RESEARCH ARTICLE

Activity and Expression Pattern of NF-ϰB /P65 in Peripheral Blood from Hepatocellular Carcinoma Patients - Link to Hypoxia Inducible Factor -1α

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Abstract

Background: Hepatocellular carcinoma is a complex and heterogeneous tumor with poor prognosis due to frequent intrahepatic spread and extrahepatic metastasis. The molecular mechanisms underlying HCC pathogenesis still remain obscure. Objectives: We aimed to investigate the abundance and the DNA binding activity of nuclear factor kappa B/p65 subunit in peripheral blood mononuclear cells from patients with HCC and to assess its prognostic significance and association with hypoxia inducible factor one alpha (HIF-1α) in blood. Subjects and methods: This study was carried out on 40 patients classified equally into liver cirrhosis (group I) and HCC (group II), in addition to 20 healthy volunteers (group III). All groups were subjected to measurement of NF-ϰB /P65 subunit expression levels by real time-PCR, and DNA binding activity was evaluated by transcription factor binding immunoassay. Serum HIF-1α levels were estimated by enzyme-linked immunosorbent assay (ELISA). Significant increase of both the expression level and DNA binding activity of NF-ϰB /P65 subunit together with serum HIF-1 alpha levels was noted in HCC patients compared to liver cirrhosis and control subjects, with significant positive correlation with parameters for bad prognosis of HCC. In conclusion, NF-ϰB signaling is activated in HCC and associated with disease prognosis and with high circulating levels of HIF-1 alpha.

Keywords: HCC - liver cirrhosis, NF-ϰB - hypoxia inducible factor one alpha - binding activity

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Introduction

Hepatocellular carcinoma (HCC) is the most common liver malignancy and rates fifth in incidence and third in mortality in the world. In Egypt, the incidence rate of HCC was doubled in the past 10 years (Abdelaziz et al., 2014). Multiple risk factors are associated with HCC disease etiology, with the highest incidence in patients with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) (Su et al., 2013), although other factors such as genetic makeup and environmental exposure are involved (Gao et al., 2012).

Nuclear factor- ϰB was originally identified as a nuclear factor specific to B cells bound to the B site of the kappa-light chain gene enhancer. However, NF-ϰB was later found to be expressed in other cell types such as the liver epithelium, where it regulates hepatic cell proliferation and survival during regeneration and development (Tiwari et al., 2013). NF-ϰB controls the expression of genes involved in the regulation of fundamental processes such as the immune response, cell adhesion, oxidative stress and cell survival (Hung et al., 2008).

NF-kB plays a crucial role in bridging the action of growth factors and inflammation to hepatic oncogenesis (Beale et al., 2008), its constitutive activation is one of the early key events involved in neoplastic progression of the liver (Luedde et al., 2011)

NF-ϰB activity comprises homo- or heterodimers formed by members of the (Reticulo Endotheliosis) Rel/NF-ϰB family of transcription factors. The functional specificity and selectivity of the NF-ϰB response arise primarily from the binding of Rel/NF-ϰB complexes to specific DNA regulatory sites (ϰB sites) of target genes in different cell types (Escarcega et al., 2007).

In humans, there are 5 subunits of this family of proteins: RelA (also called p65), NFϰB1 (also called p50), NFϰB2 (also called p52), RelB and RelC (Gonzalez-Ramos et al., 2012). NF-ϰB is kept inactive in the cytoplasm by binding to one of the inhibitory proteins called IϰB-α, IϰB-β, IϰB-ε, p105 and p100. As a result of the phosphorylation and subsequent degradation of the inhibitory subunits, NF-ϰB translocates to the nucleus, binds to ϰB sites and regulates target genes. Due to its strong transcriptional activity, the p65 subunit of NF-ϰB (NF-ϰB/p65) is responsible for most of NF-ϰB's transcriptional activity (van Loo and Beyaert 2011).

Hypoxia-inducible factors (HIFs) are essential mediators of the cellular oxygen-signaling pathway. They are heterodimeric transcription factors consisting of an oxygen-sensitive alpha subunit (HIF-α) and a

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constitutive beta subunit (HIF-β) that facilitate both oxygen delivery and adaptation to oxygen deprivation. Hypoxia-inducible factors regulate multiple aspects of tumorigenesis, including angiogenesis, proliferation, metabolism, metastasis, differentiation, and response to radiation therapy (Mimeault et al., 2013).

