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RESPONSIVENESS OF p53 EXPRESSION AND GENETIC MUTATION TO CCL_4-INDUCED DNA DAMAGE IN RAT'S LIVER

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Abstract- The present study aimed to detect the responsiveness of the tumor suppressor factor, p53, expression as well as mutational alterations to oxidative DNA damage caused by CCl_4 intoxication. Twenty rats were divided into control and CCl_4 group that subjected to hepatotoxicity by CCl_4 (2.5 ml/kg, 25%V/V in corn oil, intraperitonially biweekly for 8 weeks). blood and liver samples were taken 24 h after the last CCl_4 injection. Changes in serum AST and ALT activities as well as MDA, SOD, CAT and GSH levels with increased levels of DNA fragmentation confirmed the induction of lipid peroxidation and oxidative stress in CCl_4 group. Rats under CCl_4-oxidative damage exhibited a significantly increased p53 mRNA and protein expression by real-time PCR and immunohistochemistry, respectively. Moreover, CCl_4 group showed a marked cytoplasmic p53 translocation. PCR-SSCP mutational analysis of one of the p53 DNA-binding domain (exons 7) showed an increased frequency of p53 mutation in CCl_4 group (60%). The results of the present study indicate an association between p53 mutation and expression level and oxidative DNA damage, a mechanism that explain the increased tendency of carcinogesis in chronic liver diseases.

Keywords- CCl_4, p53, mRNA, SSCP, mutation, liver

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Introduction

Among the huge diversity in the tumor related proteins, the p53, encoded by the *TP53* gene, stands out as a key tumor suppressor and a master transcription factor regulator of various signaling pathways [1]. TP53 encodes a nuclear phosphoprotein which regulates the intrinsic cellular responses to DNA damage according to the delicate equilibrium between ROS production, and their antioxidant scavenging machinary [2]. At low levels of ROS, p53 exhibits antioxidant activity to eliminate free radicals ensuring cell survival, however, p53 can induce cell death in sever oxidative stress [3]. A myriad of genes have been shown to be transcriptional targets of p53 to perform the many roles of p53 as a tumor suppressor include the ability to induce cell cycle arrest, DNA repair, senescence, and apoptosis [4].

Tp53 gene is a frequent target for mutation with a very high prevalence of missense mutations which result in the expression of a mutant protein. Most mutations lead to proteins with impaired function and subsequently result in the deficient activation of p53 target genes [5,6]. In addition to the loss of wild-type activity, some mutants exert dominant-negative effects and/or acquire new pro-oncogenic activities [7]. Many reports have shown that p53 is accumulated in hepatocytes in several fibrotic liver diseases [8-10].

CCl_4 is an excellent model of oxidative liver injury with propagation of fibrosis. The toxigenic effects of CCl_4 depend on the generation of highly reactive free radical intermediates and stimulation of lipid peroxidation (LPO) which suggested to play important roles in the pathogenesis of irreversible cell damage [11]. In addition to these cytotoxic effects, several genotoxic activities of CCl_4 have been reported [12,13]. Although predominately negative in mutagenicity tests, CCl_4 was found to promote in vivo induced mutagenicity [14].

This study aimed to demonstrate the molecular and genetic alterations in the tumor suppressor gene, p53, in response to the cytotoxic and genotoxic action of CCl_4 in liver of rats.

Material and Methods

Chemicals

High grade CCl4 99.8% (*BDH*, *England*) was diluted into 25% (v/v) in corn oil before use. RNA extraction kit (*Qiagen, Germany*); *QIAamp* DNA Mini Kit (*Qiagen);* Rabbit polyclonal antibody against p53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); reverse transcription system (*Fermentas*) and all other chemicals used in the experiment were of analytical grade.

Animals and Experimental Design

Six week old male rats (150-170 g), provided by the laboratory of animals breeding unit, faculty of veterinary medicine, Cairo university, were kept at room temperature of 25±3°C with a 12 h dark/light cycles with free access to food and water, according to the study

protocol approved by the ethical Committee of faculty of veterinary medicine, Cairo university for animal care and experimentation. Rats were equally divided into two groups (ten rats each). To induce oxidative liver injury, rats in CCl_4 group were ip injected with 25% CCl_4 in corn oil at a dose of 2.5 ml/kg bw twice a week for eight weeks, while controls received the same isovolumetric dose of corn oil ip/8 week.

