Absence of p53 Gene Expression in Selenium Molecular Prevention of Chemically Induced Hepatocarcinogenesis in Rats
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ABSTRACT

Background/Aim: p53 pathway is thought by many researchers to be critically involved in selenium’s chemoprevention or in hepatocarcinogenesis. The aim of this study was to investigate the gene expression of p53, p21 and B-cell lymphoma-2 (bcl-2) using preventive and therapeutic approaches of selenium in chemically induced hepatocarcinogenesis in rats. Materials and Methods: Rats were divided randomly into six groups: Negative control, positive control (diethyl nitrosamine +2-acetylamino fluorene), preventive group, preventive control (respective control for preventive group), therapeutic group and therapeutic control (respective control for therapeutic group). p53, p21 and bcl-2 genes on liver tissues were measured using real-time polymerase chain reaction. Results: The expression of p53 was only significant in the therapeutic control. The expression of bcl-2 was insigniﬁcant in all the groups. p21 expression was signiﬁcant in all the groups except the preventive group. Conclusions: The selenium molecular mechanism for liver cancer prevention is not through the p53 pathway. Also, the absence of p53 is not necessary for chemically induced liver cancer in rats.

Key Words: Hepatocellular carcinoma, selenium, tumor suppressor gene

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A recent study has revealed that there are at least four possible genetic pathways that are involved in the formation of hepatocellular carcinoma (HCC):[1] the pRB/p16INK4A pathway involved in cell cycle control, the TGF-B1 pathway involved in growth inhibition and apoptosis, the B-catenin/Axin 1 pathway involved in morphogenesis and signal transduction and the p53 pathway involved in DNA damage and response. This study is only concerned with the p53 pathway.

In its normal role, p53 is a transcription factor that activates a number of genes that function in cellular responses to DNA damage with a critical role in controlling cell cycle arrest and apoptosis.[2] The exact criteria that influence p53 to stimulate the cell cycle arrest or apoptosis are not fully understood.

However, there are several factors that influence the function of p53; these include expression level of p53, the type of stress signal, the cell type and the cellular context at the time of exposure to stress.[3] The p53 gene stops a cell cycle in G1 phase when its DNA is damaged or a base mismatch happens. If this damage cannot be repaired by itself, then the cell would go to a state of apoptosis, which may occur at any stage of the cell cycle.[4] Apoptosis, or programmed cell death, is a form of cell death in which cells actively participate in their own destruction and the process is characterized by a series of specific morphological and biochemical changes and also by alterations of genomic expression.[5,6] The ability of p53 to eliminate the excess damaged or infected cells by apoptosis is vital for the proper regulation of cell proliferation in multicellular organisms.[7] Being a key component in the cellular response to stress, p53 serves as a major obstruction for carcinogenesis.[8] Among the genes that are transcriptionally upregulated by p53 are those involved in cell cycle regulation such as p21 WAF/CIP (p21) and those involved in apoptosis such as B-cell lymphoma-2 (bcl-2).

The p21 gene is a nuclear protein that induces cell cycle arrest in the G1 and G2 phases.[9] It functions as an inhibitor
of cyclin dependent kinase (CDK) complexes, which are essential for cell cycle progression.\[^{10}\] It has been revealed that the deletion of p21 from human cells reduces the ability of cell to undergo cell cycle arrest in response to p53 activation.\[^{11}\]\[^{12}\] The p21 can be activated in either a p53-dependent manner or in a p53-independent manner.\[^{13}\]\[^{14}\] Previous studies have suggested that p21 mRNA expression in non-tumor liver tissues is significantly higher than that in HCC tissues.\[^{15}\]\[^{16}\] Also, it was revealed that the low expression of p21 in HCC tissues might represent a form of CDK inhibitor dysfunction involved in carcinogenesis.\[^{16}\]

The bcl-2 is a member of the bcl-2 family.\[^{17}\] The bcl-2 family proteins play an important role in apoptosis, acting to either inhibit or promote apoptosis as well as being considered to correlate with the pathogenesis and prognosis of cancers.\[^{18}\] Although its members share close structural homologies, their biological functions differentiate into two major categories.\[^{19}\] The first category includes members that promote apoptosis such as bax, bak, bcl-xx, which are also upregulated by p53. The second category includes members that inhibit apoptosis such as bcl-2, mcl-1, and bcl-xl.\[^{20}\] High levels of bcl-2 are found in many p53 tumor cell types, suggesting that bcl-2 may block p53-dependent apoptosis in these cells.\[^{21}\]

Selenium chemopreventive activity has been reported to be involved in apoptosis and cell cycle arrest.\[^{22}\] However, the genes that are responsible for these activities remain unknown. Thus, using preventive and therapeutic approaches of selenium, the gene expression of p53, p21 and bcl-2 was investigated in this study.

