RESEARCH ARTICLE

Plasma Nuclear Factor Kappa B and Serum Peroxiredoxin 3 in Early Diagnosis of Hepatocellular Carcinoma

Saber Ismail[1], Wael Mayah[1,5]*, Hassan El Battia[4], Hanaa Gaballah[2], Asif Jiman-Fatani[6], Hala Hamouda[2], Mohamed A Afifi[6], Nehal Elmashad[3], Sherif El Saadany[1,6]

Abstract

<u>Background</u>: **Early diagnosis of hepatocellular carcinoma (HCC) is the most important step in successful treatment. However, it is usually rare due to the lack of a highly sensitive specific biomarker so that the HCC is usually fatal within few months after diagnosis. The aim of this work was to study the role of plasma nuclear factor kappa B (NF-κB) and serum peroxiredoxin 3 (PRDX3) as diagnostic biomarkers for early detection of HCC in a high-risk population. <u>Materials and Methods</u>: Plasma nuclear factor kappa B level (NF-κB) and serum peroxiredoxin 3 (PRDX3) levels were measured using enzyme linked immunosorbent assay (ELISA), in addition to alpha-fetoprotein (AFP) in 72 cirrhotic patients, 64 patients with HCC and 29 healthy controls. <u>Results</u>: NF-κB and PRDX3 were significantly elevated in the HCC group in relation to the others. Higher area under curve (AUC) of 0.854 (for PRDX3) and 0.825 (for NF-κB) with sensitivity of 86.3% and 84.4% and specificity of 75.8% and 75.4% respectively, were found compared to AUC of alpha-fetoprotein (AFP) (0.65) with sensitivity of 72.4% and specificity of 64.3%. <u>Conclusions</u>: NF-κB and PRDX3 may serve as early and sensitive biomarkers for early detection of HCC facilitating improved management. The role of nuclear factor kappa B (NF-κB) as a target for treatment of liver fibrosis and HCC must be widely evaluated.**

Keywords: Nuclear factor kappa B - peroxiredoxin 3 - hepatocellular carcinoma - early diagnosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer, thirdly leading cancer-related death worldwide (Iakova et al., 2011; Zhang et al., 2014). Chronic HBV and HCV are the major etiological factors of the disease (Cabibbo and Craxi, 2010). The mortality rates of patients with HCC remain very high (up to 94%), making HCC the third common etiology of cancer-related death. Late detection in addition to lack of effective therapies for advanced stage of the disease may be the cause of such a high mortality rate (Bosch et al., 2004).

Currently, abdominal ultrasound and serum alpha-fetoprotein (AFP) are the most widely used methods for HCC detection. However, this does not reliably detect disease in early stage (Yamamoto et al., 2010). This could be due to the facts that ultrasonography is highly dependent on the experience of its operator (Poon et al., 2009) while elevated serum AFP level is the result of altered hepatocyte-hepatocyte interface associated with loss of normal architectural arrangement rather than necrosis or active regeneration (Tyson et al., 2011).

Triphasic CT with contrast is believed to be the optimal imaging technique for the detection and staging of HCC (Yu et al., 2004). The diagnostic efficacy of CT is markedly diminished in small tumors (less than 2 cm) due to the hypo-vascularization of these small sized tumors (Gomaa et al., 2009).

Therefore, it is critical to identify and characterize sensitive and specific diagnostic biomarkers for early detection and treatment of HCC in high-risk populations (Altekruse et al., 2009; Huang et al., 2010). Recently, alternative serum biomarkers are being actively sought and proposed candidates that include nuclear factor kappa B (NF-κB) which plays a crucial role in bridging the action of growth factors and inflammation to hepatic oncogenesis (Beale et al., 2008). Activation of transcription factor NF-κB was reported as one of the early key events involved neoplastic progression of the liver (Arsura and Cavin, 2005).