Sampling

All the animals were sacrificed 24 h after the last treatment. Blood was obtained from the retro-orbital plexus, centrifuged at 4000 rpm/10 min to separate the serum for measurements of aminotransferases. Liver was immediately removed, washed with ice-cold saline and stored at -70°C until subsequent analyses.

Biochemical Analyses

Assessment of hepatotoxicity

Liver marker enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were estimated in serum according to Reitman & Frankel [15].

Assessment of Oxidative Damage

Tissue Antioxidant Activities

Frozen liver from each rat was homogenized in ice-cold normal saline and centrifuged at 10000×g for 10 min. The supernatant was used for the measurement of superoxide dismutase (SOD), catalase (CAT), and reduced glutathione content (GSH) according to methods described by Nandi & Chatterjee [16], Sinha [17], and Beutler, et al [18], respectively. The protein content of the homogenate was measured by the Lowry method [19] using bovine serum albumin as standard.

Lipid Peroxidation (LPO)

Measurement of lipid peroxidative index in term of malondialdehyde (MDA) was done according to Andersen, et al [20].

DNA Fragmentation

Hepatic DNA damage was determined by diphenylamine (DPA) assay as fragmentation percentage [21] and DNA ladder assay using agarose gel electrophoresis [22].

Histopathological Analysis

Liver samples were fixed with 10% buffered formalin, and embedded in paraffin. Thin sections (5 µm) were deparaffinized, stained with hematoxylin and eosin (H&E) and examined by light microscope.

Assessment of p53 Expression

Immunohistochemical Examination of Hepatic p53

Immunohistochemical study was performed on paraffin embedded liver tissues using polyclonal anti-p53 antibody diluted 1:50 (SantaCruz Biotech) according to the manufacturer's instructions. Stained slides were analyzed by light microscopy and photographed.

Real-time PCR Quantitation of p53 mRNA Expression Level

Expression of p53 mRNA was assessed using real-time PCR analysis standardized by coamplification of the housekeeping gene GAPDH, which served as an internal control. Briefly, total RNA was extracted from frozen liver tissues using *Qiagen* RNA extraction kit. The isolated RNA was used for cDNA synthesis using reverse transcription system (*Fermentas*). Real-time PCR was performed in a 25 µl reaction containing 12.5 µl of 2X SYBR Green Mastermix (*Applied Biosystem*), 300 nM primers, and cDNAs. p53 primer sequences were (Forward):5'-TCCCTAAGTATCCTCAGTGA-3', and (Reverse): 5'-GTAATCGAAGCGTTTGTTGA-3. For GAPDH, the forward primer was 5'-ACCACAGTCCATGCCATCAC-3' and the reverse primer was 5'-TCCACCACCCTGTTGCTGTA-3'. PCR program was as follows: 95°C for 1 min, 40 cycles of 95°C, 20 s, 60°C, 20 s, and 72°C, 20 s. final extension 72°C for 1 min. mRNA levels were expressed as fold changes after normalization with GAPDH.

Mutational Analysis of *p53* Gene

Mutations in the p53 exons 7 were analyzed by genomic PCR-SSCP analysis as described by Ibrahim, et al [23]. Briefly, DNA was isolated from frozen liver using *QIAamp* DNA Mini Kit (*Qiagen)* and PCR amplification was performed using thermocycler (*BOECO, Germany*) according to the following protocol: 94ºC for 5 min followed by 35 cycles at 94ºC for 45 s, 58ºC for 45 s, 72ºC for 45 s final extension time of 7 min at 72ºC. For SSCP analysis, the PCR-amplified product was diluted 1:1 in denaturing buffer (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanole). The samples were denatured at 98ºC (10 min), chilled on ice (10 min), loaded on a 15% nondenaturing polyacrylamide gel (PAGE) and finally electrophoresed at 120 V for 5 hrs. in a minigel electrophoresis chamber (BioRad). Visualization was performed by staining with ethidium bromide.