NF-κB and HIF-1α are of major importance for disease states associated with low-oxygen tension, such as cancer .A cross-talk between the NF-κB pathway and the HIF pathway has been recently assumed, as NFκB might be involved in redox-sensitive induction of the HIF-1through binding at a distinct element in its proximal promoter. Moreover, HIF-1 itself may contribute to the activation of the NF-κB pathway (Gorlach et al., 2008).

However, the interplay between NFκB/P65, HIF-1 in hepatocellular carcinoma has not been fully elucidated yet. Therefore, we aimed to assess the expression pattern and the DNA binding activity of NFκB/P65 subunit in the peripheral blood of patients with HCC and to evaluate their relevance to the circulating levels of HIF-1 in order to gain biological insight on the molecular mechanisms of hepatic carcinogenesis, which may be beneficial in the early diagnosis and treatment of unresectable human HCCs.

Subjects and Methods

This study was carried out on 40 patients presented to Tropical Medicine Department, Tanta University Hospital and its Outpatient Clinics as well as 20 healthy volunteers represented as control group. They were classified as follows: Group I: included 20 patients with liver cirrhosis (13 males & 7 females), aged 45 to 61 years with no radiological evidence for hepatic focal lesions. Group II: included 20 patients with HCC (15males & 5 females) aged 49 to 63 years. Diagnosis was performed by the appropriate imaging characteristics according to The American Association for the Study of Liver Diseases (AASLD) practice guidelines (Bruix and Sherman 2005). The Milan criteria was defined as the presence of a tumor 5 cm or less in diameter in patients with single HCC or no more than 3 tumor nodules, each 3 cm or less in diameter, in patients with multiple tumors, and no extrahepatic metastasis and no major hepatic vessel invasion (Lei JY et al., 2013). Group III: included 20 healthy subjects (12 males & 8 females) aged 48 to 60 years.

Patients with history of previous resection of HCC, or interferon based therapy for HCV or who have metastatic liver disease or with extra-hepatic metastasis were excluded from the study. Informed written consent was obtained from all participants. Approval was obtained from the Local Research Ethics Committee, Tanta University. Patients and controls were subjected to full history taking, thorough clinical examination and abdominal ultrasonography.

Blood sample collection

After 12 hours of overnight fasting, 7 ml of venous blood samples were taken from every investigated subject, transferred slowly into a dry sterile centrifuge tube, allowed to clot at room temperature, centrifuged at 2000 rpm for 10 minutes and serum was separated and stored at -70ºC until the time of analysis.

Laboratory investigations included

i) Routine investigations: blood sugar, blood urea and creatinine, complete blood picture, complete urine and stool analysis; *ii*) Hepatitis viral marker including anti-HCV and anti-HBV were detected by ELISA (Wilkins T et al., 2010); *iii*) Liver function tests including: serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum albumin, prothrombin time and serum bilirubin; *iv*) Estimation of serum alpha fetoprotein level (Johnson, 2001); *v*) Quantitative Determination of circulating HIF-1α level: The levels of serum HIF-1α were determined by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (R&D Systems, Minneapolis, MN 55413, USA) according to the manufacturer's instructions. Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer; *vi*) Expression of NFκB/p65 gene in peripheral blood mononuclear cells (PBMCs) was detected by using Real-time PCR (RT-PCR).

Preparation of Peripheral blood mononuclear cells (PBMCs): PBMCs were prepared by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Briefly, heparinised blood was carefully layered on Ficoll, and PBMC were harvested from the white interphase after centrifugation for 30 minutes at 400g, at room temperature and washed with phosphate buffered saline (PBS). The PBMCs samples were stored at -80°C till the samples were further processed for RNA isolation.

