Antimutagenic Efficacy of Some Natural Compounds on Cyclophosphamide-Induced *p53* Alterations

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Mutations in the *p53* tumour suppressor gene have been associated with chemical carcinogens. Natural antimutagens are promising modulators for reducing the cancer risk. The present study was carried out to assess the protective efficacy of some natural antimutagens against *p53* alterations. We investigated the ability of curcumin (100 mg/kg BW) and chlorophyllin (3 mg/kg BW) pretreatment, for three times per week for three successive weeks, to inhibit mutations induced by intraperitoneal injection of a single dose of 40 mg/kg BW of cyclophosphamide (CP). Forty male albino rats were assigned into four groups: control non-treated group, CP-treated group, curcumin-CP-treated group, and chlorophyllin-CP-treated group. Liver samples were collected for DNA isolation two days after CP injection. The isolated DNA was used in single-strand conformational polymorphism (SSCP) analysis of polymerase chain reaction (PCR)-amplified products of four regions: two in exon 5, one in exon 6, and one in exon 7. The amplified products of *p53* different regions were found to be in the expected molecular size of the designed primers. SSCP analysis of these amplified products showed that CP-induced mutation in the *p53* gene was found only in exon 7 shifting its electrophoretic mobility. Chlorophyllin treatment prior to CP injection had a more potent protective efficacy (80 %) than that with curcumin (33.3 %).

Key words: p53 Gene, Mutation, PCR-SSCP

Introduction

Alterations in oncogenes and tumour suppressor genes have been shown to be involved in the tumour development in both animals and humans. In recent years, *p53* tumour suppressor gene mutations (insertion, deletion and point mutation) have been found to be the most common genetic alterations in both human and animal cancers. Called the "guardian of the genome," *p53* is known to play a key role in regulating whether a cell will arrest, undergo apoptosis, senescence, or differentiate in response to various stresses (Lane, 1992). It contains 11 exons spanning 20 kp (Lacroix *et al.*, 2006). The *p53* function is often altered in cancer, its malfunctioning results in sustained DNA damage in cells, suggesting that it specifically could prevent tumour development (Vousden and Lu, 2002). The site and type of *p53* mutations can reflect exposure to carcinogenic agents (Harris, 1996).

One carcinogen that had potential in mutation studies is cyclophosphamide (CP), an alkylating agent which belongs to the class of nitrogen mustards. It is widely used for treating a variety of human malignancies (Colvin, 1999).

The widespread development of mutagens and their potentially dangerous effects on health have promoted the development of strategies to cope with these agents. Chemoprevention is a unique and promising strategy for reducing the cancer risk through administration of synthetic or dietary compounds that provide high-risk individuals with protection against cancer by affecting tumour incidence, mortality and morbidity. Among these natural compounds with this property, curcumin and chlorophyllin appear to be promising antimutagens (Dashwood *et al.*, 1998; Shukla *et al.*, 2002).

Curcumin found in *Curcuma longa* has been identified to reduce radiation-induced DNA damages in rat lymphocytes by modulating the SOS repair system (Thresiamma *et al.*, 1998), while chlorophyllin derived from chlorophyll appears to be one of the most promising antimutagens because of its high response against a number of substances (Dashwood *et al.*, 1991). In a previous work, treatment of rats with either curcumin or chlorophyllin revealed a lower liver microsomal MDA concentration, lower DNA fragmentation percentages and a lower concentration of 8-OHdG

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and prevented induction of mutations (Ibrahim *et al.*, 2007).

Single-strand conformational polymorphism (SSCP) is a simple and reliable technique used to analyze and detect DNA mutations. It is based on the assumption that changes in the nucleotide sequence of a polymerase chain reaction (PCR) product affect its single-strand conformation. Molecules differing by as little as a single base substitution should have different conformers under non-denaturing conditions and migrate differently (Orita *et al.*, 1989; Hayashi, 1991).

The present study was initiated with the objective of evaluating the possible *in vivo* mutation modulating effect of curcumin and chlorophyllin in CP-induced mutations in the *p53* gene using PCR-SSCP techniques.

Materials and Methods

Materials

Cyclophosphamide (CP) was obtained from ASTA Medica AG, curcumin and chlorophyllin from Sigma. Oligonucleotide primers were synthesized by MWG Research, Ebersberg, Germany. PCR kit and DNA ladder were from Abgene, UK. All other chemicals were of pure grade from Sigma.

