L, Favaudon V (2011) DNA repair and tumour radiosensitivity: focus on ATM gene. Bull Cancer 3:239–246

Kotipatruni RR, Dasari VR, Veeravalli KK, Dinh DH, Fassett D, Rao JS (2011) p53- and Bax-mediated apoptosis in injured rat spinal cord. Neurochem Res 11:2063–2074

Kruse JP, Gu W (2009) Modes of p53 regulation. Cell 137:609–622

Lee KC, Goh WL, Xu M, Kua N, Lunny D, Wong JS, Coomber D, Vojtesek B, Lane EB, Lane DP (2008) Detection of the p53 response in zebrafish embryos using new monoclonal antibodies. Oncogene 27:629–640

Liu M, Tee C, Zeng F, Sherry JP, Dixon B, Bols NC, Duncker BP (2011) Characterization of p53 expression in rainbow trout. Comp Biochem Physiol C Toxicol Pharmacol 154:326–332

Luft JC, Bengten E, Clem LW, Miller NW, Wilson MR (1998) Identification and characterization of the tumor suppressor p53 in channel catfish (*Ictalurus punctatus*). Comp Biochem Physiol B Biochem Mol Biol 120:675–682

Mai WJ, Yan JL, Wang L, Zheng Y, Xin Y, Wang WN (2010) Acute acidic exposure induces p53-mediated oxidative stress and DNA damage in tilapia (*Oreochromis niloticus*) blood cells. Aquat Toxicol 100:271–281

Olivier M, Hollstein M, Hainaut P (2010) TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harb Perspect Biol 2:1000–1008

Rau Embry M, Billiard SM, Di Giulio RT (2006) Lack of p53 induction in fish cells by model chemotherapeutics. Oncogene 25:2004–2010

Singh R, Kaur B, Sram RJ, Binkova B, Kalina I, Popov TA, Garte S, Taioli E, Farmer PB (2004) Effects of environmental air pollution on endogenous oxidative DNA damage. Mutagenesis 19:519

Tedeschi A, Di Giovanni S (2009) The non-apoptotic role of p53 in neuronal biology: enlightening the dark side of the moon. EMBO Rep 10:576–583

Warmerdam DO, Kanaar R (2010) Dealing with DNA damage: relationships between checkpoint and repair pathways. Mutat Res 704:2–11

Yu X, Sidhu JS, Hong S, Robinson JF, Ponce RA, Faustman EM (2011) Cadmium induced p53-dependent activation of stress signaling, accumulation of ubiquitinated proteins, and apoptosis in mouse embryonic fibroblast cells. Toxicol Sci 120:403–412