Real-time PCR: Total RNA from PBMC was prepared using Trizol reagent (Gibco BRL Life Technologies) according to the manufacturer's instructions. The integrity of total RNA was checked by electrophoresis through 1% agarose gel. RNA samples were then stored at -80°C. cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA Synthesis kit (#K1632, Thermo Scientific Fermentas, St. Leon-Ro, Germany) according to the manufacturer's instructions. Real-time PCR was carried out with single stranded cDNAs. PCR reactions were performed using Power SYBR Green PCR Master Mix and 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA). Primer sequences specific for the NFκB /p65 (No: NM_021975.3) are : Fwd: 5'-ATCTGCCGAGTGAACCGAAACT-3'; and Rev: 5'-CCAGCCTGGTCCCGTGAAA-3' (Sun W et al., 2012); β-actin (No:NM_001101.3): Fwd: 5'-TGGCATTGCCGACAGGATGCAGAA-3', Rev:5'-CTCGTCATACTCCTGCTTGCTGAT -3'. β-actin primers were used as an internal control. Real-Time PCR was carried out, in duplicate, by 40 cycles of 95°C for 10 sec and 60°C for 1 min. Comparative Ct (threshold cycle) method was used to determine relative amounts of the products, according to the Applied Biosystems instructions. Conventional PCR was performed with the DreamTaq polymerase (#EP0701, Thermo Scientific Fermentas, St. Leon-Ro, Germany). All expression data were normalized by dividing the target amount by the amount of β-actin used as internal control for each sample.

NFκB activation was examined by using transcription factor binding assay kit as follows:

Isolation of nuclear proteins Nuclear proteins were isolated from PBMCs extract using Nuclear Extract kit (Cat#40010, Active Motif, Carlsbad, CA, USA) according to the protocol of the manufacturer. Briefly, the sample was placed in 0.8mL of ice-cold hypotonic buffer [10mmol/L HEPES (pH7.9), 10 mL KCL, 0.1 mmol/L EDTA, 0.1 mmol/L ethylene glycol tetraacetic acid, 1 mmol/L DTT; Protease inhibitors (aprotinin, pepstatin and leupeptin, 10 mg/L each). The homogenates were incubated on ice for 20 mins, vortexed for 20s after adding 50μL of 10% Nonidet p-40, and then centrifuged for 1 minute at 4°C in an Eppendorf centrifuge. Supernatants were decanted, the nuclear pellets after a single wash with hypotonic buffer without Nonidet p-40 were suspended in an ice-cold hypertonic buffer (20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCL, 1 mmol/L EDTA, 1 mmol/L DTT; Protease inhibitors), incubated on ice for 30 mins at 4°C , mixed frequently and centrifuged for 15 mins at 4°C. The supernatants were collected as nuclear extracts and stored at -70°C (Gong JP et al., 2002). Concentrations of total proteins in the samples were determined according to the method of Bradford (#Cat no.500-0006, Bio-Rad Protein Assay) (Bradford, M. 1976).

Evaluation of NF-kB/p65 DNA-binding activity NFκB/p65 DNA-binding activity was evaluated using the ELISA-based TransAM™ NFκB/p65 protein assay (#Cat no. 40096, Active Motif, Carlsbad, CA, USA), following the protocol of the manufacturer. In this commercial kit, a duplexed NF-κB oligonucleotide containing a κB consensus sequence is attached to the surface of 96-well plates. Activated NF-κB dimers in 20 μg nuclear extract bound to the attached oligonucleotide is specifically and quantitatively detected by subsequent incubation with antibodies against the activated forms of NFκB/p65 followed by an enzyme-linked (horseradish peroxidase) secondary antibody for colorimetric scoring (Van Laere SJ et al., 2006). The absorbance was measured on an ELISA reader at 450 nm with a reference wavelength of 655 nm.

Statistical analysis

The data were analyzed using statistical package for the social science (SPSS) version 20.0 software (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as mean ±Standard deviation. Qualitative data expressed as number and percentage and analyzed by Chi -square test (X2). Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. Correlations were analyzed using the Pearson test.

Results

The demographic data and associated risk factors are demonstrated in Table 1. There were no significant differences in age, sex and associated risk factors between the liver cirrhosis and HCC groups. Table 2 shows the clinical and ultrasonographic characteristics of the studied groups. There was no significant difference in portal vein diameter between cirrhotic and HCC patients, while portal vein thrombosis (PVT) was found in 3 (15%) cirrhotic patients and 4 (20%) HCC patients. Concomitantly, there was significant difference as regards MELD score between cirrhotic and HCC groups (p-value <0.05). Regarding Milan Criteria, 9 (45%) HCC patients lay within, while 11 (55%) patients lay beyond the criteria. Tumor size was <3 cm in 8 (40%) patients, while 12 (60%) patients had tumor size >3 cm. Also, 9 (45%) patients had single tumor while multiple tumors were demonstrated in 11 (55%) HCC patients. Portal vein invasion was found in 4 (20%) HCC patients.

