Molecular and Biochemical Changes of Mice Hepatic Cancer Induced by Diethylnitrosamine and Treated with Curcumin

Metwally K. Mahfouz1, Hosny A. Hassan2, Hussein A. Ali3, Yakout A. El-Senosi3, Mohamed A. Abdelaziz4

1 Department of biochemistry, Animal Health Research Institute, 2 Department of biochemistry, Faculty of Medicine Assiut University, 3 Department of biochemistry, Faculty of Veterinary Medicine, Benha University, 4 Department of biochemistry, faculty of pharmacy, Al-Azhar University (Assiut)

A B S T R A C T

Hepatocellular carcinoma is one of the most common malignancies worldwide. Diethylnitrosamine (DENA) is a well known potent hepatocarcinogenic agent. DENA is known to induce damage in many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models. Curcumin is a potent anti-inflammatory agent with strong therapeutic potential against a variety of cancers. Curcumin has been shown to suppress transformation, proliferation, and metastasis of tumors. These effects are mediated through modulation of various inflammatory and angiogenic cytokines. It also inhibits proliferation of cancer cells by inducing apoptosis through modulation of oxidative stress.

KEY WORDS: Hepatocellular carcinoma, Curcumin, Diethylnitrosamine

1. I N T R O D U C T I O N

Liver cancer is one of the most common malignancies worldwide, especially in Asia and Africa [1]. Hepatocellular carcinoma accounts for about 80%-90% of all liver cancer and is the fourth most common cause of cancer mortality [2]. Major risk factors for liver cancer include hepatitis viral infection, food additives, alcohol, aflatoxins, environmental and industrial toxic chemicals, air and water pollutants [3]. Diethylnitrosamine (DENA) is a well known potent hepatocarcinogenic agent. DENA is known to induce damage in many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models [4]. Although there are many strategies for the treatment of liver cancer [5] the therapeutic outcome of this cancer remains very poor. Therefore, prevention seems to be the best strategy for lowering the incidence of this disease. Recently, considerable research has been carried out in the search for natural or synthetic compounds as a means of chemically preventing liver cancer [6]. In this regard, many compounds have been tested with proved efficacy against experimentally-induced hepatocarcinogenesis. Curcumin is one of such agents; derived from turmeric (Curcumin longa), it has been used for thousands of years in the orient as a healing agent for variety of illnesses [7]. Concerning angiogenesis previous studies cancer and role of curcumin in controlling angiogenesis it was explained [8] by through studying some angiogenic molecules, among these molecules, VEGF and bFGF appear to be the most important for sustaining tumor growth. VEGF and bFGF are produced by many kinds of cancer cells (and by certain types of normal cells) while there is no enough studies concerning TGF-β. The precise mechanism that leads to angiogenesis is not fully understood, but growth factors
that cause proliferation of endothelial cells have been shown to play a critical role in this process. Regarding to the relation between apoptosis and curcumin in cancer liver, Curcumin has been shown to induce apoptosis in a variety of cells, but no comprehensive study focused on angiogenesis and apoptosis-inducing activities of curcumin is available so far. Oxidative stress signals induced by the formation of reactive oxygen species (ROS) and glutathione depletion are also considered as important activators of apoptosis [9]. Being lipophilic, curcumin can collapse transmembrane mitochondrial potential and increase permeability for proton and interfere with energy the coupling system in the mitochondria. Therefore, this work aims to study the inhibiting role of curcumin on mice hepatic cancer induced by DENA. Moreover, study the mechanism of this inhibitory role which may be either through the anti-angiogenic and/or fibrinogen inhibitory action of some inflammatory cytokines or through the apoptotic inducer action of curcumin and finally through modulating the oxidative stress state in the mice liver.

2. MATERIALS AND METHODS

2.1. Chemicals

RNA extraction kit from (BioFlux, Bioer Technology Co., Ltd.). RT using RevertAidTM First strand cDNA synthesis kit (Fermentas). PCR using Tag master/high yield (Jena Bioscience, Germany). DNA ladder using low range DNA ladder 50-1kb linear scale (Jena Bioscience, Germany). Protein marker using page RulerTM prestained protein ladder (Fermentas). PVDF membrane (Amersham HybondTM-P GE Healthcare) was used. All the other chemicals and solvents used in the study were of analytical grade and were obtained either from Sigma Chemical Company or commercial suppliers, unless otherwise mentioned.