Statistical Analysis

All data were expressed as mean ± standard error (SE). Significance of the differences between control and CCl4 groups are statistically calculated by using independent t-test using SPSS version 16 (IBM Co., Armonk, NY, USA). Densitometric analysis of electrophoretic images was performed using GelPro software (Media Cybernetics Inc., USA).

Results

Assessment of Hepatotoxicity

Administration of CCl4 for 8 weeks markedly increased the activity of serum hepatic marker enzymes such as AST and ALT as compared with the control group (P<0.001) [Table-1].

Table 1- changes in hepatic marker enzymes activities in CCl4-intoxicated rats

	ALT ((U/L))	AST ((U/L))
Control	19.17±0.40	43.33±0.42
CCl4	1396.7±36.66***	1140±22.65***

*** very highly significant at p < 0.001 with respect to control group

Assessment of Oxidative Stress

Antioxidant Enzymes

CCl4 toxicity resulted in a marked reduction of SOD and CAT activities in liver tissues compared with the control group [Table-2]. The hepatic GSH concentration decreased significantly in CCl4 group [Table-2].

Lipid Peroxidation

Oxidative stress resulting from the metabolism of CCl4 in the liver plays a critical role in damaging the liver and promoting lipid peroxi-

dation. The MDA level was significantly higher in liver homogenates of CCl4-intoxicated rats compared with the control group [Table-2].

Table 2- Changes in the activities of SOD, CAT, and levels of GSH and MDA in liver homogenates of experimental animal groups.

Groups/ Parameter	MDA (nM/g liver)	CAT (μM H_2O_2 utilized/ mg protein)	SOD (U/mg protein)	GSH (μM/g liver)
Control	65.85 ± 1.89	2.29 ± 0.08	89.95 ± 2.24	12.36 ± 0.21
CCl_4	134.47± 3.60***	1.07± 0.04***	22.48±1.30***	2.57 ± 0.20***

Values are expressed as means (±SE).
*** very highly significant at p<0.001 with respect to control group.

DNA Fragmentation

Exposure to CCl4 elicited the hepatic DNA fragmentation that showed a marked increase to 47.9% compared to 14.82% in control group [Fig-1](A). Moreover, DNA ladder assay showed conformity to the DNA fragmentation assay [Fig-1](B).

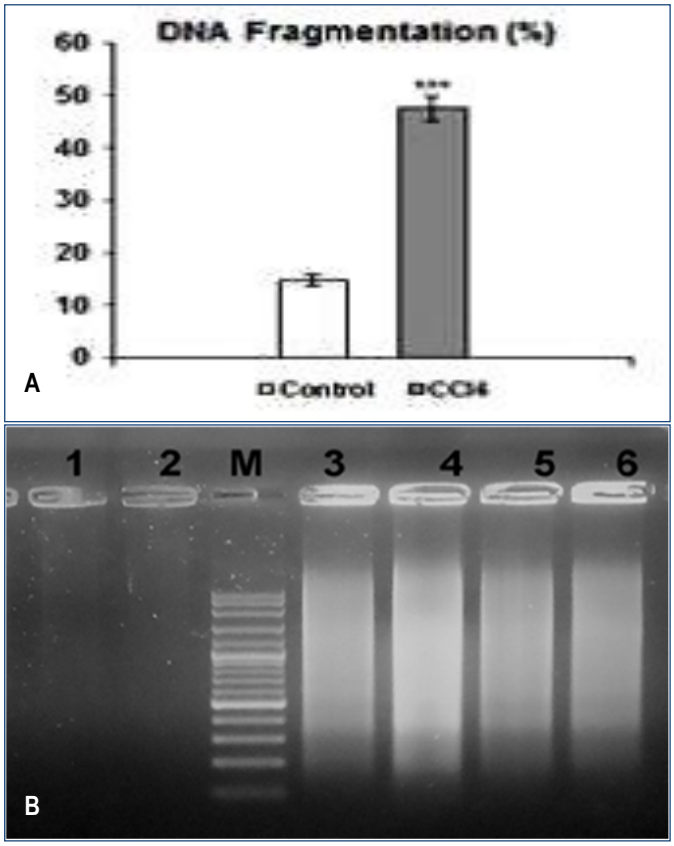

Fig. 1- Effect of CCl4 on hepatic DNA fragmentation, **(A)** DNA fragmentation% estimated by DPA assay; **(B)** DNA laddering on 1% agarose gel stained with ethydium bromide. *Lane M*: 100 bp DNA ladder; *Lane 1,2*: DNA of control rats; *Lane 3-6*: DNA of CCl4 group

