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Telomerase Activity, Survivin and Caspase-3 Expression During Malignant Transformation in Experimental Hepatocarcinogenesis

[Deneysel Hepatik Kanser Oluşumunda Kötü Huyluluğa Geçiş Evresinde Telomeraz Aktivitesi, Survivin ve Kaspaz-3 Ekspresyonu]

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ABSTRACT

The present study aimed to evaluate telomerase activity, survivin and caspase-3-expression during the multistep process of hepatocarcinogenesis initiated by nitrosamine precursors in male albino rats and to dissect their potential prognostic value and discuss the relationship between survivin and caspase-3 expressions. The study was performed on 150 Wistar albino rats, Telomeric Repeat Amplification Protocol assay was used to detect the telomerase activity and Western blot technique was used to detect both survivin and caspase-3 expressions as well as histopathological examination of the collected liver samples. Hepatocellular carcinoma was developed after 12 months of initiation. A significant increase was observed in telomerase activity and survivin expression level while; caspase-3 expression was reduced in hepatocellular carcinoma cases in comparison with the normal ones. Also, inverse and non linear correlation was observed between survivin and caspase-3 expression. Reactivation of telomerase is a cancer-specific event and may contribute to the development of Hepatocellular carcinoma. Up regulation of survivin and down regulation of caspase-3 expression suggesting that the suppression of apoptosis may be a major step involved in hepatocarcinogenesis. We conclude that telomerase and survivin may be used as a potentially prognostic marker for hepatocellular carcinoma and survivin may inhibit caspase-3 by indirect pathway.

Key Words: Hepatocellular carcinoma, Telomerase, Survivin, Caspase-3, Nitrosamine

ÖZET

Bu çalışmanın amacı erkek albino sıçanlarda nitrozamin öncülleri ile başlayan çok basamaklı hepatokarsinogenez sürecinde telomeraz aktivitesi, survivin ve kaspaz-3 ekspresyonunu değerlendirmek, takipsel potansiyellerini incelemek ve survivin ile kaspaz-3 arasındaki ilişkiyi tartışmaktır. 150 Wistar albino sıçan ile yapılan bu çalışmada, telomeraz aktivitesi telomeric tekrar amplifikasyon protokolü metodu ile survivin ve kaspase-3 ekspresyonları ise Western blot ve karaciğerlerin histopatolojik incelenmesi ile belirlenmiştir. Başlangıçtan 12 ay sonra hepatik hücresel kanser gelişmiştir. Hepatik hücresel kanser olgularında normallerle karşılaştırıldığında telomeraz aktivitesi ve survivin ekspresyon düzeyinde belirgin bir artış gözlenmekle birlikte kaspaz-3 ekspresyonu düşmüştür. Ayrıca survivin ve kaspaz-3 ekspresyonları arasında zıt ve doğrusal olmayan bir korelasyon gözlenmiştir. Telomerazın tekrar aktive olması kansere özgü bir olaydır ve hepatik hücresel kanser gelişiminde payı olabilir. Survivin ekspresyon regulasyonundaki artış ve kaspaz-3 ekspresyon regulasyonundaki azalma, apoptozun baskılanmasının hepatokarsinogenezdeki önemli basamaklardan olduğunu düşündürmektedir. Telomeraz ve survivinin hepatik hücresel kanser için potansiyel bir takip belirteci olarak kullanılabileceği ve survivinin kaspaz-3'ü indirekt bir yol ile inhibe ettiği kanaatindeyiz.