2.2. Animals

Thirty Male albino mice with average body weight (30 ± 5g) were obtained from Animal House of Assiut University. The animals were taken as the following: I- DENA group (n = 10): Each animal of the carcinogenic groups received a single dose of DENA (200 mg/kg body weight) intraperitoneal (I.P) and left for 20 weeks (time of sacrificing ). II -Curcumin- treated group (n = 10). Each animal of the curcumin groups was received single dose of DENA (200 mg/kg body weight) I.P then treated orally for 15 days with a dose of 250 µg curcumin /100 g body weight in volume of 0.5 ml saline, curcumin was injected after the appearance of ulcerative skin lesion (after 90 days) and left till the end of the 20 weeks (time of sacrificing ). The time of curcumin administration was adjusted to be 3 hour before sunset. III - Control group (n = 10) Received 0.5 ml saline orally. In all group: the animals were sacrificed after 20 weeks from the beginning of the experiment. Blood samples were collected and livers were excised rapidly and used for RNA preparation or homogenization in 20 mM Tris, 100 mM NaCl, 1mM EDTA and 0.5% Triton X100 buffer. Protein content of liver homogenate was determined using Biuret reagent and bovine serum albumin as standard. The protease inhibitors mix was added, aliquot and stored at -80°C tell use.

2.3. Detection of Caspase-3 by RT-PCR

2.3.1. RNA isolation

Total RNA fractions were prepared using total RNA Kit (Omega Bio-tek).

2.3.2. RT-PCR
This step was done using RT/PCR preMix kit (Bioron Cat No.:122020-96) which consists of premix tubes that contain all the components necessary for cDNA synthesis and amplification in one tube as reverse transcriptase and DNA polymerase enzymes.

2.4. Detection of TGF-β by Western blotting analysis

30µg from each sample homogenate were denatured by boiling for 5min in 2% SDS (Sodium Dodecyl Sulphate) and 5% 2-mercaptoethanol and loaded into separate lanes of a 12% SDS-PAGE gel. The samples were separated electrophoretically at 100 volts for 2 hours. The separated proteins were electrically transferred onto PVDF membrane using T-77 ECL semi-dry transfer unit from Amersham Biosciences for 2 hours. The membrane was blocked in TBS buffer containing 0.05% Tween and 5% non-fat milk for one hour. The membranes were then incubated either with rabbit polyclonal anti rat TGF-β (Abcam) or monoclonal anti Rat (SANTA CRUZ Biotechnology, INC). Polyclonal goat anti-rabbit or anti-mouse immunoglobulin conjugated to alkaline phosphatase (Sigma–Aldrich, Schelldorf, Germany) diluted 1:5000 in the 10x diluted blocking buffer served as secondary antibody. Detection of proteins bands was done by adding alkaline phosphatase buffer (100 mM tris pH 9.5; 100 mM NaCl; 5 mM MgCl2) containing substrate, 6.6 µl NBT/ml and 3.3 µl BCIP/ml from (stock of 50 mg/mL nitroblue tetrazolium NTB and 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate BCIP in 70 % formmamide). Color reactions can be stopped by rinsing with stop buffer (10 mM Tris-Cl, pH 6.0, 5 mM EDTA).

2.5. Biochemical analysis

2.5.1. Determination of nitric oxide

The method used is described by previous study [10]. Tissue levels of NO were determined as total nitrite concentration after reduction of nitrate to nitrite using cadmium and reaction with Griess reagent to give yellow color complex, which measured at 550 nm. Briefly, 260 µL tissue extract were added to 26 µL ZnSO4 (30%, w/v in H2O). Then it was mixed well and incubated for 15 minutes at room temperature and centrifuged at 10000 rpm for 15 minutes, then activated cadmium with 2 % HCl was prepared. 200 µl of deproteinized tissue extract were added to 80 ul of a mixture of [3 parts of NH4Cl 2.6 % w/v in H2O + one part of sodium borate (Borax, 2.1 % w/v in H2O)] and "activated" cadmium. 125 ul of freshly prepared Griess reagent was added to an equal volume of the reduced clear tissue extracts, then the absorbance was measured at 550 nm against reagent blank using (State Fax ELISA reader, Model 2000). Calculations were done using the standard curve.