**MATERIALS AND METHODS**

**Chemicals**

Trizol (Cat. No.: 15596-026), SuperScript\textsuperscript{\textregistered} III First-strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) (Cat. No.: 18080-05) and RNase and DNase free water (Cat. No.: 10977-015) were bought from Invitrogen, Carlsbad, California, USA. iQ\textsuperscript{\textregistered} Sybr Green Supermix (Cat. No.: 170-8880) was from Bio-Rad, Berkeley, California, USA. p21, p53, bcl-2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TBE buffer (Cat. No.: 060816) were purchased from Bio-Basic Inc, Markham, Canada.

**Animals and diet**

Male Sprague-Dawley rats (6–8 weeks) were obtained from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia. They were housed in plastic cages (3–4 rats per cage) with wood chips for bedding. The animals were acclimatized to standard laboratory conditions (temperature 22–25°C, humidity 55 ± 10% with a 12-hour light–dark cycle) for 1 week before the commencement of the experiments. During the entire period of study, the rats had free access to food and water. The rats were maintained on a basal diet (22% crude protein, 5% crude fiber, 3% fat, 13% moisture, 8% ash, 0.85–1.2% calcium, 0.6–1% phosphorus and 49% nitrogen free extract) (mouse pellet was 702-P from Gold Coin Co., Ltd., Kuala Lumpur, Malaysia). The recommendations of Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) for the care and use of animals were strictly followed throughout the study (UKMAEC No.: FSKB/2006/ Jamaludin/22- August/170-December-2006).

**Experimental design**

Forty-four rats were randomly divided into six groups, with six or eight rats in each group, as follows.

- **Group 1** (negative control): Rats were given normal rat chow and drinking water. Also, a single intraperitoneal (IP) injection of saline (0.9%) was given.
- **Group 2** (positive control): Liver tumors were induced with a single IP injection of diethyl nitrosamine (DEN) at a dose of 200 mg/kg body weight in saline.\[^{23}\] Two weeks after DEN administration, the carcinogen effect was promoted by 2-acetylaminofluorene (2-AAF) (0.02%). The promoter was incorporated into the rat chow for 10 weeks.
- **Group 3** (preventive group): 4 weeks before DEN administration, the rats were fed with sodium selenite (4 mg/l) through drinking water and this was stopped at week 4 (the day of commencement of DEN administration).
- **Group 4** (preventive control): The rats in this group served as controls for group 3. The rats were given sodium selenite for 4 weeks only. No DEN or 2-AAF was given. Instead, a single IP injection of saline (0.9%) was given.
- **Group 5** (therapeutic group): 4 weeks after starting DEN administration (as in group 2) was started, the rats were treated with sodium selenite (4 mg/l) through drinking water and this continued until the completion of the experiment (8 weeks).
- **Group 6** (therapeutic control): The rats in this group served as controls for group 5. They were given sodium selenite for eight consecutive weeks. No DEN or 2-AAF was given. Instead, a single IP injection of saline (0.9%) was given.

16 weeks after the initiation of the experiment, all the rats were fasted overnight and then killed by cervical dislocation under ether anesthesia. Sodium selenite supplementation in drinking water and normal drinking water was renewed every 2–3 days. Diet with 2-AAF was freshly prepared and wood chips for bedding were changed weekly.
Liver tissues
Immediately after killing, all livers were removed, sliced into various pieces, frozen in liquid nitrogen, wrapped in aluminum foil in sealed bags and stored at −80°C until use. Prior to RNA extraction, liver tissues were brought to room temperature and weighed to have 100 mg each. Then, they were cut into small pieces before homogenization. All liver tissues for RT-PCR were taken from the largest lobe in the rat liver. Portions of the livers were also fixed in 10% neutral buffered formalin for histoprocessing and staining with hematoxylin and eosin (H and E) for histopathologic examination.