Anahtar Kelimeler: Hepatik hücresel karsinoma, Telomeraz, Survivin, Kaspaz-3, Nitrozamin

INTRODUCTION

Carcinogenesis is a multistage process, initiation, promotion, transformation and progression (1). Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide (2). It is associated with alterations in several genes due to the infection by hepatitis B or C or chronic alcohol intake or exposure to mycotoxin aflatoxin B-1 (3). Nitrosamine, a well known hepatocarcinogenic agent, can lead to a preneoplastic lesion like the low and high grade displastic nodule / adenomatous hyperplasia (4). The activation of oncogenes and inactivation of tumor suppressor genes have an important role in the mechanisms of cancer development (5). Telomerase is a ribonucleoprotein enzyme complex (a cellular reverse transcriptase) that has been referred to as a cellular immortalizing enzyme. It stabilizes telomere length by adding hexameric (TTAGGG) repeats onto the telomeric ends of the chromosomes. Gonzalez et al. (6) reported that telomerase shortening apparently has a dual role in tumor development and progression. It induces chromosomal instability and the initiation of cancer and stabilization of telomeres. The predominant mechanism of telomere stabilization in tumor cells is the activation of the telomerase, the telomere synthesizing enzyme (7). Matthew, (8) reported that shortened telomere in mitotic cells may be resp onsible for some of the changes associated with normal aging. In most normal tissues, telomerase activity is not expressed in somatic tissues adjacent to the tumor tissues; accordingly, telomerase activity has proved to be a reliable marker for detecting tumor cells in resection margins (9).

Survivin is a protein that inhibits apoptosis and regulates cell division. It inhibits apoptosis by either directly or indirectly interfering with the function of caspases by targeting the caspase-3 which located at the end of the cascade. Caspase-3 acts as both initiators and executors in the apoptotic process. Therefore, survivin and caspase-3 have been the focus of debate regarding apoptosis (10). The present work focuses on the evaluation of telomerase activity, survivin as well as caspase-3 expression during experimental multistep hepatocarcinogenesis.

MATERIALS AND METHODS

Experimental design

The experiments were performed on 150 Wistar Albino rats kept on a standard diet consisting of 24 % protein, 5.5 % fibers, 5.5 % carbohydrates and water. Standard commercial diet (control diet) was purchased from The Egyptian Company of Oils and Soaps. The animals were divided into two groups, as the control (n = 50) and treatment (n = 100) groups. The treatment group received hepatocarcinogenic agent (nitrosamine precursors) in the drinking water in a concentration of (2000 ppm sodium nitrate and 1000 ppm dibutylamine) for three months to initiate hepatocarcinogenesis and then they received standard diet and water for the 12 months (11). Ten rats of the control for each treatment groups and 20 rats of the treated group were sacrificed each 3, 6, 9, and 12 months from the start of the study. The last 30 animals (10 untreated rats and 20 treated rats) were died during the period of treatment. The liver specimens were extracted and collected, and shock-frozen in small pieces at - 80 ºC. The study protocol was approved by local ethical committee of the National Cancer Institute, Cairo University.

Telomerase activity (TRAP Assay)

PCR-ELISA Kit from Boehringer Mannheim (GmbH, D- 68305 Mannheim) was used to detect telomerase activity of tissue samples.

(1) Elongation / Amplification

25 µl of the reaction mixture and 5 µl of the internal standard were transferred into a tube suitable for PCR amplification. Samples were added as 10 µg total protein. Extracts were prepared from thin slices of frozen tissue specimens in sterile disposable Petri dishes, then transferred to homogenization tubes contain 200 µl ice-cold lysis buffer. Homogenized on ice with a motorized pestle (Velcorin, Bayer AG, Leverkusen, Germany) until uniform consistency is achieved, Incubation on ice for 30 min. The lysate was centrifuged at 16,000 x g for 20 min at 2-8 ^{o}C. Carefully the supernatant was transferred to a new tube. The protein concentrations were measured by standard Bradford method (12). Heat-treated cell extracts were used as negative controls. The reaction mixtures were completed to 50 µl with nuclease-free water and then according to the kit used protocol, the PCR elongation / amplification was done.

(2) Detection by ELISA

The PCR products were divided into two aliquots, denatured and hybridized separately to digoxigenin-labelled detection probes, specific for the telomeric repeats and for the internal standard, respectively. The resulting products were immobilized via the biotin label to a streptavidin-coated microtiter plate. Immobilized amplicons are then detected with an antibody against digoxigenin which is conjugated to horseradish peroxidase and the sensitive peroxidase substrate trimethylbenzidine.