2.5.2. Determination of thiobarbituric acid reactive substances (TBARS)

The method used is described by Buege [11]. The procedure is based on the reaction of one molecule of malondialdehyde (MDA) with two molecules of thiobarbituric acid (TBA) at low pH (2-3), and a temperature of 95°C for 45 min. Equal volumes (200 µL) of the TBA reagent and samples or standards + 10 µL BHT solution were mixed, incubated at 100 °C for 15 minutes and left to cool. The mixture was Centrifuged 3000 rpm for 10 minutes. Absorbance at 535 nm of the supernatant was recorded against bidistilled water blank. Calculations were done using the standard curve of MDA.

2.5.2. Estimation of anti oxidants
2.5.2.1. determination of serum catalas concentration

50 ml serum incubated with 1 ml substrate at 37°C for 1 min. the substrate solution prepar as 65 mmol per substrat ml H2O2, 60 mmol/l sodium potassium phosphate buffer ph 7.4. The reaction stopped by addition of 1 ml 32.4 mmol /l Molybadat, measure the yellow complex at 405 nm against blank [12]

2.5.2.2. Determination of Serum Glutathione S Tranferase

Reagent
Phosphate buffer, Ph 7.4, Glutathione Redused GSH, Chloro 2.4- dinitrobenzene (CDNB) and Trichloroacetic acid.
Procedure
Buffer (R1): Sample 1.0ml and Blank 1.0ml.
Sample: Sample 0.05ml and Blank 0.05ml.GSH (R2): 0.1ml Incubate at 37c for 5 min. then add CDNB (R3) 0.1ml mix well. Incubate at 37c for exactly 5min. Terminate the reaction by adding: TCA (R4) 0.1 ml sample and 0.1 ml blank, CDNB (R3) 0.1 ml blank mix well, centrifuge at 3000 r.p.m. for 5 min. Measure the absorbance of sample (A sample) against the blank at 340 nm.

2.6. Statistical analysis

Statistical analysis was achieved using Graph Pad In Stat. software Inc, Program, version 4.0 Philadelphia, Data were presented as mean ± SD and the levels of significance were accepted with p <0.05. Multiple comparisons were done using one way ANOVA followed by Tukey-Kramer test as multiple comparison post ANOVA test.

3. RESULTS

3.1. TGF-β by We stern blotting analysis

Figure (1) show TGF-β expression of rat hepatocellular carcinoma homogenate from control, DENA and curcumin groups. Rabbit polyclonal antibodies for TGF-β were used in 1:200 dilutions Polyclonal goat anti-rabbit or anti-mouse immunoglobulin conjugated to alkaline phosphatase (Sigma–Aldrich, Schelldorf, Germany) diluted 1:5000 in the 10x diluted blocking buffer served as secondary antibody.

3.2. Detection of Caspase-3 by RT-PCR

Figure (2) shows the expression of Caspase-3 in the all of experimental groups (control, DENA and curcumin treated group). As appears in this figure Caspase-3 decreased in DENA group then increased in curcumin treated group.

3.3. Histopathological study

To assess the changes in parenchymal cells of the liver after DENA administration and Curcumain treatment, HE stained slides were examined 16 weeks after DENA administration, as shown in figure (7). Administration of DENA
induced morphological deformations in the liver pronounced with chronic hepatitis with hydropic degeneration and macroglobular nodules. These nodules show focal prominence of bile ductular proliferation and after Curcumain administration treatment the diseased liver had some improvements in its histological structure which Included epithelium of central vein become intact (arrow head), architecture of hepatocyte arrangement become normal (arrow) in some parts of diseased area and hydropic degeneration decreased.