Nuclear factor kappa B (NF-κB) was originally identified as a nuclear factor specific to B cells bound to the B site of the k-light chain gene enhancer. However NF-κB was later found to be expressed in other cell types

[1]Departments of Tropical Medicine & Infectious Diseases, [2]Medical Biochemistry, [3]Clinical Oncology, Faculty of Medicine Tanta University, Tanta, [4]Department of Tropical Medicine & infectious Diseases, Faculty of Medicine, Kafrelsheikh University, Kafr Elsheikh, Egypt, [5]Faculty of Dentistry, [6]Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia *For correspondence: waelmayah@gmail.com

such as the liver epithelium, where it regulates hepatic cell proliferation and survival during regeneration and development. NF-κB controls the expression genes involved in the regulation of fundamental processes such as the immune response, cell adhesion, physical stress (e.g. liver regeneration), oxidative stress and cell survival (Hung et al., 2008).

Viral hepatitis causes a general inflammatory response in the liver, which is often followed by cirrhosis and HCC development. NF-κB seems to be crucially involved both in fibrogenesis and in the initiation and promotion of HCC in the chronically inflamed liver. Overall, activation of the NF-κB signaling pathway by the action of proinflammatory mediators such as TNF-α, chemokines, interleukins, and structural and non-structural viral proteins likely promotes survival of precancerous hepatocytes, thereby favoring their conversion to a malignant phenotype through subsequent genetic lesions (Bouchard et al., 2011).

The interplay between NF-κB and growth factors implicated in invasion and metastasis has raised the hypothesis that NF-κB might also play a role in the promotion of hepatic metastasis (Nugler et al., 2008).

Majority of studies have demonstrated that the progression of HCC is closely correlated to oxidative stress (Jo et al., 2011). Cancer cells usually exhibit changes in key mitochondrial regulators of cell death or mitochondrial structure and function which are different from those of normal cells. This enables them to use antioxidant systems to maintain the redox balance under high levels of oxidative stress (Trachootham et al., 2009).

Classical antioxidant genes related to cancer include superoxide dismutase (SOD), catalase, glutathione peroxidase, peroxiredoxins (PRDXs), and thioredoxin (Trx) (Basu et al., 2011; Marra et al., 2011). Peroxiredoxin 3 (PRDX3) was identified as a member of the peroxiredoxin protein family (Prx), which acts as a cellular defense system against oxidative stress and play important roles in peroxide detoxification, and belong to a family of thiol-specific antioxidant enzymes catalyzing the reduction of hydrogen peroxide (H_2O_2) in the presence of thioredoxin. Its catalytic activity and protein sequences are different from those of other antioxidants (Dietz et al., 2002). Overexpression of PRDX3 has been reported in mesothelioma, HCC, breast cancer, and ovarian cancer, suggesting that it may be involved in tumorgenesis and cancer progression (Huh et al., 2012).

The aim of the work is to study the role of plasma nuclear factor kappa B (NF-κB) and serum peroxiredoxin 3 (PRDX3) as sensitive and specific diagnostic biomarkers for early detection and treatment of early HCC in high-risk populations.

Materials and Methods

This study was carried out on 136 patients presenting to Tropical Medicine Department or its outpatient clinics in Tanta University Hospital, after ethical committee approval. In addition 29 healthy subjects represented the control group. They were 97 males and 68 females, their ages ranged between 33-65 years. They were classified as follows: Group I (control): included 29 healthy individuals. They were 16 males and 13 females. Their ages ranged between 33-48 years; Group II (liver cirrhosis): included 72 patients with liver cirrhosis. Diagnosis of liver cirrhosis was based on clinical picture, biochemical, ultrasonographic criteria, and pathological data, without any evidence of hepatic focal lesions. They were 43 males and 29 females. Their ages ranged between 46 -65 years; Group III (HCC): included 64 patients with HCC diagnosed according to AASLD practice guidelines for HCC diagnosis (Bruix and Sherman, 2005). They were 38 males and 26 females. Their ages ranged between 42 -63 years.

Exclusion criteria

Patients who refuse to sign consent, patients with previous treatment of HCC, presence of extra-hepatic metastasis, patients with other causes of chronic liver disease (e.g. Hemochromatosis, Wilson's disease autoimmune hepatitis), patients with metastatic liver disease, patients with sepsis, acute infections, rheumatoid arthritis, systemic lupus erythematous, chronic obstructive pulmonary disease, pulmonary tuberculosis and Cushing syndrome.