Hybridization and ELISA step action

For each sample, 10 µl of denaturation reagent was pipetted into 2 separate reaction tubes. For each tube, 2.5 µl of the amplification product was added and incubated at 15 ºC ~ 25 ºC for 10 min. Hundred microliter hybridization buffer were added to one vial and 100 µl hybridization buffer IS (internal standard) to the other vial and was mixed thoroughly by briefly vortexing. Hundred microliter of each mixture were transferred into each well of the precoated microplate modules supplied with the kit. After the incubation at 37 ºC with shaking at 300 rpm for 2 hr, the solutions were removed and the wells were washed with buffer. Treatment with anti DIG-HRP

working solution was done and then incubated at 15 ºC ~ 25 ºC for 30 min at a rotation 300 rpm.

Detection of survivin and caspase-3 by western blot technique

The homogenized samples (One gram of each sample was homogenized in phosphate buffered saline (PBS) 1:1 (w/v) pH 7.4 using a homogenizer (OMNI TH, international S/N TH-1060). Then total protein of each sample was detected using Bradford method with protein content of 200 µg per 15 µl were loaded on 30 % acrylamide gel. After electrophoresis, the gels were blotted on nitrocellulose sheets (0.25 µm positively charge) using the western blot technique. Survivin monoclonal antibody, dilution (1:1000) from Santa Cruz Biotechnology (D-8 : sc-17779) was used for detection of survivin, caspase-3 monoclonal antibody, dilution (1:1000) from Oncogene Research Products for detection of caspase-3, the secondary antibody, Anti-goat IgG, whole molecule, Peroxidase conjugate, (Oncogene Research Products), dilution (1:400) was used. Following visualization of the protein bands using diaminopenzedine, the intensity of the bands was measured using Dual-Wavelength flying spot scanning densitometry to calculate the protein content of interest.

Histopathological examination

Rats were sacrified and a piece of their liver tissues were fixed in formalin and paraffin embedded subsequently, tissue sections were stained with haematoxylin and eosin (Magnification 200).

Statistical analysis

Statistical package for social sciences, version 12, was used for statistical analysis. Using Friedman test, association between two independent qualitative variables was done by Chi 2 test, Fisher's exact whenever appropriate. Differences were considered significant when p-value $\leq$ 0.05 and highly significant when p-value $\leq$ 0.01 (11).

RESULTS

Telomerase activity

No telomerase activity was detected in the liver tissues of the control group. The results revealed that a statistically significant difference in relative telomerase activity (RTA) detected in the treatment group compared to the untreated control group, $p < 0.001$, (Table 1). On the other hand, after 3 months of treatment the nitrosamine-treated group showed telomerase activity was detected in 4/20 (20 %) of the rat liver tissues in the range of 0.00 to 43.2 % with mean activity of 8.3 % $\pm$ 3.5 whereas no telomerase activity was detected in the control group. After 6 months of treatment, four out of 20 liver tissue samples (20 %) showed relative telomerase activity with slightly higher mean activity than that found after 3 months (12 % $\pm$ 4.3), has appeared with range 0.00 to 60.5 %. After 9 months of treatment, twelve out of 20 (60 %) of liver tissue samples showed telomerase activity with mean 47.8 % $\pm$ 4.2 has appeared with range 0.00 – 88 %. The 12 months, the relative activity of telomerase was detected in 15/20 (75 %) of the liver tissue samples in the range of 0.00 to 95 % with mean activity 72.1 $\pm$ 3.8. All the samples were analyzed histopathologically (Figure 1), following 3 months of treatment, 33 % of the samples that expressed detectable telomerase activity showed some pathological changes represented by hemorrhage accompanied with necrotic cells. After 6 months of treatment period, partial inflammation was observed in fatty livers, the cells were greatly affected with pycknotic nuclei, congested with inflammatory changes and cellular infiltration has appeared. Following 9 months, 85.7 % of the samples showed some anaplastic changes as binucleation, large nucleus and anaplasia, additionally preservation of hepatic lobular pattern signs of cellular death illustrated in densely stained pycknotic nuclei and hyalinization with less degenerated vacuolated cytoplasm, small hemorrhagic areas. At the end of 12 months of treatment, 93.75 % of the samples showed telomerase activity and a clear evidence of HCC.