Histopathological Analysis

Liver sections from control rats stained with H&E showed normal hepatic architecture [Fig-2](A). Liver sections from the CCl4 group showed degenerated, ballooned and necrotic hepatocytes with acidophilic hyaline inclusions. Features of mononuclear cell infiltration, steatosis and congestion marked by distended sinusoidal spaces filled with erythrocytes were also more pronounced in CCl4 group [Fig-2](B).

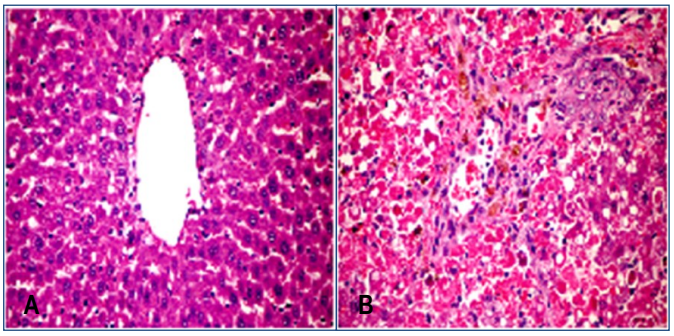

Fig. 2- Photomicrographs of liver histological sections stained with H&E stain, **(A)** control rat liver with histological structures of normal hepatic lobules; **(B)** CCl4-induced liver damage with hepatocellular vacuolization, focal hepatic necrosis and congestion of hepatic sinusoids.

Effect of CCl_4 Oxidative Damage on p53 Expression

p53 Immunohistochemistry

The expected CCl4-induced hepatocytotoxic effects were accompanied by a significantly elevated p53 protein level. p53 immunopositive cells reached up to 56.9% of the stained area compared to 16.9% observed in the control group as detected by immunohistochemical staining [Fig-3]. In addition, CCl_4 treatment caused a marked cytoplasmic translocation of p53 protein.

Real Time PCR Quantitation of Tp53 mRNA

The quantitative analysis of Tp53 mRNA level by real-time PCR [Fig -4] showed that Tp53 was excessively expressed in the CCl4 group reaching about 18 fold of that of control rats.

Tp53 Mutational Analysis

The PCR-amplified products of Tp53 exons 7 were found to be in the expected molecular size [Fig-5](A). PCR-SSCP analysis using 15% PAGE revealed a mobility shift pattern [Fig-5](B) in the studied Tp53 exon 7 indicating polymorphism/point mutation with up to 60% frequency in CCl_4 treated group [Fig-5](B&C).

Discussion

Xenobiotics biotransformation mostly involves the generation of reactive oxygen species (ROS), which play a major role in the emergence of cancer and other health disturbances [24,25]. In addition to the cytotoxic effects of ROS, several genotoxic actions occur subsequently including formation of DNA adducts, altering genes expression and mutational changes [26,27]. There is a considerable body of evidence indicating that the tumor suppressor gene Tp53 is the central regulator of cellular response to genotoxic stress [28].

The present study aims to provide the evidence that oxidative damage of hepatocytes triggers p53-dependant apoptotic cell death through various genetic alterations. To clarify the above idea, CCl_4 model was used. Tp53 mRNA level was assessed by Real-time PCR, p53 protein expression and subcellular localization were determined by immunohistochemistry and finally Tp53 mutational was analyzed using SSCP technique.

CCl_4 is extensively used as an experimental model of hepatotoxicity because it has several similarities with human cirrhosis [8,29]. CCl_4 toxicity is a multifactorial process produces a wide array of dysfunction and injury. CCl_4 toxigenic sequence initiates with its metabolism primarily by the hepatic cytochrome P450 2E1 to highly reactive

trichloromethyl (•CCl3) and peroxy trichloromethyl radicals (•OOCCl3) which covalently bind to proteins, lipids and nucleic acids causing oxidative modifications [30], LPO which propagate inflammatory response and necrosis [31], in addition to induction of oxidative DNA damage including formation of DNA adducts, genetic mutations, strand breakage and chromosomal alterations [32].