Laboratory biochemical findings for the studied groups are demonstrated in Table 3. Hepatic profile namely ALT, AST, serum albumin, prothrombin activity, international normalized ratio (INR) and direct bilirubin showed statistically significant differences between patients and control groups, with having statistically significant higher levels in HCC group compared to liver cirrhosis group.

On the other hand, there were no statistically significant differences between the studied groups as regards hemoglobin %, or total lecuocytic count.

The mRNA expression levels of NF-KB/p65 subunit

Table 1. Demographic and Associated Risk Factors in The Patients Groups

		Group I, Liver cirrhosis (No.=20)	Group II, Hepatocellular carcinoma (No.=20)	F/X^2	p-value
Age		52.8±7.8	55.6±7.6	F=0.258	0.117
Sex	Male	13 (65%)	15 (75%)	X^2=0.599	0.362
	Female	7 (35%)	5 (25%)		
HCV antibodies only					
	Positive	16 (80%)	13 (65%)	X^2=1.889	0.028
	Negative	4 (20%)	7 (35%)		
HCV&HBV antibodies					
	Positive	4 (20%)	7 (35%)	X^2=1.132	0.288
	Negative	16 (80%)	13 (65%)		

*Group I: patients with liver cirrhosis; Group II: patients with HCC; HCV: hepatitis C virus, HBV: hepatitis B virus, HCC: hepatocellular carcinoma

Table 2. Clinical and Ultrasonographic Characteristics of The Two Patients Groups

	Group I, Liver cirrhosis (No.=20)	Group II, Hepatocellular carcinoma (No.=20)	t-test /X^2	p-value
Portal vein diameter (PVD)	14.1±1.8	15.8±1.8	1.32	0.32
Portal vein thrombosis (PVT)				
Yes	3 (15%)	4 (20%)	0.25	0.14
No	17 (85%)	16 (80%)		
Portal vein invasion:				
Yes	-	4 (20%)	-	-
No	-	16 (80%)		
Biliary radical dilatation (IHBRD)				
Positive	20	0	40	0.001*
Negative	0	20		
MELD score	18.2±4.9	24.5±9.7	3.62	0.01*
Milan criteria:				
Within	-	9 (45%)	-	-
Beyond	-	11 (55%)		
Tumor size	<3cm	-	8 (40%)	- -
≥3cm	-	12 (60%)		
Tumor number				
Single	-	9 (45%)	-	-
Multiple	-	11 (55%)		

*Group I: patients with liver cirrhosis; Group II: patients with HCC; MELD score: Model For End-Stage Liver Disease score; *Significant P was considered at <0.05

Table 3. Statistical Comparison of The Routine Laboratory Findings of The Studied Groups

	Group I liver cirrhosis	Group II HCC	Group III Control	ANOVA		Tukey's test		
				F. test	p. value	P1	P2	P3
Serum Albumin (S. Albumin)	2.6±0.3	2.4±0.2	4.0±0.2	2.52	0.04*	0.03*	0.04*	0.02*
Total bilirubin (T. bilirubin)	2.4±1.7	1.9±0.6	0.5±0.17	2.69	0.02*	0.04*	0.01*	0.01*
Direct bilirubin	0.9±0.7	0.9±0.3	0.1±0.06	2.32	0.008*	0.04*	0.01*	0.001*
Alanine amino transferase (ALT)	34.1±16.1	37.87±20.11	22.27±8.15	5.63	0.007*	0.02*	0.04*	0.02*
Aspartate amino transferase (AST)	54.9±21.7	59.4±47.4	24.9±7.0	3.85	0.007*	0.03*	0.01*	0.001*
International normalized ratio (INR)	1.3±0.23	1.9±0.3	1.00±0.05	3.63	0.019*	0.01*	0.02*	0.04*
Prothrombin activity	70.4±14.9	64.7±6.1	100.1±0.08	0.58	0.02*	0.020*	0.04*	0.01*
Platelets cells	90074.0±46203.8	82813.3±48738.8	199600.0±56753.6	5.62	0.05	0.03*	0.02*	0.04*
Hemoglobin%	10.16±1.5	10.33±2.1	14.08±0.9	0.32	0.96	0.41	0.07	0.32
Total leucocytic counts (TLC)	6533.3±5890.1	7018.6±5436.3	5660.0±1545.8	0.75	0.14	0.65	0.09	0.52

*Significant P was considered at <0.05; P1 comparison group I vs group II; P2 comparison group I vs group III; P3 comparison group II vs group III.