Survivin expression

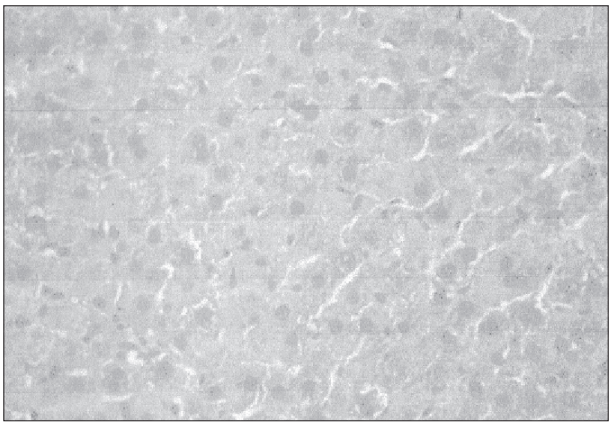
Survivin protein expression was detected in the collected liver tissues by Western blot technique using anti-survivin antibody. No survivin protein expression was detected in the untreated control liver tissues (Figure 2a). In the nitrosamine-treated group, (Table 1) the 3 months period revealed that survivin was detected in 30 % (6/20) of cases (113 % $\pm$ 12). We considered the

Table 1. Telomerase activity and survivin, caspase-3 expressions during hepatocarcinogenesis

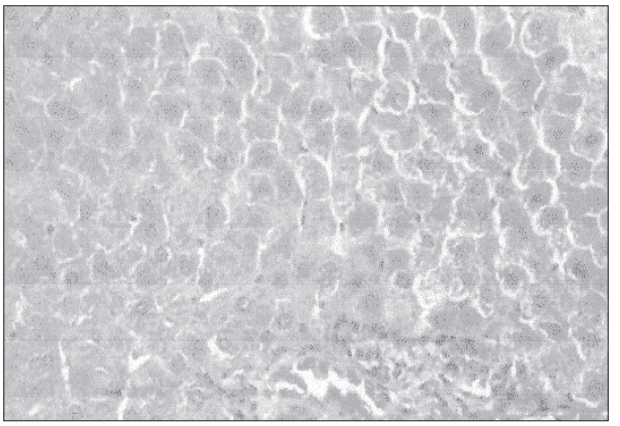
Treatment Period	Telomerase activity (%)	Survivin (%)	Caspase-3 (%)
Untreated Controls	1.6 $\pm$ 0.7	0 $\pm$ 0	100 $\pm$ 0.0
Treatment Groups			
3 months	8.3 $\pm$ 3.5*	113 $\pm$ 12*	87 $\pm$ 12*
6 months	12.0 $\pm$ 4.3*	148 $\pm$ 27*	83 $\pm$ 21*
9 months	47.8 $\pm$ 4.2*	210 $\pm$ 21*	28 $\pm$ 11*
12 months	72.1 $\pm$ 3.8*	419 $\pm$ 32*	15 $\pm$ 3*

The data are expressed as (mean $\pm$ SD)%.