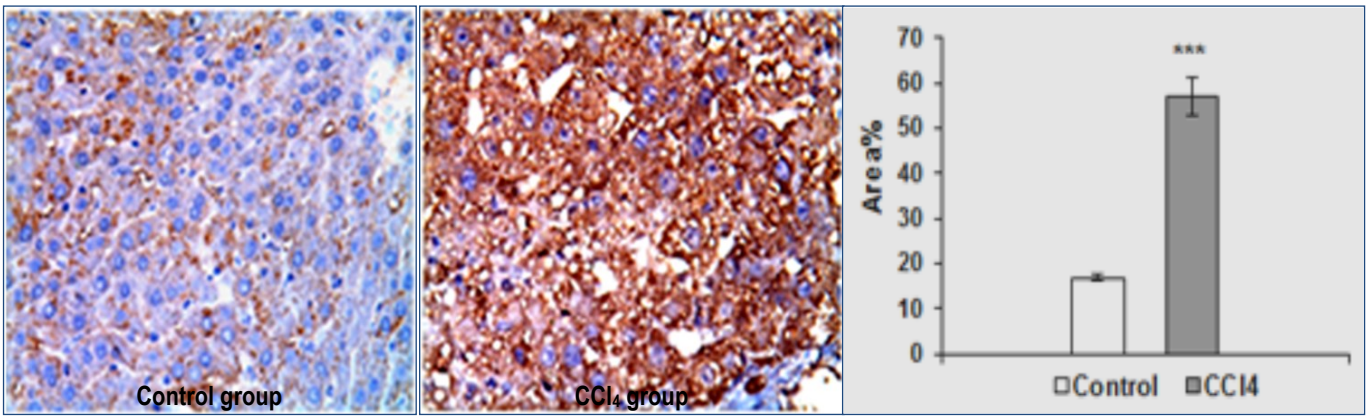

Fig. 3- Immunohistochemiscal detection of p53 protein expression in liver sections of the tested groups. CCl4 group shows intense brown staining of cytoplasmic translocated p53. The bar chart represents p53 immunopositive cells/field (mean of 6 fields). *** very highly significant difference

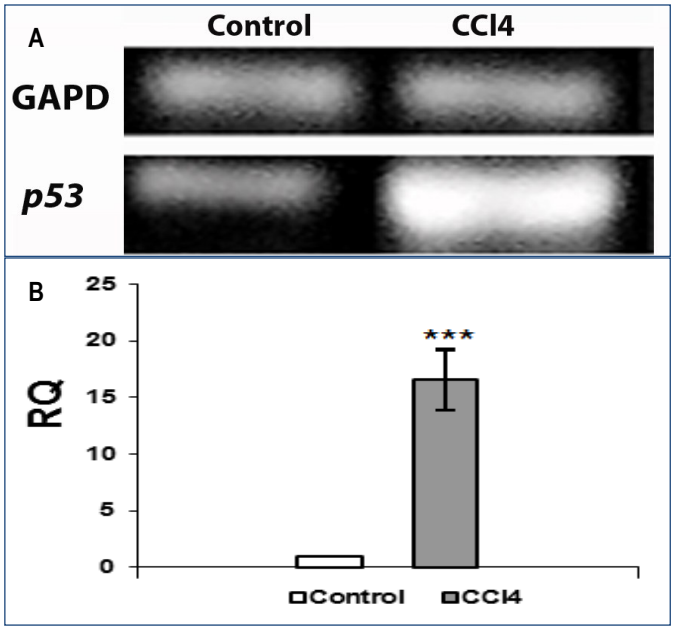

Fig. 4- Change in mRNA expression level of Tp53 gene in liver of CCl4-treated rats. RQ is the fold change (means±SE). Normalization is to GAPDH mRNA level. *** significant increase from the control group

As expected, Hepatic indicators of oxidative damage in this study were markedly changed after 8 weeks of CCl4 intoxication and in accordance with Domitrović, et al [33]. A significant increase in the serum levels of AST and ALT were observed in CCl_4 group [Table-1]. Elevation in serum concentration of these enzymes by CCl4 has been attributed to hepatic structural damage leading to release of such cytoplasmic entities into the circulatory system after cellular damage and necrosis have occurred [33-35].