**Fig.3 Control group Normal histology of liver with polygonal hepatocytes with prominent nucleus and maintained sinusoidal space.**

**Fig.4 DENA group shows morphological deformations in the liver included dissociation of hepatic cord, dilated bile duct (arrow) with proliferation of its epithelium, hydropic degeneration of hepatocytes with intracytoplasmic inclusion and dilated central venu with degenerated epithelium lined it (arrow head).**

**Fig.5 Curcumin Treated group after curcumin treatment the diseased liver had some improvements in its histological structure which Included epithelium of central vein become intact (arrow head), architecture of hepatocyte arrangement become normal (arrow) in some parts of diseased area and hydropic degeneration decreased. (H&E, 400×).**

4. DISCUSSION

HCC is the main form of liver cancer, Apart from chronic infections with hepatitis B and Hepatitis C viruses, which are the main causes of HCC, contamination of foodstuff with aflatoxins [1]. HCC in mice model was best studied using DENA, which is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication and is normally used as a carcinogen to induce liver cancer in animal models [4]. DENA has been shown to be metabolized to its active ethyl radical metabolite and the reactive product interacts with DNA causing mutation, which would lead to carcinogenesis [13]. Curcumin is one such agent; derived from turmeric (Curcumin longa), it has been used for thousands of years in the orient as a healing agent for variety of illnesses. Research over the last few decades has shown that curcumin is a potent therapeutic potential against a variety of cancers through suppressing the activation of several transcription factors that are implicated in carcinogenesis [14].

In the current study we focus on studying the possible mechanisms of this anticancer effect of curcumin, from these mechanisms; modulation of angiogenesis...
and fibrogenic effect of TGF-β, apoptotic effect of caspase-3 and modulation of oxidative stress. Regarding to the study of the regulation of the angiogenic effect of some inflammatory cytokines, tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into a cancerous growth, supplying nutrients and oxygen and removing waste products. For most solid tumors, angiogenesis is essential for tumor growth and metastasis [15]. In the current study, mice hepatic cell homogenate showed over expression of TGF-β protein in the DENA animal group, this expression was significantly higher than that of the healthy group. The expression of TGF-β in curcumin treated group was significantly reduced when compared with that of DENA group.

The increase of TGF-β and its release in the liver cancer mice was agreed with a study made by Bertran [16], they explained that tumor angiogenesis actually starts with cancerous tumor cells releasing molecules that send signals to surrounding normal host tissue. In another study made by Mazzocca [17] they explained that VEGF is a strong angiogenic factor in HCC and is mainly responsible for neovascularization in HCC. Another role of TGF-β in HCC was explained by Giannelli [18] through its profibrinogenic effect, as it initiates a signaling cascade which is closely linked to liver fibrosis, cirrhosis and subsequent progression to HCC. Because of its role in these stages of disease progression, TGF-β appears to play a unique role in the molecular pathogenesis of HCC. Thus, it is a promising target for pharmacological treatment strategies.

Curcumin has been shown to suppress the proliferation of human vascular endothelial cells in vitro [19] and to abrogate the FGF-2-induced angiogenic response in vivo, [20] suggesting that curcumin is also an antiangiogenic factor, not only it was effective in preventing and reversing cirrhosis but also probably by its ability of reducing TGF-β expression [21]. Also explained the effect of curcumin on TGF-β through its antifibrotic and fibrolitic action.

In the current study, the expression of TGF-β in curcumin treated group was significantly reduced when compared with that of DENA group. Curcumin’s antiangiogenic property was explained in [22] they explained this role due in part to its inhibitory action on the serine proteinase family; urokinase plasminogen activator system (uPA). uPA interacts with a specific receptor via the EGF-like domain in the urokinase amino-terminal fragment. Its angiogenic effect is due to its effect on the migration of endothelial cells and through activation and/or release of several angiogenic factors, such as FGF, TGF, TNF, HGF, and VEGF.