RNA extraction
100 mg of liver tissue was homogenized with 1 ml of Trizol in a homogenizer (FastPrep 24, MP Biomedicals, Carlsbad, California, USA) for 2 minutes at 60 seconds interval. It was then centrifuged at 1200 g for 5 minutes at 4°C. About 800–850 ml was pipetted into a new Eppendorff tube and incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added and mixed vigorously by hand for 15 seconds prior to incubation at room temperature for 5 minutes. The sample was centrifuged at 1200 g for 15 minutes at 4°C. The upper aqueous phase, which contained the RNA, was carefully removed and transferred to a fresh Eppendorff tube. 0.5 ml of isopropanol was added and mixed with the aqueous solution and 10 ml of poly-carrier was added to this mixture to precipitate the RNA. After centrifugation at 1200 g for 10 minutes at 4°C, the supernatant was removed and visible RNA pellet was collected and washed with 1 ml of 75% ethanol, and then centrifuged at 7500 g for 5 minutes at 4°C. After ethanol was completely removed, the RNA was allowed to dry for 25 minutes at room temperature. The total RNA was finally dissolved in 20 ml of diethyl phosphorocyanidate (DEPC)-treated water and kept at −20°C.

Reverse transcription
Reverse transcription (RT) of total RNA to cDNA by Superscript III was performed by mixing 5 ml of total RNA, 1 ml of 50 mM oligo(dT), 1 ml of 10 mM dNTP mix and 3 ml of DEPC-treated water and incubated at 65°C for 5 minutes. It was then placed on ice for at least 1 minute. 10 μl of cDNA synthesis mix containing 2 ml of 10 RT buffer, 4 ml of 25 mM MgCl₂, 2 ml of 0.1 m Dithiothreitol (DTT), 1 ml of RNase OUT (40 U/ml) and 1 ml of superscript III RT (200 U/ml) was added and mixed with the prepared RNA mixture. This mixture was incubated at 50°C for 50 minutes. The reaction was terminated at 85°C for 5 minutes. It was then placed on ice for at least 1 minute and 1 ml of RNase H was added, mixed and incubated at 37°C for 20 minutes. At this stage, cDNA was ready to be used for PCR. RT was carried out in 9700 PCR System, GeneAmp, Applied Biosystems, Carlsbad, California, USA.

Real-time polymerase chain reaction
Real-time PCR was performed with the iQ™ Sybr Green Supermix RT-PCR kit using the iQ™ Real-time PCR detection system (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. Mixtures were prepared in a total volume of 25 ml containing 1 ml of cDNA sample, 1 ml of forward primer gene, 1 ml of reverse primer gene [Table 1], 12.5 ml of Sybr Green Supermix and 9.5 ml of RNase/DNase free sterile water. All samples were run on 96-well reaction plates using sterile strips with their attached caps. The conditions of PCR were set according to the recommendations in the manufacturer’s instructions. The PCR reaction consisted of six cycles. Cycle one involved incubation at 95°C for 30 seconds to activate DNA tag polymerase. Cycle two was set at 95°C for 3 minutes to pre-denature and further activate DNA tag polymerase. Cycle three contained 40 cycles of amplification, with each cycle consisting of 10 seconds of denaturation at 95°C, followed by 1 minute of annealing and extension at 61°C. Cycles four, five and six were used to measure the melting curve analysis. Cycle four was set at 95°C for 1 minute, followed by 1 minute at 60°C (Cycle five). Cycle six, which contained 70 cycles, ranging from 60°C to 94.5°C, with temperature increasing steps of 0.5°C every 10 seconds, was carried out to analyze the melting curve. Baseline and threshold values were automatically determined for all plates using the Bio-Rad iQ5 software 2.0. The obtained data were analyzed using the following relative gene expression formula:[24]

\[
\Delta \Delta CT = 2^{-\Delta CT} (\text{housekeeping gene}) - CT (\text{target gene})
\]

Ct (cycle threshold) indicates the fractional cycle number at which the amount of amplified target reaches a fixed

Table 1: Primer sequences

| Genes     | Accession no. | Primes          | Base pair |
|-----------|---------------|-----------------|-----------|
| GAPDH     | XM-001067852.1| Forward: 5′-gct cac taa agg cca tcc tg-3′ | 189       |
| p21       | NM-001002831.1| Forward: 5′-ctg gcc cac aga gaa cac ca-3′ | 210       |
| p53       | NM-030989.3   | Forward: 5′-gtt ccc aga gct gaa tga gg-3′ | 125       |
| Bcl-2     | NM-016993.1   | Forward: 5′-cga ctt tgc aga gat gtc ca-3′ | 223       |

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Bcl-2: B-cell lymphoma-2
threshold. In addition to this, the use of relative expression quantification requires no standard curve or DNA gel electrophoresis.