Experimental design

Forty 6-week-old male albino rats, 100–120 g, were reared at Biochemistry Department, Faculty of Veterinary Medicine, Cairo University, Egypt and kept under good hygienic conditions with food and water *ad libitum*. Animals were allocated into 4 groups, each of ten rats:

(1) Control non-treated group: This group was kept without any treatment throughout the study.

(2) CP-treated group: Animals of this group were injected intraperitoneally (i.p.) with a single dose of 40 mg of CP/kg body weight (BW) (Shukla *et al.*, 2002) at the 3rd week from the beginning of the study.

(3) CP-curcumin-treated group: Rats were orally administered 100 mg of curcumin/kg BW (Shukla *et al.*, 2002) three times a week for three successive weeks, then injected by a single dose of 40 mg of CP/kg BW i. p. at the third week of curcumin treatment (Shukla *et al.*, 2002).

(4) CP-chlorophyllin-treated group: This group was given 3 mg of chlorophyllin/kg BW three times a week for three successive weeks (Madrigal-Bujaidar *et al.*, 1997), then administered CP like the previous group.

Sampling

2 d post CP injection, rats were dislocated from cervical region under general anesthesia, and livers were collected and stored at −40 °C until use for DNA preparation.

Genomic DNA isolation

High-molecular weight genomic DNA was isolated from hepatic tissues using the phenol/chloroform method as described by Sambrook *et al.* (1989). The concentration of DNA was spectrophotometrically estimated by measuring its optical density (OD) at 260 nm, and the quality was assessed as the ratio of OD at 260/280 nm.

Synthesis of oligonucleotide primers

Primers flanking exons 5, 6 and 7 of the rat *p53* gene were synthesized. These primers were designed according to the published sequences for *p53* exons (Soussi *et al.*, 1988) and introns (Hulla and Schneider, 1993). The amplified fragments were less than 200 bp long which provides optimal sensitivity for SSCP analysis (Orita *et al.*, 1989).

The sequences of the used primers, the target exons and the expected fragment sizes are listed in Table I.

Polymerase chain reaction (PCR)

The primer-directed enzymatic amplification of specific rat *p53* gene fragments was performed as follows: PCR products were generated in a total volume of 50 μl containing: 10 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 25 pmol of each primer; 200 μM of each dNTP; 1 μg of genomic DNA; and 2.5 U of *Taq* DNA polymerase. The amplification was carried out in an automated thermal cycler as follows: 35 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 53–59 °C, and extension for 1 min at 72 °C, with initial denaturation at 95 °C for 5 min and final extension for 5 min at 72 °C.

Amplification was verified by electrophoresis on 2 % (w/v) agarose gel in 1 × TAE buffer (2 mM of EDTA, 40 mM of Tris-acetate), using a 100 bp ladder as a molecular weight marker for confirmation of the PCR products length. Gels were stained

Table I. Sequences of the oligonucleotide primers for exons 5 to 7 of the rat *p53* gene for PCR-SSCP analysis.

Primer	Sequence	Target	Fragment length [bp]
F1	5′-GAT TCT TTC TCC TCT CCT AC-3′	Portion of exon 5 (5′ end)	158
R1	5′-TGT AGA TGG CCA TGG CAC GG-3′		
F2	5′-GTC ACC TCC ACAACA CCT CCA CC-3′	Portion of exon 5 (5′ end)	165
R2	5′-GTG GTA CCG TAT GAG CCA CC-3′		
F3	5′-GCC TCT GAC TTA TTC TTG C-3′	Exon 6	158
R3	5′-GTG GTA TAG TCG GAG CCG AC-3′	Exon 6	
F4	5′-GTG GTA CCG TAT GAG CCA CC-3′	Exon 7	157
R4	5′-CAA CCT GGC ACA CAG CTT CC-3′	Exon 7	