Another explanation for the antiangiogenic action of curcumin was explained in [23] and [24], they explained that curcumin inhibits Gelatinase A (MMP-2) and gelatinase B (MMP-9), which are implicated in the formation of loose and primitive-looking meshwork formed by aggressive cancers such as melanoma and prostate cancers. MMP-2 and MMP-9 are metalloproteinases that cause the formation of new capillaries by activating growth factors, and curcumin has been shown to inhibit the gelatinolytic activities of MMP and to suppress expression and transcription of MMP, indicating its inhibitory effects at both the transcriptional and posttranscriptional levels.

Apoptosis helps to establish a natural balance between cell death and cell renewal in mature animals by destroying excess, damaged, or abnormal cells [25]. Apoptotic signalling within the cell is transduced mainly via two molecular pathways: the death receptor pathway (also called the extrinsic pathway) and the mitochondrial pathway (also called the intrinsic pathway). Both of them activate a variety of proteases, mainly the group of proteases called caspases (cysteiny
aspartate-specific proteases), and endonucleases, which finally degrade cellular components [26]. Caspases are constitutively expressed as inactive proenzymes, generally require proteolytic processing for their activation and are capable of self-activation as well as activating each other in a cascade-like process[27].

In the current study mRNA expression of caspase-3 through PCR technique was significantly higher in curcumin treated group than that of DENA group, these results were in agree with a study in [28], they explained the mechanism through the decreases of the expression of antiapoptotic members of the Bcl-2 family and elevates the expression of p53, Bax, and procaspases-3, -8, and -9.

The link between TGF-β and apoptosis in HCC was studied in [28] as they found disruption of the TGF-β pathway occurs in HCC and might cause dysregulation of apoptosis; as the tightly controlled homeostatic mechanisms between cell growth and apoptosis that exist in normal liver tissue are disrupted during hepatocarcinogenesis. TGF -β signaling system is a central component of the mechanisms by which cell growth and apoptosis are controlled in the liver. The recent delineation of the TGF-β signaling pathway has provided a unique framework for analysis of the impact that disruption of individual components of this signaling pathway can have on apoptosis during hepatocarcinogenesis. Regarding to the oxidative stress status, in the current study tissue levels of both nitric oxide and lipid peroxide were significantly increased in DENA group (p<0.05 and p<0.001) when compared to the control group and also in curcumin treated group reduced (p<0.05 and p<0.001 respectively) in curcumin treated group when compared to control group whereas serum levels of both catalase and glutathione-s-transferase were significantly decreased in DENA group (p<0.05 and p<0.001 respectively) whereas the levels were significantly increased (p<0.05 and p<0.001 respectively) in curcumin treated group.

These results were in agree with a study in [29]and [30]. Curcumin up-regulates enzymes such as catalase, glutathione transferase, glutathione peroxidase, and superoxide dismutase, and their mRNAs. It has been reported to scavenge free radicals, increase antioxidant status, inhibit lipid peroxidation, and elevate levels of glutathione and sulfhydryl groups.

The link between curcumin and apoptosis in relation to oxidative stress was explained by a study in [31]. They clarified that curcumin induces reactive ROS-mediated depletion of GSH, which leads to caspase-dependent and independent apoptosis in mouse fibroblast cells. Curcumin suppresses the growth of human leukemic cells via ROS-independent GSH depletion, which leads to caspase activation, inhibition of sphingomyelin synthase (SMS) activity, and induction of ceramide (Cer) generation.

5. CONCLUSION

Our investigation reported the biochemical changes associated with the administration of curcumin in liver cancer induced by DENA. It demonstrated its ability to (a) minimize the frequency of DENA-induced carcinomas (b) decrease the expression of some angiogenic cytokines mainly TGF-β (c) increase mRNA expression of some apoptotic markers mainly caspase-3 (d) Decrease level of detrimental oxidative stress. These findings suggest possible therapeutic implications in hepatic carcinogenesis and a promising target for pharmacological treatment strategies.