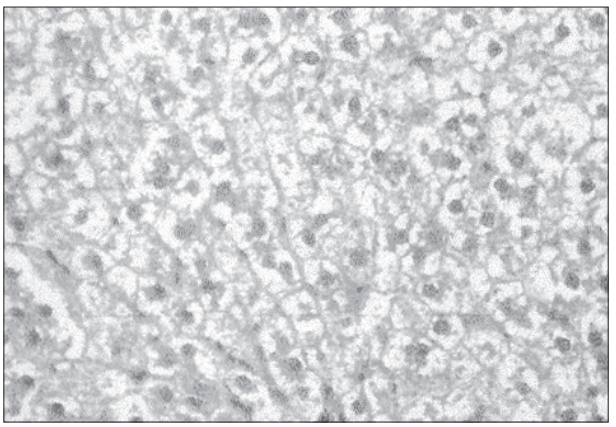
* Significant to the untreated control p-value < 0.001.



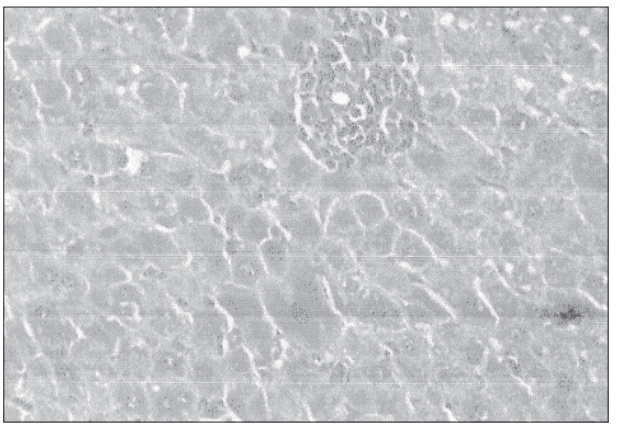
(a)
Control



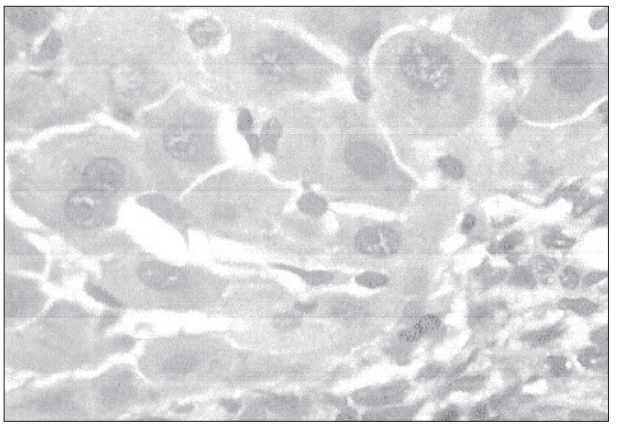
(b)
Hemorrhage accompanied with necrotic cells after 3 months of treatment



(c)
After 6 months, partial inflammation, fatty liver the cells were greatly affected with pycknotic nuclei, congested with inflammatory changes and cellular infiltration



(d)
After 9 months showed some anaplastic changes as binucleation, large nucleus, anaplasia and preservation of hepatic lobular pattern signs of cellular death, small hemorrhagic areas were observed



(e)
After 12 months of treatment, showed evidence of HCC development

Figure 1. Pathological report for each micrograph of liver section of albino rat (H & E Magnification 200)

expression of the minimal signal of survivin as 100 % expression, case # 3 in this group (Figure 2b). Similarly, after 6 months of treatment, 30 % (6/20) of samples showed slightly increased survivin expression (148 % ± 27) (Table 1, Figure 2c). In the 9 months treatment group, 60 % (12/20) of cases showed survivin expression (210 % ± 21) (Table 1, Figure 2d) whereas, 80 % (16/20) of the cases showed mean expression (419 % ± 32) in the 12 months group (Table 1, Figure 2e). These results showed statistically significant increase in the mean expression level of survivin during hepatocarcinogenesis. Fifty percent of the liver samples showed survivin expression after 3 months which associated with some pathological changes from normal livers presented by hemorrhage accompanied with necrotic cells. Similarly, 50 % of samples after 6 months showed survivin expression, also showed some deviations from normal pathological pattern as partial inflammation, fatty liver, the cells were greatly affected by pycknotic nuclei, congested with inflammatory changes and cellular infiltration. After 9 months, 85.7 % of the liver samples which showed survivin expression also showed some premalignant changes represented by binucleation, large nucleus and anaplasia. After 12 months, histopathological analyses showed sever anaplasia, displasia, binucleation, complete destruction of portal tract which surrounded by inflammatory cells (lymphocytes and plasma cells), the blood vessels were enlarged and ill-defined, supporting that HCC was developed (Figure 1).