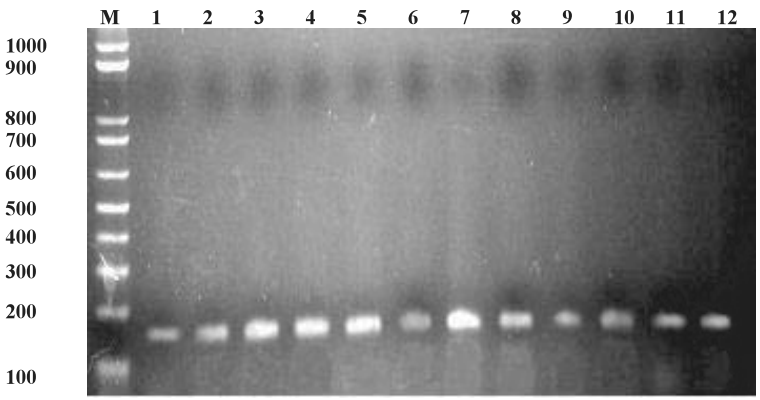

Fig. 1. PCR products of the exon 5 of *p53* gene with F1 and R1 primers. Lane M, 100 bp ladder size marker; lanes 1–3, control non-treated rats; lanes 4–6, CP-treated rats; lanes 7–9, CP-curcumin-treated rats; lanes 10–12, CP-chlorophyllin-treated rats. All PCR products are less than 200 bp long.

with ethidium bromide (1 μg/ml), visualized under a UV Tran-illuminator and photographed.

SSCP analysis

A mixture of 10 μl of PCR product and 10 μl loading buffer (50 % w/v sucrose, 0.25 % bromophenol blue in 50 % formamide) was prepared. The mixture was heated to 100 °C for 7 min and then plunged into ice for 5 min prior to loading onto the gel. The mixture was inoculated into 12.5 % polyacrylamide gel with 20 % formamide and electrophoresed in 1 × TBE buffer (89 mM Tris-boric acid and 2 mM EDTA) on a minislab gel at room temperature at 150 V for 5 min, then at 80 V until the DNA bands migrated about 2/3 of the gel (Kumeda and Asao, 1996). The gel was stained with ethidium bromide (1 mg/ml) for 2 min and then destained in water for 15 min. Gels were visualized under a UV illuminator and photographed using a digital camera.

Results and Discussion

p53 gene amplicons

The PCR-amplified products of *p53* different regions (exons 5 to 7) were found to be in the expected molecular size of the designed primers; Fig. 1 shows some examples of such products. The examined regions of the *p53* gene in the present study were considered the highly conserved domains in which most mutations occurred (Hollstein *et al.*, 1991). The existence of rat *p53* pseudogenes and their amplification using exonic primers only were detected according to Hulla (1992). The presence of these pseudogenes interfered the mutational analysis of the *p53* gene. Therefore, a combination between intronic primers and exonic ones was used to exclude the possible detection of rat *p53* pseudogenes (Hulla, 1992).

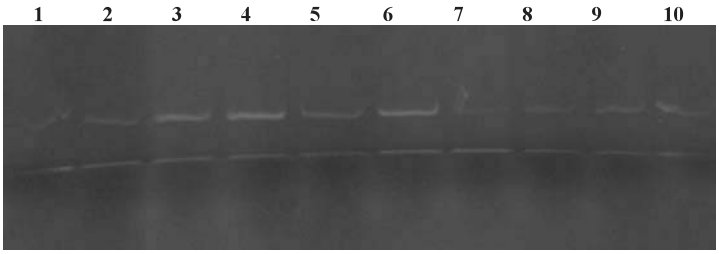

Fig. 2. SSCP of PCR products of exon 5 with F1 and R1 primers run on 12.5 % PAGE containing 20 % formamide. Lanes 1 and 2, control non-treated rats; lanes 3 and 4, CP-treated rats; lanes 5–7, CP-curcumin-treated rats; lanes 8–10, CP-chlorophyllin-treated rats. No alterations in the electrophoretic mobility are detected in either of the DNA bands.

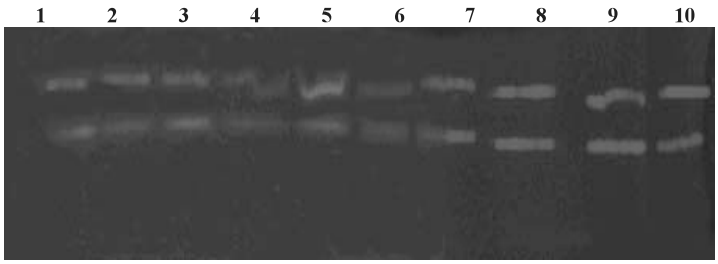

Fig. 3. SSCP of PCR products of exon 5 with F2 and R2 primers run on 12.5 % PAGE containing 20 % formamide. Lanes 1 and 2, control non-treated rats; lanes 3 and 4, CP-treated rats; lanes 5–7, CP-curcumin-treated rats; lanes 8–10, CP-chlorophyllin-treated rats. No alterations in the electrophoretic mobility are detected in either of the DNA bands.