**Statistical analysis**
Data were expressed as means ± standard deviation (SD). The data were analyzed using statistical package for social sciences (SPSS) version 13, and Microsoft Office Excel 2003 was used to draw graphs. Shapiro–Wilk test was used to check the normality of the variable. Accordingly, Student’s $t$ and Mann–Whitney’s $U$ tests were used to analyze data that follow normal or non-normal behavior of distribution pattern, respectively. Differences in statistical analysis of data were considered significant at $P < 0.05$.

**RESULTS**
Histopathologic examination of the liver in the positive control showed a completely disrupted architecture. The normal liver cords were displaced with variably sized neoplastic nodules. The hepatocytes were more than two cells thick, paler and showed enlarged vesicular nuclei with prominent nucleoli [Figure 1]. However, the preventive and therapeutic groups revealed that the liver was nodular, but with largely preserved architecture.

![Figure 1: Neoplastic liver showing enlarged nuclei with prominent nucleoli, as seen in the positive control (received a single IP injection of DEN at a dose of 200 mg/kg body weight in saline and 2 weeks later, the carcinogen effect was promoted by 2-AAF (0.02%) and continued for 10 weeks). Hematoxylin and eosin (×20)](image1)

![Figure 2: Hyperplastic liver with largely preserved architecture as seen in the preventive group (received sodium selenite which was stopped at week 4, the day of commencement of DEN administration as in group 2) and the therapeutic group (received a single IP injection of DEN as in group 2, and 4 weeks later, rats were treated with sodium selenite for 8 weeks). Hematoxylin and eosin (×20)](image2)

![Figure 3: Normal liver architecture as seen in the negative (received normal rat chow and drinking water), preventive (treated with sodium selenite alone for 4 weeks and served as control for group 3) and therapeutic controls (treated with sodium selenite alone for 8 weeks and served as control for group 5). Hematoxylin and eosin (×20)](image3)

![Figure 4: Relative expression of p53 in all experimental groups, using RT-PCR. Group 1 received rat chow and drinking water. Group 2 received a single IP injection of DEN at a dose of 200 mg/kg body weight in saline, and 2 weeks later, the carcinogen effect was promoted by administering 2-AAF (0.02%) which was continued for 10 weeks. Group 3 received sodium selenite (4 mg/l in drinking water) which was stopped at week 4 (the day of commencement of DEN administration as in group 2). Group 4 rats were treated with sodium selenite alone for 4 weeks (served as control for group 3). Group 5 received a single IP injection of DEN as in group 2, and 4 weeks later, rats were treated with sodium selenite (4 mg/l in drinking water) for 8 weeks. Group 6 rats were treated with sodium selenite alone for 8 weeks (served as control for group 5). Results are expressed as means ± SD. Values were analyzed using Student’s $t$ and Mann–Whitney’s $U$ tests](image4)
The majority of variably sized nodules were hyperplastic (one cell thick) [Figure 2]. The negative, preventive and therapeutic controls were free of any abnormality [Figure 3].

The relative expression of p53 was only significant in the therapeutic control, which had continuous administration of sodium selenite for 8 weeks, when compared with both the negative and positive controls. However, all other experimental groups showed no significant expression of p53 when compared with either negative or positive controls [Figure 3]. There was no significant expression of bcl-2 among all the experimental groups, including the positive group (group 2), when compared with the negative control (group 1). In addition, the preventive group (group 3) and its respective control (group 4) and the therapeutic group (group 5) and its respective control (group 6) showed no significant dysregulation of bcl-2 when compared with the positive control [Figure 5].

Real-time RT-PCR showed that the relative gene expression of p21 in the positive control was significantly lower when compared with the negative control. Also, the therapeutic group and its respective control showed lower significant expression of p21 when compared with the negative control. Interestingly, the preventive group, which had selenium for 4 weeks only, followed by DEN and 2-AAF, showed that the expression of p21 was not significant when compared with both the negative and positive controls. However, the preventive control, which had selenium only without chemical carcinogens, showed a significant lower expression when compared with the negative control [Figure 6].