Caspase-3 expression

Western blot was used to assess the expression of caspase-3 for each treatment group as well as controls, using anti-caspase-3 antibody (Figure 3a). All control livers showed detectable caspase-3 activity. We considered expression of normal controls as 100 %. So, after 3 mon-

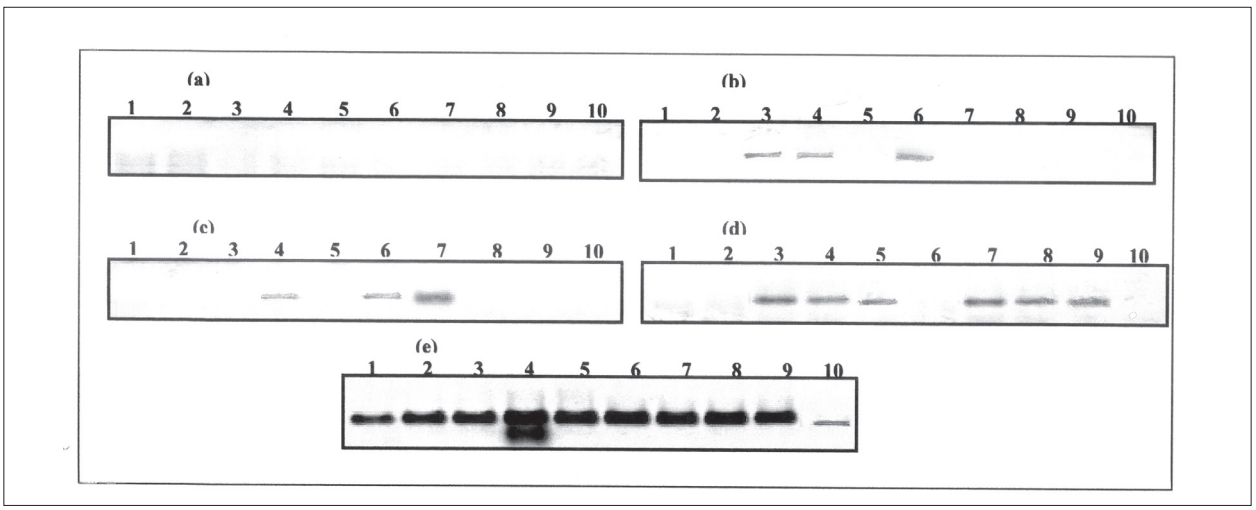


Figure 2. Expression of survivin (MW 16.3 KDa) during hepatocarcinogen administration using Western Blot. a) No survivin was detected in normal liver samples, b) Survivin expression in 10 cases after 3 months; showing 3 cases expressing survivin, signal of survivin for case # 3 (lane 4) was taken as 100 % expression. c) Survivin expression in 10 cases after 6 months. d) Survivin expression in 10 cases after 9 months. e) Survivin expression of 10 cases after 12 months of nitrosamine precursor's administration.