All patient and control groups were subjected to the following: i) Thorough history taking; ii) Clinical examination: with special stress on signs of liver cell failure, hepatomegaly, splenomegaly and/or ascites; iii) Routine laboratory investigations, including complete blood picture, blood urea and serum creatinine; iv) Detection of hepatitis C virus by 3rd generation ELISA and Polymerase Chain Reaction (PCR) for HCV RNA (Wilkins et al., 2010); v) Detection of hepatitis B virus by HBV surface antigen (Chan et al., 2002); vi) Liver function tests including total and direct serum bilirubin, serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum albumin and prothrombin time and activity; vii) Estimation of alpha fetoprotein level (AFP) (Macias-Rodriguez et al., 2000); viii) Abdominal Imaging: Abdominal ultrasonography was done for all patients & controls, while Triphasic CT scans were done for patients groups (GII & GIII); ix) Blood sampling: Peripheral venous blood samples were taken from all patients and controls under complete aseptic condition after overnight fasting. The blood sample of each subject was divided into two halved; the 1st half was collected into the available EDTA-treated tubes; the tubes were centrifuged for 20 minutes at 1000×g to obtain plasma (for plasma NF-κB level measurement). The other half was collected into sterile tubes without additive and centrifuged at 3500×g for 5 minutes at room temperature. Supernatants were transferred into Eppendorf tubes and centrifuged at 12000×g for 10 min to remove cellular fractions to obtain serum (for serum PRDX3 measurement). All obtained plasma and serum samples were aliquoted, frozen and stored at (-80°C) till the time of biochemical examination.

Measurement of plasma nuclear factor Kappa B level

NF-κB level was estimated by ELISA kit supplied by Unique product Superb quality Client favorite Nicest service (USCN) made in USA, purchased from new test

company. The microtiter plate provided in this kit has been pre-coated with an antibody specific to nuclear factor Kappa B (NF- KB). Standards or samples are then added to the appropriate micro titer plate wells with a biotin-conjugated antibody preparation specific for NF-κB. Next, Avidin conjugated to horseradish peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain NF-κB, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of NF-κB in the samples is then determined by comparing the optical density (O. D.) of the samples to the standard curve (Punsawad et al, 2012).

Measurement of serum peroxiredoxin 3 (PRDX3)

PRDX3 levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (BioVender research and Diagnostic Products, USA, Canada and Mexico (Nonn et al, 2003).

Assay Procedure

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use. All standards, controls and samples should be run in duplicate for confirmation of reproducibility. Determine the number of 16-well strips needed for assay. Insert these in the frame(s) for current use. Add 300μl of Incubation buffer

to all wells and incubate the plate for 5 minutes at room temperature. Thoroughly aspirate the solution from the wells. Wash wells 2 times. For the standard curve, add 100μl of the standard to the appropriate microtiter wells. Add 100μl of the Standard/Sample Dilution Buffer to zero wells. Serum requires at least 30 fold dilution in the Standard/Sample Dilution Buffer. And add 100 μl of diluted samples to each well. Cover the plate with the plate cover and incubate for 2h at room temperature. Thoroughly aspirate from the wells. Wash the wells 3 times. Pipette 100μl of "Working Secondary Antibody Solution" into each well. Cover the plate with the plate cover and incubate for 1h at room temperature. Thoroughly aspirate from the wells. Wash the wells 3 times. Add 100μl "Working AV-HRP Solution" to each well. Cover the plate with the plate cover and incubate for 30 minutes at room temperature. Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times. Pour enough Substrate you need into a tube or reagent boat. Add 100μl of Substrate to each well. The liquid in the wells should begin to turn blue. Incubate the plate at room temperature. Avoid exposing the microtiter plate to direct sunlight. Do not cover the plate with aluminum foil (or other metal), or color may develop. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. O.D. values should be monitored and the substrate reaction stopped before O.D. of the positive wells exceeds the limits of the instrument. O.D. values at 450nm can only be read after the Stop Solution has been added to each well. Keep the plate away from sun light

Table 1. Comparison of Liver Function Tests, Alpha-fetoprotein (AFP), Nuclear Factor Kappa B (NF-κB) and Peroxiredoxin 3 (PRDX3) in the Studied Groups