Table 4. Statistical Comparison of The Levels of AFP, HIF1α, NFKB/p65 Expression and Binding Activities between The Studied Groups

	Group I liver cirrhosis	Group II HCC	Group III Control	ANOVA		Tukey's test		
				F. test	p. value	P1	P2	P3
alpha-fetoprotein (ng/ml)	27.9±5.3	654.8±91.1	2.01±0.5	9.3	0.001*	0.001*	0.001*	0.001*
HIF-1α (μg/L)	93.1±13.2	145.9±24.2	33.7±4.1	12.3	0.001*	0.001*	0.001*	0.001*
NFKB/p65 mRNA	0.099±0.006	0.18±0.006	0.023±0.006	11.3	0.001*	0.002*	0.001*	0.003*
NFKB/p65 binding	0.34±0.04	0.77±0.05	0.11±0.02	12.6	0.003*	0.001*	0.001*	0.001*

*P was calculated by one way ANOVA test followed by Tukey's post-hoc test. P was considered significant at <0.05; *Significant; P1 comparison between group I and II; P2 comparison between group I and III; P3 comparison between group II and III; HIF-1α: hypoxia inducible factor one alpha, NFKB/p65: nuclear factor kappa B/p65 subunit

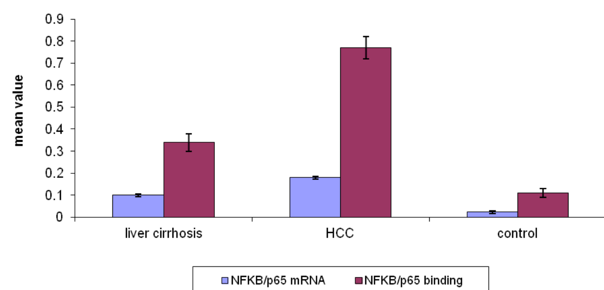

Figure 1. NFKB/P65 mRNA Expression Levels and Binding Activity in The Studied Groups, they were significantly increased in hepatocellular carcinoma group compared to the other groups

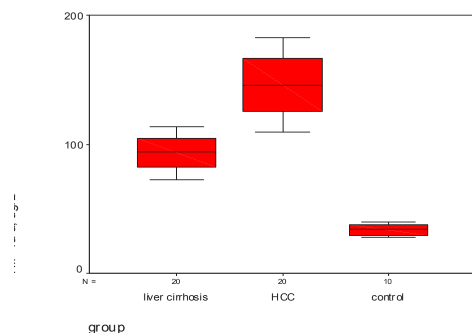

Figure 2. Hyoxia Inducible Factor-1α Serum Levels in The Studied Groups, showing a significant increase in hepatocellular carcinoma group compared to the other groups

were significantly up regulated in PMNCs from HCC patients (0.18+0.006) compared to those from liver cirrhosis patients (0.099+0.006) or control group (0.023+0.006) with p-value < 0.01. Moreover, the DNA binding capacity of NF-ϰB/p65 subunit in the nuclear fractions of PMNCs was found to be significantly different among the studied group (p<0.01) (Figure 1 and Table 4). Additionally, serum levels of hypoxia inducible factor 1 α were significantly elevated in HCC patients (145.9+24.2 μg/L) as compared to liver cirrhosis patients (93.1+13.2μg/L) and the control group (33.7+4.1μg/L), p-value <0.01 (Figure 2 and Table 4).

Using multiple comparisons test (Tukey's test), the above mentioned parameters were markedly higher in HCC group compared to the liver cirrhosis or to the control group; they were also higher in the liver cirrhosis group compared to the control group (p-value <0.01).

To further define the role of the HIF-1α, NFϰB/p65 expression and DNA binding activity in the progression of HCC, Pearson correlation test was used to test their correlations with age, sex, serum alpha fetoprotein and other prognostic determinants of HCC in the studied groups. The mRNA expression levels and binding activity of NFϰB/p65 subunit were significantly correlated with serum alpha fetoprotein, MELD score, tumor size, portal vein invasion, but not correlated with age, sex or tumor number as shown in Table 5. On the other hand, serum levels of HIF 1a showed the same pattern of correlation with these prognostic parameters, together with being significantly correlated with NFϰB/p65 mRNA expression levels (r=0.547, p<0.01).