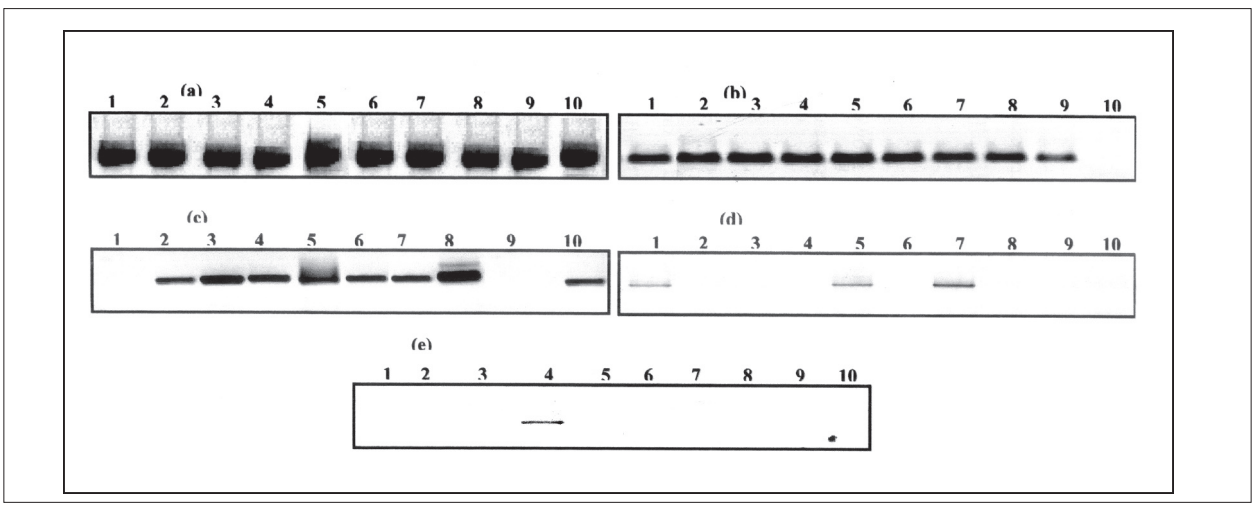


Figure 3. Expression of Caspase-3 (MW 32 KDa) during hepatocarcinogen administration using Western Blot. a) Caspase-3 expression in normal liver samples, b) Caspase-3 expression in 10 cases after 3 months; showing 9 cases expressing caspase-3. c) Caspase-3 expression in 10 cases after 6 months. d) Caspase-3 expression in 10 cases after 9 months. e) Caspase-3 expression of 10 cases after 12 months of nitrosamine precursor's administration.t

ths of the treatment (Figure 3b), 65 % (13/20) of the livers were detectable with mean expression 87 % ± 12. Interestingly, 76.9 % (10/13) of cases showed caspase-3 expression didn't show any survivin expression. After 6 months (Figure 3c), 60 % (12/20) of livers showed caspase-3 expression with a mean expression of 83 % ± 21. However, 66.6 % (8/12) which showed caspase-3 expression didn't show any survivin expression. After 9 months of treatment (Figure 3d), 15 % (3/20) of livers showed caspase-3 expression with significantly decreased mean of expression (28 % ± 11, $p < 0.01$). The detectable three caspase-3 expressions showed no survivin expression. After 12 months, of treatment (Figure 3e), a weak signal of caspase-3 expression was detected in one case with 28 % ± 11. Histopathological examinations were as reported previously in the survivin case.

In general, there is an inverse relationship between survivin and caspase-3 expressions. Expression of survivin showed a negative but not linear correlation with that of caspase-3 ($p = - 0.547$).