	GI (control)	GII (liver cirrhosis)	GIII (HCC group)	Tukey's test
Total bilirubin (mg/dl)	0.53 ± 0.23	2.46 ± 1.91	1.96 ± 0.63	p1<0.001* p2=0.002* p3=0.538
Direct bilirubin (mg/dl)	0.15 ± 0.06	0.91 ± 0.73	0.91 ± 0.43	p1<0.001* p2<0.001* p3=1
ALT (I.U/L)	22.27 ± 8.76	34.13 ±16.92	37.87 ± 20.71	p1=0.122 p2=0.023* p3=0.893
AST (I.U/L)	24.93 ± 7	54.9 ± 21.71	59.4 ± 47.45	p1=0.016* p2=0.004* p3=0.96
Albumin (g/dl)	4.05 ± 0.26	2.65 ± 0.32	2.49 ± 0.25	p1<0.001* p2<0.001* p3=0.52
Prothrombin activity (%)	95.8 ± 4	70.4 ± 14	64.7 ± 6.1	p1<0.001* p2<0.001* p3=0.345
Alpha-fetoprotein (AFP) (ng/mL)	6.3 ± 1.2	10.55±5.7	993.8 ± 965.58	p1=0.99 p2<0.001* p3<0.001*
Nuclear factor kappa B (NF-κB) (ng/mL)	10 ± 1.54	145.8 ± 53.5	255.5 ± 54	p1<0.001* p2<0.001* p3<0.001*
Peroxiredoxin 3 (PRDX3) (ng/mL)	52.3 ± 26.8	132.3 ± 96.5	304.3 ± 204.48	p1=0.026* p2<0.001* p3<0.001*

*Significant values (p<0.05); P1: GI *vs* GII; P2: GI *vs* GIII; P3: GII *vs* GIII

because the Substrate is light sensitive. Typically, reaction is stopped 5~10 minutes after treatment of Substrate, but this time can be adjusted as the user desires. Add 100μl of Stop Solution to each well. The solution in the wells should change from blue to yellow. Read the absorbance of each well at 450 nm. Read the plate within 20 minutes of adding the Stop Solution (Nonn et al, 2003).

Statistical analysis

Statistical analysis was performed using Sigma Stat software version 2. The results were expressed by mean±standard deviation (SD) the comparison was made by one way ANOVA on rank test (Tukey's test). p value less than 0.05 was considered statistically significant. The diagnostic accuracy of each of the candidate biomarkers was evaluated using receiver operating characteristic (ROC) curve analysis, reporting the area under the curve (AUC) and its 95% confidence interval (CI). The diagnostic cut-off values and the related sensitivity and specificity were determined.

Results

Table (1) showed comparison of liver function tests, alpha-fetoprotein (AFP), plasma nuclear factor kappa B (NF-κB) and serum peroxiredoxin 3 (PRDX3) in the studied groups. Significant differences were observed between the diseased groups (II &III) and the control group (GI) as regards liver function tests (p<0.05) except for ALT levels between the control (GI) and the cirrhotic liver group (GII) (p>0.05).

Significant differences were observed between all studied groups regarding measured NF-κB and PRDX3 (p<0.05).

Serum alpha-fetoprotein levels showed significant differences between HCC group (GIII) in comparison to the control group (GI) and the cirrhotic liver group (GII) (p<0.05) and a non-significant difference was detected between the control group (GI) and the cirrhotic liver group (GII) (p>0.05).

Receiver operating characteristic (ROC) curve analyses suggested that serum PRDX3 and plasma NF-κB expression are useful biomarkers for detecting patients with HCC. Both serum PRDX3 and plasma NF-κB showed a higher area under curve (AUC) of 0.854 (95% CI for PRDX3 and 0.825(95% CI) for NF-κB. At a cutoff value of 156.3 ng/mL, PRDX3 sensitivity and specificity was 86.3% and 75.8% respectively. While, NF-κB at a cutoff value of 193.24 ng/mL showed sensitivity and specificity of 72.4% and 75.4% respectively. On the other hand AFP showed AUC of (0.65) with sensitivity of 72.4% and specificity of 64.3% (Table 2).