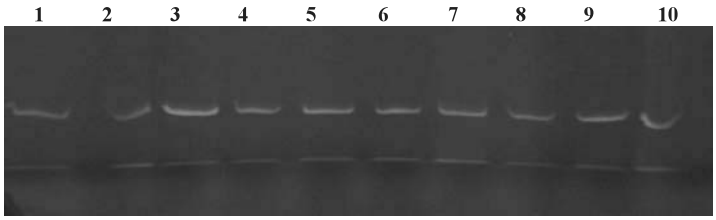

Fig. 4. SSCP of PCR products of exon 6 run on 12.5% PAGE containing 20% formamide. Lanes 1 and 2, control non-treated rats; lanes 3 and 4, CP-treated rats; lanes 5–7, CP-curcumin-treated rats; lanes 8–10, CP-chlorophyllin-treated rats. No alterations in the electrophoretic mobility are detected in either of the DNA bands.

SSCP analysis

SSCP analysis of the amplified PCR products using 12.5 % polyacrylamide gel (PAGE) containing 20 % formamide revealed that cyclophosphamide had no mutation effect on exons 5 and 6 in rats (Figs. 2, 3, 4). At the same time, SSCP analysis revealed that there was 80 % mutation frequency in exon 7 of the tested rats (Figs. 5, 6).

In spite of using different mutation analysis methods, the mutation found in exon 7 of the *p53* gene in the present study was in line with previous investigations where more than 90 % of mutations of the *p53* gene occurred in exons 5 to 8 (the highly conserved domains). Sequence-specific DNA binding activity of the *p53* protein was found in these conserved domains; therefore mutations in these domains resulted in loss a of DNA-binding capacity (Weinberg *et al.*, 2004). Most studies had focused on these domains in spite of the possibility of *p53* gene mutations in other regions could not be completely excluded (Lens *et al.*, 1997; Pettitt *et al.*, 2000; Sturm *et al.*, 2003).

In fact, a close association between pretreatment with alkylating agents and mutation of the

p53 gene has been noted, as the tumour suppressor *p53* gene is considered a major target of CP (Appella and Anderson, 2001). Cyclophosphamide, the alkylating agent that was used as a model teratogen in the present study, was converted by the liver cytochrome P450 system to 4-hydroxycyclophosphamide. The cleavage of this product produced highly cytotoxic agents (acrolein and phosphoramide mustard). This cytotoxicity is mediated by the alkylation of DNA at the N7 position of guanine and the formation of intra-/interstrand cross-links, DNA adducts, DNA-protein cross-links, single-strand breaks and inhibition of strand separation (Anderson *et al.*, 1995).

Pretreatment with 100 mg of curcumin/kg BW produced a 33.3 % protection against the CP-induced mutation of exon 7 in the current study (Fig. 5). In this respect, Shukla *et al.* (2002) reported that curcumin can inhibit CP-induced mutagenic damages in a dose-response manner. They also showed that the frequency of different kinds of chromosomal aberrations, fragmentations and exchanges induced by CP was reduced by the pretreatment with curcumin.

The role played by curcumin in the prevention of cancer may arise from the *in vitro* inhibition of proteins that are important for cancer progression, *e.g.* NF-κB, lipoxygenase and cyclooxygenase isoenzymes (Brennan and O'Neill, 1998; Plummer *et al.*, 1999; Shureiqi and Lippman, 2001). Moreover, curcumin had *in vivo* protection against colon tumours in models of chemical carcinogenesis at high concentrations (Kawamori *et al.*, 1999; Singh *et al.*, 1998) as it contains α- and β-unsaturated ketones, which react with NF-κB repressing transactivation. Furthermore, curcumin has also been found to protect from cisplatin-induced clastogenesis by acting as a free radical scavenger (Antunes *et al.*, 2000). In addition, curcumin reduced radiation-induced DNA damages in rat lymphocytes by modulating the SOS repair system (Thresiamma *et al.*, 1998). On the other hand, many authors claimed that curcumin itself might induce a genetic mutation and alter the functions of the *p53* tumour suppressor gene (Moos *et al.*, 2004).