MDA, the main product of lipid peroxidation and important index of oxidative stress [36], was significantly elevated in CCl_4-challenged rats indicating an excessive lipid peroxidation compared with that in the normal control [Table-2]. These results are in line with those of Khan, et al [31] and Ebaid, et al [34].

The current study showed that CCl4 induced significant decrease in free radical scavenging enzymes, such as SOD and CAT [Table-2]. The depletion of these antioxidant enzymes is attributed to either amplified consumption by the enhanced LPO or downregulation of their gene expression due to CCl_4 toxicity [37]. This would cause an increased accumulation of superoxide radicals, which could further stimulate lipid peroxidation.

GSH is the main endogenous nonenzymatic antioxidant that maintains the intracellular redox balance and protects cells against oxidative stress-caused damage [38]. A significant reduction of GSH was observed upon CCl_4 chronic administration in this study [Table-2]. These results match with previous reports for GSH exhaustion during CCl_4 metabolism [31,34]. This GSH depletion may be resulted from the direct requisition of GSH by glutathione peroxidase to scavenge the production of hydrogen peroxide (H2O2), and suppress LPO [39].

The histological findings of liver sections [Fig-2] revealed severe liver cell damage in rats after administration of CCl_4, supporting the observed biochemical changes. The presence of necrosis, fibrosis, inflammatory cell infiltration, and steatosis are characteristics after intoxication with CCl_4 [34,35].

With respect to CCl_4 genotoxicity, a number of *invivo* studies in mammalian systems found that metabolism of CCl_4 causes DNA damages, gene mutation, breaking and alteration of chromosomal induces mutations and deletions probably through the production of oxidative stress [32]. Using radiolabeled, CCl4 showed a modest binding to hepatocyte DNA with increases in the oxidative DNA adducts formation, 8-oxodeoxyguanosine, and MDA deoxyguanosine adduct [12]. The current study revealed a considerable level of DNA fragmentation (3.2 fold increase) in CCl_4-treated rats verified by both spectrophotometric and DNA ladder assay [Fig-1]. In agreement with previous reports that showed the oxidative DNA damaging effect of CCl_4 in liver [31] verified by DNA ladder assay.

Two mechanisms of oxidative stress-induced DNA fragmentation are reported. First, A role of LPO in chromosomal DNA fragmentation through the loss of membrane integrity that might proceed not only in plasma membranes but also in the nuclear membranes

close to chromosomes, and thereby may make suitable circumstances for ROS such as ·OH radicals to attack chromatin DNA. Also, products of LPO such as MDA or 4-hydroxynonenal, are highly reactive aldehydes that can form protein and DNA adducts [40]. A second mechanism involves the depletion of mitochondrial energy through loss of NAD+ and deletion of ATP. Mitochondrial impairment is induced by myriad stimuli including GSH depletion. Under these stress conditions, an estimated 1-5% of electrons escape the respiratory chain and participate in formation of ROS and LPO accompanied with a decrease in the mitochondrial reducing capacity for NAD(P). The decrease in NAD(P)H compromises the activity of antioxidant enzymes, which further increases the deleterious effects of ROS [38]. In addition, decreased NAD(P)H permeabilizes the inner mitochondrial membrane and favors the release of Ca2+ and uncouples oxidative phosphorylation resulting to depletion of ATP and impairment of most Ca2+ regulation system and finally DNA fragmentation and apoptosis [41].