DISCUSSION

Nitrosamine precursors were used to initiate hepatocarcinogenesis in rats. In accordance to previous study (14), our results revealed that, regarding the histopathological examination, the anaplastic changes were observed in 70 % of the livers of sacrified rats after 9 months and HCC was developed in 80 % of the samples after 12 months of treatment. These results provide evidence that nitrosamine precursors (sodium nitrate and dibutylamine) can be used as carcinogens since nitrosamine can be formed in vivo from its precursor which induces initiation and progression of the malignant transformation. It can directly react and insert damage on DNA and accelerate the cell cycle entry as reported previously (15). Telomerase has been found to be expressed in most immortal cell lines, but it undetectable in adult normal tissues, so in malignant tumors telomerase is thought to be activated to maintain their immortality. It has been suggested that it is one of the critical steps in malignant transformation and its strong enhancement consider an important part of hepatocarcinogenesis (4). In accordance to Nagao et al. (16), no telomerase activity was found in the liver of normal controls. In the animals treated for 3 and 6 months, very low telomerase activity was detected with some hemorrhage accompanied with necrotic cells and inflammation and these results were consistent with Kyo et al. (17). This may be due to the presence of telomerase positive infiltrating lymphocytes and resident sinusoidal cells in these non-malignant tissues as reported by Youssef et al. (18). Nine months after treatment, telomerase activity was detected in 60 % of the samples with significantly higher activity compared to those treated for 3 and 6 months. This was associated with some anaplastic changes in the liver like hyalinization and pycknotic nuclei. High telomerase activity was detected in HCC specimens developed after 12 months of treatment, suggesting that increase in telomerase activity is highly associated with malignant transformation. This is a universal event in HCC in correlation with previous reports (19,20). On the other hand, telomerase activity was not detectable in one of the HCC samples, which might indicate the presence of a telomerase-independent mechanism for telomere length maintenance in these tumors as explained previously (21). The low telomerase of the early stage of HCC may lead to telomere shortening which limits the proliferative capacity of the cells and restrains the regenerative capacity of organ systems; also induce chromosomal instability and initiation of cancer. Reactivation of telomerase at late stages of HCC (9 and 12 months) is necessary to prevent ongoing telomere shortening, chromosomal instability and genetic disarrangement, and is necessary for tumor cell survival and cancer progression in accordance to Maser et al. (22).

Survivin, inhibitor of apoptosis may prolong cell survival by targeting the caspase-3 (10). It was reported that the over expression of survivin in neoplasm may obliterate apoptosis and allow aberrant progression of transformed cells through mitosis. The disruption of survivin microtubule interaction results in loss of anti-apoptotic function of survivin and increase caspase-3 activity during mitosis (23). The present study revealed that survivin is highly expressed in HCC, while no expression in control livers, which may reflect significant association with anaplastic changes. Low survivin in the early stages of treatment (3 and 6 months) indicating that up regulation of survivin may be an early event, this finding is in consistence with Nadia et al. (24). On the other hand, caspase-3 has been reported to be over expressed in B-cell chronic lymphocytic leukemia (25) and acute myelogenous leukemia (26). The present results revealed that one out of 16 HCC samples expressed low level of caspase-3 compared to its level in normal liver. A significant decrease in caspase-3 expression was observed in the livers after 3, 6, 9 and 12 months of the treatment. This finding supports that down regulation of caspase-3 is a vital step in the process of hepatocarcinogenesis, this is in accordance with that reported by Sun et al. (27). Thus, permanent loss of homeostatic equilibrium between cell proliferation and cell death may be a critical determinant in the transition from the reversible stage of tumor promotion to the irreversible commitment to tumor progression. Once apoptotic competence is lost, the heritable oncogene-activated proliferative advantage effectively precludes recovery of cell number homeostasis and a point of no-return establishes the malignant state (28). In general, our results showed an inverse correlation between survivin and caspase-3 ($p = -0.547$) indicating that survivin expression may inhibit apoptosis via its direct or indirect inhibition of caspase-3 protein expression. The non-linear correlation found here can be explained by the indirect pathway as

survivin is characterized by non-zinc binding domain (known as ring finger), thus survivin cannot bind caspase-3 directly. Sasaki et al. (29) showed that survivin induced caspase-9 deactivation first and then caspase-3 could not be activated at the end of the cascade. The obtained results revealed that normal liver lacked telomerase activity and survivin expression whereas caspase-3 is well expressed. The development of HCC increased the telomerase activity and survivin expression while caspase-3 was decreased.

In conclusion, telomerase might be a potentially prognostic marker for HCC, additionally survivin may be an indicator for the developing and prognosis of HCC. Survivin affects terminal effector of apoptosis (caspase-3) and as it exists in tumor cells; it would be an ideal target of apoptosis-based therapy.

Further studies are needed to investigate the possible mechanism of caspase-3 inhibition by survivin.

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