Table 5. Correlation Matrix between all The Studied Parameters

	Serum HIF-1α, ug/L		NFϰB/ p65 mRNA		NFKB/ p65 binding	
	r	p	r	p	r	P
Age	-0.087	0.296	-0.163	0.126	0.015	0.017
Sex	0.017	0.205	0.276	0.596	-0.038	0.41
MELD score	0.421	0.009*	0.225	0.04*	0.0269	0.04*
Portal vein invasion	0.425	0.01*	0.523	0.04*	0. 285	0.04*
Tumor size	0.311	0.01*	0.526	0.009*	0.374	0.01*
Tumor number	0.058	0.658	0.164	0.16	-0.023	0.17
Alpha-fetoprotein (AFP) ng/ml	0.557	0.001*	0.493	0.001*	0.632	0.001*
HIF-1α, ug/L			0.547	0.001*	0.68	0.001*

*Significant; **r=Pearson's correlation coefficient, P was considered significant at <0.05

Discussion

Hepatocellular carcinoma (HCC) is the most common form of liver cancer, usually triggered by chronic inflammation (Zekr et al., 2012) and continuous liver injury (Sakurai et al., 2013). It is a highly vascular tumor characterized by fast infiltrating growth, early metastasis, high-grade malignancy (Lee et al., 2014) and poor therapeutic efficacy (Zhou et al., 2012).

The NFκB family of transcription factors has recently been identified as a critical mechanistic link between chronic inflammation and cancer (Hoesel and Schmid, 2013), however its contribution to the molecular pathogenesis of hepatic carcinogenesis is not fully elucidated.

The results of the current study revealed that NFκB/P65 subunit expression levels were significantly increased in PMNCs of patients with HCC as compared to those with liver cirrhosis and to the control subjects. These findings are in agreement with the study by Zhang et al. (2013) who reported that the mRNA expression level of NFκB/p65 was significantly increased in human HCC tissues with invasion than that of without invasion, where this overexpression contributed to HCC metastasis through activation of NFκB dependent matrix metaoproteinasse-9 expression. Moreover, Wu et al. (2009) observed that hepatic NFκB mRNA was aberrantly overexpressed during the course of rat hepatocarcinogenesis development.

Regarding the DNA binding capacity of the p65 subunit of NF-κB, the present study showed it was significantly increased in PMNCs from HCC patients compared to those with liver cirrhosis and to the control subjects. This finding is consistent with the study of Yokoo et al. (2011) who reported constitutive activation of NF-κB in human HCC tissue samples compared to surrounding liver tissues.

The viewpoint that NFKB activation has a prominent role in hepatic carcinogenesis was underscored by the study done by Wu et al. (2010) who proved that small interference RNA (siRNA) mediated inhibition of nuclear factor-κB/p65 resulted in suppression of human hepatoma cell growth; they also concluded that nuclear factor-κB is a potential molecular target for HCC gene therapy. Similarly, Wong et al. (2013) achieved inhibition of growth of liver cancer cells through suppressing the activity of NF-κB by p21-activated protein kinase 1-inhibitor. Furthermore, Haruki et al. (2013) showed that inhibition of NF-κB enhances the antitumor effect of tumor necrosis factor-α gene therapy for HCC in mice.

Concomitantly, the finding that NF-κB/p65 was significantly activated in liver cirrhosis patients compared to the control subjects is in harmony with the study by Ren S et al., 2013 who observed that NF-κB/p65 was activated in macrophages in cirrhotic patients with hypersplenism, as it was found to promote phagocytosis and cytokine secretion by splenic macrophages. Well in line, Li et al. (2012) reported that elevated expression of NF-κB/p65 is closely associated with the occurrence and development of hepatic fibrosis, through increasing secretion of collagens I and III after the activation of hepatic stellate cell.