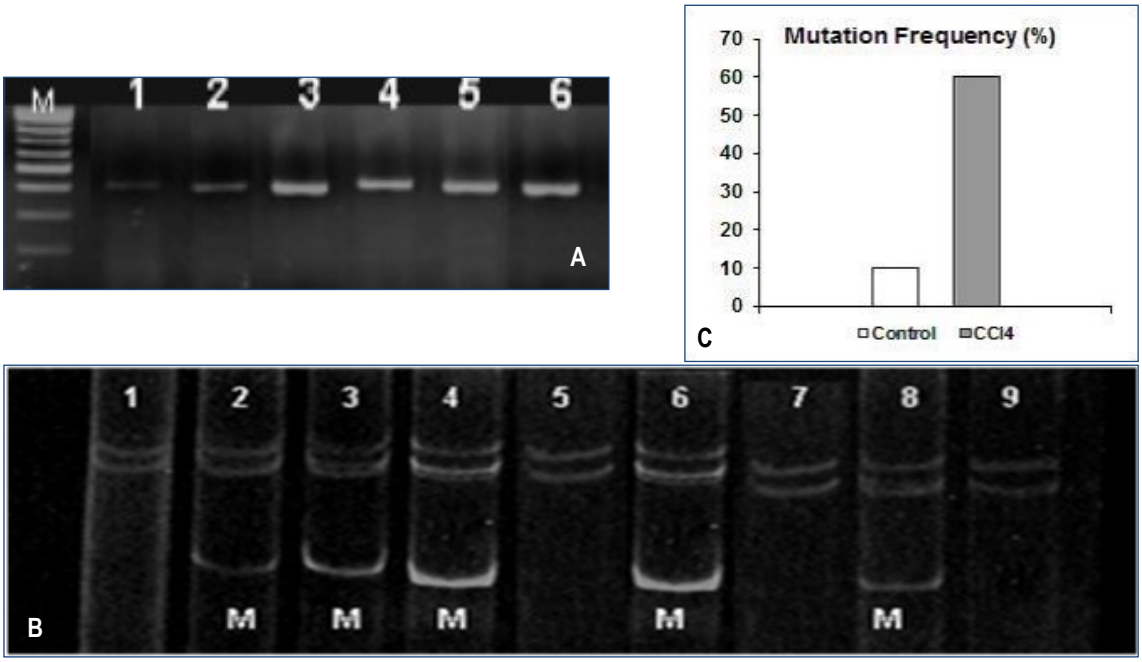

Fig. 5- Mutational analysis of Tp53 gene exon7, **(A)** representative PCR products for Tp53 exon7 (157 bp), M: 50 bp ladder; **(B)** representative SSCP analysis of Tp53 exon7 on 15% PAGE; *Lanes 1, 5, 7* and *9* show normal SSCP pattern; *Lanes 2-4, 6* and *8* show mutant SSCP pattern marked with M; **(C)** Tp53 exon7 mutation frequency in control and CCl4 groups.

One of the most important regulators of the intrinsic cellular responses to DNA damage is the tumor suppressor factor, p53 [2]. p53, is a nuclear phosphoprotein which is critical for cell cycle control against replication of damaged DNA and uncontrolled cell proliferation that can lead to cancer [1]. ROS are potent activators of p53 [42] directly through DNA damage [31], or indirectly by activation of other signaling pathways such as p38, *c-jun*-NH_2-kinase (JNK), or NF-κB [43]. In unstressed cells, p53 expression is maintained at a low level through the ubiquitin/ proteasome pathway [44]. The intensity of inflammation induces the pro-apoptotic protein p53 and inhibits the anti-apoptotic Bcl-2 [45]. Once the stress signal is conducted to the mitochondria, increased cell membrane permeability, release of apoptosis-related proteins, induction and overexpression of p53 occur [46,47].

One possible outcome of p53 overexpression is G1/S cell cycle arrest, allowing the repair of damaged DNA before replication, while a second possible outcome is induction of apoptosis through p53-regulated genes such as *P21*, *PUMA*, *NOXA* and *Bax*. In either case replication of damaged DNA is prevented [5,31].

The present study showed, that p53, at the protein level, was sharply up-regulated in the CCl4 group compared to control group as detected by immunohistochemical staining [Fig-3]. This indicates that p53 was overexpressed upon CCl_4 toxicity and comes in line with Guo, et al, [8], El-kott [48] and Qu, et al [49]. But, a remarkable finding was the cytoplasmic translocation of p53 protein in hepatocytes of CCl_4 group in contrast to previous studies that revealed a clear nuclear localization of p53 in CCl_4 toxicity [Fig-3] [8,48,49]. Moll and colleges [50] attributed the abnormal cytoplasmic sequestration to the inability of p53 to translocate to the nucleus that presumably prevents the protein from functioning as a tumor suppressor.