Table (3) showed that both NF-κB and PRDX3 were positively correlated with each other and with the size of HCC mass. Moreover, a significantly positive correlation was reported between AFP and NF-κB but not between AFP and PRDX3 or the size of mass.

Discussion

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer, thirdly leading cancer-related death worldwide (Iakova et al., 2011; Zhang et al., 2014). HCC develops on a background of chronic liver disease, such as chronic inflammation or cirrhosis (Zekri et al., 2013). The incidence and mortality rates for HCC are nearly identical, indicating the overall poor survival of patients with this tumor. Therefore, the most effective treatment relies on the early diagnosis of hepatocellular carcinoma (Bruix and Sherman, 2005). The early detection of HCC is usually rare making it fatal within few months after diagnosis (Thomas and Zhu, 2005). Most imaging techniques help to discover HCC after considerable time of onset. In most instances, oncologists depend on AFP as the commonest and feasible marker for assessing HCC in addition to imaging. However, AFP is not completely reliable marker in early HCC diagnosis, prevention or therapy due to its low specificity and sensitivity (Teofanescu et al., 2010; youns et al., 2013). Liver biopsy is always considered as an invasive procedure, so biochemical findings are still greatly appreciated (Makuuchi et al., 2008).

The ideal hepatic tumor biomarkers should possess high specificity and sensitivity, should be easily accessible, easily measurable, minimally invasive, inexpensive, accurate, and acceptable to patients and physicians (Mendy and Walton, 2009).

In the current work, we tried to detect the clinical value of both plasma nuclear factor kappa B (NF-κB) and serum peroxiredoxin 3 (PRDX3) and their role as valuable biomarkers for early HCC detection. Peroxiredoxin 3 (PRDX3) is a c-Myc target gene that is required for mitochondrial homeostasis and neoplastic transformation and predominantly localizes in the mitochondria (Wonsey et al., 2002). Moreover, PRDX3 might serve as a first-line of host defense against reactive oxygen species and in protecting cells from H_2O_2-induced apoptosis (Rabilloud et al., 2002).

We found AFP significantly elevated in the HCC group in relation to the other two groups (p<0.05) without any significant difference between the group of liver cirrhosis and healthy control (p>0.05). This is in agreement with Nagaoka et al. (2003) who found that the mean plasma concentration of alpha fetoprotein was significantly higher in patients with hepatocellular carcinoma as compared to

Table 2. ROC Curve Analysis, Sensitivity and Specificity of Peroxiredoxin 3, NF-κB and AFP

	AUC	Sensitivity (%)	Specificity (%)
PRDX3 (ng/mL)	0.854	86.3	75.8
NF-κB(ng/mL)	0.825	84.4	75.4
AFP (ng/mL)	0.65	72.4	64.3

Table 3. Correlation between the Studied Parameters

	PRDX3 (ng/mL)	AFP (ng/mL)	Size (cm²)
NF-κB (ng/mL)	r=0.257 P=0.04*	r=0.353 P=0.004*	r=0.376 P=0.002*
PRDX3 (ng/mL)	-	r =0.058 P=0.64	r=0.35 P=0.0045*
AFP (ng/mL)	-	-	r=0.181 P=0.15

*Significant values (p<0.05); r=Correlation coefficient

patients with chronic liver disease.

However, 18 out of 64 HCC patients (28.1%) showed AFP value less than or equal to 400 ng/dl which strengthen that, AFP is not completely reliable marker in early HCC diagnosis, prevention or therapy due to its low specificity and sensitivity (Teofanescu et al., 2010; youns et al., 2013).

In our study we tried to evaluate the value of nuclear factor kappa B (NF-κB) and peroxiredoxins (PRDX3) as sensitive biomarkers for early and reliable detection of HCC. We found both NF-κB and PRDX3 to be significantly elevated in the HCC groups in relation to the other groups. Moreover, serum PRDX3 and plasma NF-κB showed a higher AUC of 0.854 (for PRDX3) and 0.825 (for NF-κB) with sensitivity of 86.3% and 84.4% and specificity of 75.8% and 75.4% respectively compared to AUC of AFP (0.65) with sensitivity of 72.4% and specificity of 64.3%.