Regarding the pretreatment of rats with chlorophyllin, it was found to have a powerful protective efficacy (80 % protection) against the mutation effect of CP on exon 7 of the *p53* gene (Fig. 6). Several mechanisms have been hypothesized to explain the antimutagenic activity of chlorophyllin, including its antioxidant properties and its ability to form complexes with mutagens (Ardelt *et al.*, 2001).

The proposed preventive mechanism of chlorophyllin is that it can act as an "interceptor molecule" through the formation of tight molecular complexes with carcinogens (Breinholt *et al.*,

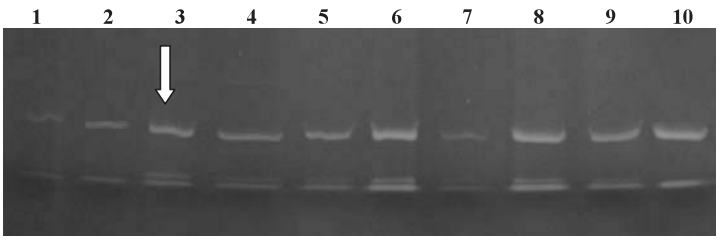

Fig. 5. SSCP of PCR products of exon 7. Lanes 1 and 2, negative control; lanes 3 and 4, CP-treated rats; lanes 5–10, CP-curcumin-treated rats. The arrow indicates the pattern of shifted mobility of amplified mutant bands.

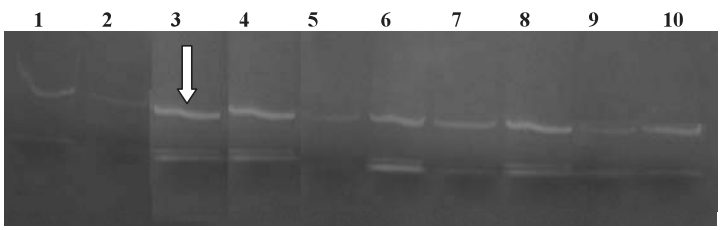

Fig. 6. SSCP of PCR products of exon 7. Lanes 1 and 2, negative control; lanes 3 and 4, CP-treated rats; lanes 5–10, CP-chlorophyllin-treated rats. The arrow indicates the pattern of shifted mobility of DNA.

1995). Thus, chlorophyllin may diminish the bioavailability of dietary carcinogens by impeding their absorption and by shuttling them through the fecal stream, leading to reduced DNA adducts and tumour burden (Kensler *et al.*, 1998; Breinholt *et al.*, 1999). Therefore, chlorophyllin can be considered the most effective anticarcinogen in experimental models when given in a large dose. The chlorophyllin anticarcinogenic spectrum is its *in vitro* powerful inhibition of cytochrome P450 enzymes that are involved in the bioactivation of several environmental carcinogens (Yun *et al.*, 1995). Chlorophyllin makes the conversion of carcinogens into harmless products more efficient by lowering the cytochrome P450 enzyme activity in the first place and by reacting with carcinogens to produce harmless complexes, just as the glutathione transferases do. Thus, chlorophyllin mimics glutathione transferase activity but not induces its activity. At the same time, chlorophyllin acts as an antioxidant inhibiting lipid peroxidation (Egner *et al.*, 2001) by reducing hydroxyl radicals and the DNA strand breaks production in a concentration-dependent manner (Park *et al.*, 2003). Curcumin and chlorophyllin prevents the DNA polymorphism effect of CP resulting in a normal pattern of restriction digestion (Ibrahim *et al.*, 2007). The antioxidant activity of curcumin and chlorophyllin was shown to inhibit the mutagenic effect of CP (Azuine and Bhide, 1992; Botelho *et al*., 2004).

Taken all results into account, we can conclude that both curcumin and chlorophyllin exhibit protective effects against carcinogen exposure. Clearly, there is a little doubt that nutrition is involved in cancer prevention, nevertheless optimizing nutrition by the use of foods and their bioactive components represents a non-invasive and cost-effective strategy for reducing the risk of cancer induction in many regions of the world especially in developing countries where these materials are available for most people at low prices.

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