The regulation of p53 functions is tightly controlled through several mechanisms including p53 transcription, translation, protein stability, post-translational modifications, and subcellular localization [51]. Several nuclear localization signals are clustered on the C terminus of p53 molecule to mediate its migration into the cell nucleus. In normal proliferating cells, the p53 protein has a short half-life [52] and it continuously shuttles between the nucleus and the cytoplasm. p53 exports from the nucleus to the cytoplasm, where p53 undergoes degradation via the proteasome pathway [53]. Under stresses, p53 is stabilized and accumulates in the nucleus, where it activates expression of the stress response genes, resulting in cell cycle arrest and apoptosis [47]. The exclusion of p53 protein from nucleus to cytoplasm was proposed as a novel mechanism to inactivate p53 function with or without mutation. Mutant p53 in human hepatoma cell lines accumulates in the cytoplasm, and lose its transcriptional function [54].

Real time-PCR quantitation of Tp53 gene expression [Fig-4] confirms the results of immunohistochemistry [Fig-3], with a significantly increased Tp53 mRNA level in CCl4-hepatotoxic group compared to normal control rats.

Regarding p53 activity, Khan, et al [31] reported a marked reduction in % activity of p53 after CCl_4 toxicity. Our results regarding p53 revealed a marked increase in p53 expression level at both protein and mRNA levels. These results are in harmony with those of Khan, et al [31] and could be explained on the basis that Tp53 overexpression in some cirrhotic livers as CCl_4-induced liver fibrosis [34], may be due to a normal Tp53 gene response to ROS or, alternatively, to an abnormally functioning or mutated Tp53 gene [7]. In addition, the presence of mutant TP53 allele, even in the heterozygous situation, antagonizes p53 tumor suppressor functions by interference with the formation of tetramers essential transcriptional activity and DNA binding function of p53 [55].

Tp53 gene mutations (insertion, deletion and point mutation) have been found to be the most common genetic alterations in both human and animal cancers. The site and type of *p53* mutations can reflect exposure to carcinogenic agents [56]. The vast majority of Tp53 mutations are missense resulting in loss of its ability to bind DNA in a sequence-specific manner and consequently a mutant p53 protein with oncogenic properties [7].

By the use of SSCP-PCR mutation analysis, we found that 60% of CCl_4-treated rats bearing mutation in the DNA-binding domain of Tp53 (exons7) [Fig-5]. This percentage is in line with previous investigations, where about 80% of mutations of the Tp53 exon 7 occurred in response to cyclophosphamide-induced DNA damage [23]. Year published:DOI:Mutations of Tp53 gene were frequently found in cirrhotic livers compared with livers of patients with chronic hepatitis, suggesting that p53 mutations at the stage of cirrhosis may be a causative factor that may potentially lead to hepatocellular carcinoma [57].

The examined region of Tp53 gene is considered one of the highly conserved domains and also contains the p53 sequence-specific DNA binding activity; therefore mutations in these domains resulted in loss of DNA-binding capacity [58].

Conclusion

Taken together, the results allow us to propose a model where CCl_4 - induced oxidative DNA damage modulates p53 expression, either by upregulation of Tp53 mRNA transcription, overexpression of p53 protein, translocation of p53 to cytoplasm, or by mutation in p53 DNA binding domain. We suggest that the tumorogenesity of CCl_4 is attributed to these p53 alterations.

Abbreviations

ALT: Alanine Aminotransferase

AST: Aspartate aminotransferase

ROS: Reactive Oxygen Species

LPO: lipid peroxidation

SOD: superoxide dismutase

CAT: catalase

GSH: reduced glutathione

MDA: Malondialdehyde

PCR-SSCP: Polymerase chain reaction-single strand conformational polymorphism

GAPDH: Glucose 6 phosphate dehydrogenase

Conflicts of Interest : There was no conflict of interest for any of the authors of this paper.

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