These results are in agreement with Arsura and Cavin (2005) who reported an increased NF-κB level in HCC patients which may be due to its constitutive activation of the early key events involved in neoplastic progression of the liver. It has been revealed that a number of pro-inflammatory mediators such as TNF-α, chemokines, interleukins contribute in the progression of chronic liver disease, many of which are either targets or activators of nuclear factor-KB (NF-κB) (Bonacchi et al., 2003; Seki et al, 2007; Dominguez, et al, 2009; Guo, et al., 2009 and Miura, et al, 2010).

Wu et al. (2009) observed that hepatic NF-κB mRNA was aberrantly overexpressed during the course of rat hepatocarcinogenesis. Moreover, Yokoo et al. (2011) reported a constitutive activation of NF-κB in human HCC tissue samples compared to surrounding liver tissues. This is supported by the recent study of Gaballah et al which provided evidence that active NF-ϰB signaling has a major contribution to the molecular pathogenesis of human hepatocellular carcinoma. They also concluded that, the association between NF-ϰB activity and expression levels with the bad prognostic parameters for HCC potentiates their value as prognostic markers to predict treatment outcome, metastasis or recurrence (Gaballah et al., 2014).

Luedde and Schwabe (2011) proposed that NF-κB acts as a central link between hepatic injury, fibrosis and HCC, and that it may represent a target for the prevention or treatment of liver fibrosis and HCC.

Concomitantly Sunami et al. (2012) revealed that hepatic activation of NF-κB is sufficient to induce liver fibrosis by a way of macrophage-mediated chronic inflammation. Therefore, agents controlling the hepatic NF-κB system represent attractive therapeutic tools to prevent fibrosis development in chronic liver diseases. Moreover, it was reported that Inhibition of NF-κB may enhances the antitumor effect of tumor necrosis factor-α gene therapy for hepatocellular carcinoma in mice (Haruki et al., 2013).

A number of different factors may trigger activation of NF-κB during hepatocarcinogenesis. In HBV-induced HCC, it has been suggested that the oncogenic HBV X protein activates the NF-κB signaling pathway. This up regulation might promote progression of HCC via

proliferative and anti-apoptotic effects, as well as the spreading of liver tumor cells (Zhang et al., 2010).

NF-κB activation is also detected in HCV via core protein. Moreover, many patients with advanced liver disease display increased activation of NF-κB in the liver (Tai, et al., 2000; Schwabe et al., 2006).

It was confirmed that PRDX3 expression may play an important role in hepatocarcinoma progression. Qiao et al. (2012) has analyzed the comparative proteomic profiles between HCC tumors and adjacent non-tumor tissues using two-dimensional gel electrophoresis and MALDI-TOF MS. They indicated that over-expression of PRDX3 was associated with 94.9% HCC, which was significantly higher than that in non-tumor tissues. Moreover, this over-expression of PRDX3 was correlated with poor differentiation of HCC suggesting that PRDX3 has substantial clinical impact on the progression of hepatocarcinoma, and may be a potential therapeutic target for HCC.

Several studies have showed that PRDX3 is over-expressed in many cancers like prostate cancer, cervical cancer, colorectal cancer, and ovarian adenocarinoma. Aggressively growing cancer cells produce high levels of ROS that disturb the redox balance. PRDX3 is an active responder to oxidative stress, and its expression is augmented to remove cellular ROS and inhibit apoptosis, which is beneficial to cancer growth and invasion (Kim et al., 2009; Li et al., 2009; Liu et al., 2010; Wu et al., 2010).

Our findings were in agreement with that of Shi et al. (2014) who found serum PRDX3 to be significantly higher in HCC patients than in the patients with liver cirrhosis and healthy control. The sensitivity and specificity of serum PRDX3 for the diagnosis of HCC were 85.9% and 75.3%, respectively. They concluded that, serum PRDX3 can be used as a noninvasive biomarker for the diagnosis and/or prognosis of HCC.

In conclusion, both nuclear factor kappa B (NF-κB) and peroxiredoxin 3 (PRDX3) may serve as early and sensitive biomarkers for early detection of HCC making early management and control more possible. The role of nuclear factor kappa B (NF-κB) as a target for prevention and treatment of liver fibrosis and HCC must be widely studied.

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