Taken together, this observed activation of NF-κB may be attributed to the fact that HCC commonly arises in a setting of chronic inflammation and subsequent liver fibrosis and cirrhosis (Ramakrishna et al., 2013). It was found that in HBV-induced hepatocarcinogenesis, the oncogenic HBV X protein activates the NF-κB signaling pathway through the upregulation of TANK-binding kinase 1 (TBK1), thus promoting progression of HCC via proliferative and antiapoptotic effects, as well as triggering the spread of tumor cells (Kim et al., 2010 and Zhang et al., 2010). The same mechanism can be displayed in HCV induced HCC, where HCV core protein was found to mediate activation of NF-κB. Moreover, many patients with advanced liver disease have increased levels of lipopolysaccharides which are known activators for NF-κB in the liver (Luedde et al., 2011).

It is worth noting that the reported association between overexpression and activation is in accordance with the study of Wang L et al., 2013 who documented an overexpression of NFκB/p65 in human breast cancer cell line causing increased NF-κB transcriptional activity. Consequently, active NF-κB signaling can promote cancer progression and metastasis through induction of several chemotactic and anti-apoptotic and cell cycle regulatory genes, controlling epithelial to mesenchymal transition and enhancing tumor vascularization (Xia et al., 2014).

Among the upregulated NF-κB -responsive genes, hypoxia inducible factor one alpha (HIF-1α) is a master regulatory transcription factor controlling cellular adaptation to hypoxia, activating metabolic and signaling pathways promoting cell survival (Lenihan et al., 2013).

The current study reported a significant increase in serum HIF-1 α levels in HCC patients compared to those with liver cirrhosis and control subjects. This finding is in agreement with the study of Li et al. (2011) who concluded that hepatic HIF-1α expression is associated with the development and prognosis of HCC and proved that circulating HIF-1α level can be a useful marker for HCC diagnosis and prognosis. Furthermore, these results are in line with the fact that hypoxia is a characteristic feature of solid tumors, where cells respond to hypoxia by generating mature and functional HIF-1α (Semenza, 2011) which plays a pivotal role in the growth, infiltration and metastasis of tumor cells (Unwith et al.,2014).

Of note, the observed significantly higher levels of circulating HIF-1α in patients with liver cirrhosis compared to control subjects can be explained on the basis of its role in the hepatic inflammation-fibrosis axis (Rosmorduc et al., 2010). Hypoxic areas represent a major feature of cirrhotic liver where hypoxia inducible factors help to counteract the decrease in oxygen tension through upregulation of angiogenesis and enhancement of liver fibrogenesis (Troeger et al., 2011).

Concerning its relationship with NF-κB/P65, serum HIF-1α was significantly correlated with nuclear factor-κB/P65 expression levels; this finding is in keeping with the notion of an existing crosstalk or interplay between NF-κB and HIF-1α particularly in carcinogenesis. NF-κB is a critical transcriptional activator of HIF-1α and basal nuclear factor-κB activity is required for HIF-1α protein accumulation under hypoxia (Taylor et al., 2009). Moreover, a hypoxia-dependent transcriptional

upregulation of hypoxia-inducible factor-1α by nuclear factor-κB was reported in several types of cancer (Yoshida et al., 2013), stimulating epithelial to mesenchymal transition (Cheng et al., 2014), thus promotinralg tumor growth and angiogenesis.

Furthermore, the present study contributed information towards establishing a significant association of the aberrant activation and over expression of NF-κB/p65 with bad prognostic parameters for HCC as MELD score, tumor size, portal vein invasion, suggesting an important role for NF-κB/p65 in the progression of HCC and highlighting its significance as a prognostic marker for HCC. In harmony with this note, are the previous studies which confirmed the prognostic significance of NF-κB in various types of cancers such as breast cancer (Sarkar et al., 2013) and squamous cell carcinoma of head and neck (Balermpas et al., 2013).

In conclusion, the current study provided unequivocal evidence that active NF-κB signaling has a major contribution to the molecular pathogenesis of human hepatocellular carcinoma. Besides, the association between NF-κB/p65 activity and expression levels with the bad prognostic parameters for HCC potentiates their value as prognostic markers to predict treatment outcome, metastasis or recurrence. The present study has also shed light on the interplay between NF-κB/p65 and HIF1-α in hepatic carcinogenesis as proved by the impact of NF-κB/p65 overexpression on the circulating levels of HIF1-α. Finally, the activation of NF-κB/p65 in HCC has implicated its pivotal role as a novel potential therapeutic target for treatment of patients with HCC.

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