**DISCUSSION**

Previous studies from different geographic origins reported that bcl-2 expression in HCC cases did not play a role in hepatocarcinogenesis. [27-29] In agreement with these studies, bcl-2 expression showed no difference in all the experimental groups, even with the carcinogenic group (positive group) in our study. Despite the fact that bcl-2 is an antiapoptotic protein and is upregulated in various tumor types, and increased bcl-2 expression has been linked to chemoresistance and unfavorable prognosis, [30] in this study, bcl-2 was not affected by chemical carcinogens. Thus, it might be suggested that during chemical carcinogenesis in rats, bcl-2 expression is not influenced by either DEN or 2-AAF. On the other hand, it has been noted that the downregulation of bcl-2 has been proven to be a promising treatment approach in various experimental trials including prostate, pancreatic and breast cancers. [31-33] This study has shown that the preventive and therapeutic selenium treatments did not have an effect on bcl-2 expression. Thus, it can be suggested that bcl-2 is not a target gene for selenium treatment on chemically induced hepatocarcinogenesis in rats.

Despite the fact that alterations in the tumor suppressor gene (p53) seem to be critical for human hepatocarcinogenesis and that mutations in the p53 gene are the most common genetic events in human cancers, [34] as well as their frequent occurrence in HCC, [35] this study has shown that p53 expression in the positive control is not significant.
when compared with the negative control. However, the therapeutic control, which had a continuous sodium selenite treatment but without chemical carcinogens, showed a significant reduced expression of p53 when compared with both the negative and positive controls. This could be due to the sub-toxic dose of selenium (4 mg/l) that was used in this study. Interestingly, the preventive control, which had the same dose of selenium for 4 weeks only, showed no significant value when compared with either the positive or negative controls. This could be due to the rat’s utilization of sodium selenite. The finding of this study (absence of p53 gene alteration) is in line with other previous studies,[36-38] which collectively reported that there were no mutations or deletions in p53 gene alterations in rat hepatic tumors induced by different carcinogens such as DEN, 3’-methyl-4-dimethylaminoazobenzene, aflatoxin B1 (AFB1) and nitrosomethyleurea.

It is important to note that most of the p53 mutations occur in the late stage of HCC. One study investigated p53 mutations in hepatocarcinogenesis, which were induced by the hepatitis B virus (HBV) and AFB1 in three shrew animals. It revealed that alterations of p53 may be a late event in the development of HCC, appearing at the 105th week of initiated experiments.[59] Furthermore, it was observed that p53 mutations occur more frequently in poorly differentiated (54%) than in well- or moderately differentiated (21%) HCCs and not at all in early HCCs.[60] Also, in nodule-in-nodule lesions, p53 mutations were shown in the inside of the nodule, as an advanced HCC marker, but not in the outside portion, as an early HCC component, providing direct evidence that p53 is involved in the late stage of HCC.[61] In addition, it has been reported that p53 has a very short half-life and thus could be undetectable.[42] It is noteworthy that although quantitative RT-PCR is a sensitive method, the signals of p53 could be too weak to be specifically detected by this instrument. In summary, the absence of p53 gene expression on chemically induced liver cancer by DEN and 2-AAF may suggest that the loss of p53 is not necessary for hepatocarcinogenesis in rats.

The significant low and unchanged gene expressions of p21 and p53, respectively, as shown in the positive control when compared with the negative control may suggest that p21 is regulated by the independent pathway of p53 on chemically induced hepatocarcinogenesis in rats. One of the interesting findings in this study was that the therapeutic group showed a significantly low p21 gene expression compared to the negative control. Thus, it might suggest that combinations of DEN, 2-AAF and sodium selenite suppress the functions of p21. Also, supplementation of selenium alone as in the preventive and therapeutic controls (without addition of DEN or 2-AAF) may have an effect on p21 function. However, histopathologic examination showed no abnormality of the liver tissues in these two groups.

In conclusion, using preventive and therapeutic approaches, this study showed that the selenium molecular mechanism for liver cancer prevention is not through the p53 pathway. Also, the absence of p53 is not necessary for chemically induced liver cancer in rats.

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