6. ACKNOWLEDGMENTS

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Mahfouz et al. (2014)

Table (1) mean values of biochemical

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<th>Control</th>
<th>DENA</th>
<th>Curcumin</th>
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<tr>
<td>Tissue Lipid Peroxide</td>
<td>7.4270 ± 0.62</td>
<td>38.70± (p&lt;0.001)**</td>
<td>20.440±5.51 (p&lt;0.05)*</td>
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<tr>
<td>(n mol/ml)</td>
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<tr>
<td>Tissue Nitric oxide</td>
<td>3.6730 ± 0.6798</td>
<td>15.960 ± 7.722 ± 1.364 (p&lt;0.05)*</td>
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<tr>
<td>(n mol/ml)</td>
<td></td>
<td>2.037(p&lt;0.001)*</td>
<td></td>
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<tr>
<td>Serum Glutathione</td>
<td>408.4± 56.01</td>
<td>270.8 ± 142.2 ± 33.16 (p&lt;0.001)**</td>
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<tr>
<td>-S- transferase (U/L)</td>
<td></td>
<td>25.18(p&lt;0.001)**</td>
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<tr>
<td>Serum Catalase</td>
<td>42.89± 5.812</td>
<td>16.42 ± 2.89 24.57 ± 5.033 (p&lt;0.001)**</td>
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<td>(Ku/l)</td>
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7. REFERENCES


Effect of Curcumin on hepatic cancer


التغير في دلالات البيولوجيا الجزئية والبيوكيميائية لسرطان الكبد المحدث تجريبياً في الفئران بواسطة داية

أيثيل نيتروز أمين والمعالج بالكركم

متوالي قطب محفوظ 1 حسن علي حسن 2 حسن عبد المقصود علي 3 باقوت عبد الفتاح السنوسي 4 محمد احمد عبد العزيز 4 معهد بحوث صحة الحيوان، 2 قسم الكيمياء الحيوية -كلية الطب -جامعة إسماعيل، 3 قسم الكيمياء الحيوية كلية الطب البيطري - جامعة بها، 4 قسم الكيمياء الحيوية الطبية كلية الصيدلة - جامعة الأزهر

المتخصص العربي

يعتبر سرطان الكبد واحد من الأورام السرطانية الأكثر انتشاراً في العالم. لذلك تم دراسة تأثير الكركم على سرطان الكبد في الجرائز من خلال دراسة الميكانيكية التي تعمل بها هذه المركيبات في حالة ثبوت فاعليتها كمضادات لسرطان الكبد وتتلخص هذه الميكانيكية اما في تقليل تكوين اوعية دموية جديدة في خلايا الكبد المصابة أو في زيادة الاندماج الخلوي الذاتي أو في نقصان الشوارد الطلبلية. لذا فقد تم عمل هذه الدراسة على مجموعة من جرائز التجربة لدراسة تأثير الكركم على سرطان الكبد وذلك بعد حقن الجرائز بالمادة السرطانية (داية أيثيل نيتروز أمين ) جرعة واحدة في البطن ثم بعد مرور تسعة أيام من الحقن تم تمت معالجة الجرائز بالكركم لمدة أسبوعين. ثم بعد ذلك دراسة مستوى تعبير بروتين عامل النمو الظهاري الذي يعتبر أحد عوامل بناء اوعية دموية جديدة في خلايا الكبد المصابة بالسرطان حيث وجد نقص ذو دلالات جوهرية في مجموعة الكركم مقارنة بالمجموعة السرطانية. كذلك تم دراسة مستوى تعبير الحمض النووي الرسول للكسب -3 في الأنسجة التي يعتبر واحد من دلالات الاندماج الخلوي الذاتي حيث وجدت زيادة جوهرية في مستوى في مجموعة الكركم مقارنة بالمجموعة السرطانية. وتم أيضاً دراسة مستوى الشوارد الطلبلية (فوق أكسيد الدهون وأكسيد النيترات) حيث وجد نفس ذو دلالات جوهرية في مجموعة الكركم مقارنة بالمجموعة السرطانية. مما يفسر عمل الكركم كمواد مضادة للأورام من الممكن ان يكون نتيجة اتفاقهم بمساعدته على زيادة الاندماج الخلوي الذاتي وتقليل بناء اوعية دموية جديدة وتقليل مستوى الشوارد الطلبلية (فوق أكسيد الدهون وأكسيد النيترات) التي لا تعتبر تأثير مباشر في حدوث السرطان.

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