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EXPRESSION OF Bcl2, P53, AND TNF-α GENES IN EGYPTIAN PATIENTS WITH HEPATOCELLULAR CARCINOMA

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ABSTRACT

In Egypt, hepatocellular carcinoma (HCC) is the second most common malignancy in males and fifth in females. Various proteins or oncogenes and suppressor gene are involved in the process of apoptosis, including Bcl-2, p53, and the Fas/Fas system. This work aimed to investigate the expression of Bcl-2, p53 and TNF-α in patient with HCC and to compare their levels of expression with those of the patients with liver cirrhosis. This work included 38 patients with liver cirrohosis, 42 patients with HCC; in addition to 20 healthy individuals whose were served as control group. The levels of Bcl2 and p53 were estimated in the blood of HCC and LC patients by using flowcytometric analaysis. In addition, level of TNF-α was determined by using enzyme-linked immunosorbent assay (ELISA). In comparison to controls, Bcl2, p53 and TNF-α level were significantly higher in cirrhosis and HCC. Results indicated that p53 play important roles in hepatocarcinogenesis. Apoptosis-related genes p53, bcl-2, are related to HCC occurrence, and their expression renders an increasing tendency during the formation of HCC. In HCC, p53 is not noticeably related to cell apoptosis, and its high expression may relate to the proliferation of liver cells. Therefore p53 status and the expression of Bcl-2 by tumor cells might be good indicators of sensitivity to chemotherapy for patients with HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common types of malignant tumors that carry a poor prognosis worldwide (El-Serag et al., 2008). In Egypt, it is the second most common malignancy in males and fifth in females. Major risk factors of inducing HCC include various chemicals and viruses (El-Zayadi et al., 2005). Previous studies have provided evidence that p53 tumor suppressor gene plays a major role in hepatocarcinogenesis. It is well known that the inactivation of p53 is the most common genetic alterations in human cancers including HCC (Li et al., 1993). The p53 has a critical role in regulation of cell cycle, DNA repair and synthesis as well as in apoptosis (Gottlieb, and Oren, 1998). The p53 stimulates a wide network of signals that act through two major apoptotic pathways. The extrinsic, death receptor pathway triggers the activation of a caspase cascade, and the intrinsic, mitochondrial pathway shifts the balance in the Bcl-2 family towards the pro-apoptotic members, promoting the formation of the apoptosome, and consequently caspase-mediated apoptosis (Benchimol, 2001). The impact of these two apoptotic pathways may be enhanced when they converge through Bid, which is a p53 target (Eferl et al., 2003). The majority of these apoptotic effects are mediated through the induction of specific apoptotic target genes. The prevention of cancer is profoundly dependent on the p53 tumor suppressor protein. The ability of p53 to eliminate excess, damaged or infected cells by apoptosis is vital for the proper regulation of cell proliferation in multi-cellular organisms (Fisher, 2001).

Tumor necrosis factor-α (TNF-α) is a pleitrophic cytokine with immunoregulatory and metabolic functions (Chen and Goeddel, 2002). TNF-α exerts a stimulatory effect on natural killer cells and plays a potential role in antitumor cytolytic responses. Elevated serum concentration of TNF-α have been described in patients with HCC (Mervat et al., 2005). The current work aims to

investigate the expression of bcl2, p53 and TNF-α in hepatocellular carcinoma and liver cirrohosis patients.

MATERIALS AND METHODS

The present study was conducted on 80 patients who were admitted to the National Liver Institute, Minufiya University, during the period from September 2008 to May 2009. According to clinical examination and disease history, patients were divided into two groups: the first group included patients with liver cirrhosis (n=38; 5 females and 33 males, with mean age of 51 ± 10 years), while the second group included patients with liver hepatocellular carcinoma (n=42; 7 females and 35 males with mean age of 52 ± 10 years). In addition to 20 healthy individuals (8 females and 12 males, their mean age was 46 ± 12 years), were served as control group. All patients were subjected to routine clinical examination, conventional laboratory investigations and abdominal ultra-sonography.

Determination of Bcl2 and P53 expression

Flow cytometric analysis of bcl2 and P53 expression was performed using method of Dean and Jett (1974). Briefly, cells were incubated with either anti-Bcl-2 or anti-P53 fluorescein isothiocyanate for 15 min. at RT. Cells were washed, and data were acquired using Becton Dickinson FACScalibur and CellQuest software (Becton Dickinson). Fluorescence intensity was standardized using isotype-matched negative control antibodies. Data were analyzed using CellQuest software (Becton Dickinson).

Determination of serum level of TNF-α

Serum level of TNF-α was quantified by ELISA which based on the quantitative sandwich immunoassay technique that uses immobilized monoclonal antibody and biotin-linked polyclonal antibody.

Statistical analysis

Data were subjected to an analysis of variance using the general model procedure .Variables having a significant F-test ($p < 0.05$) were compared using the least significant difference (LSD) test.

RESULTS

1) Levels of expression of Bcl-2, P53 and TNF-α

Table (1) shows the level of expression of bcl-2, p53 and TNF- in the population of the study. Results demonstrated a significant increase in the level of expression of bcl-2, p53 and TNF-α as compared to those of the normal controls. Meanwhile, results showed significant increases in the level of bcl-2 and TNF-α expression in the HCC as compared to those patients with LC. Level of p53 expression in the patients with HCC was insignificantly increased as compared to that of LC.

Table 1: Level of expression of Bcl-2, P53 and TNF-α

Group	Bcl2 (%)	P53 (%)	TNF-α (pg/ml)
Control	5.8 ± 2.3	4.9 ± 1.6	3.3 ± 1.2
LC	11.2 ± 6.4*	45.0 ± 20.2*	10.4 ± 3.8*
HCC	23.7 ± 11.6*,#	50.8 ± 23.7*	51.3 ± 25.6*,#

(*) significant as compared to the control; (#) significant as compared to LC

2) Levels of expression of Bcl-2, P53 and TNF-α in relation to tumor size

As shown in table 2, the level of bcl2 in patients with tumor size less than 3 cm was higher than those with tumor size more than 3 cm. No significant differences in level of bcl2 was found between patients with tumor size less than 3 cm and those with tumor size more than 3 cm ($p = 0.3$). Also, the level of p53 in patients with tumor size more than 3 cm was higher than those with tumor size less than 3 cm. No significant differences in levels of p53 was found between patients with tumor size less than 3 cm and those with tumor size more than 3 cm ($p = 1.0$). In addition, the level of TNF-α in patient with tumor size less than 3 cm was found to be higher than those with tumor size more than 3 cm. No significant difference in levels of TNF-α was found between patients with tumor size less than 3 cm and those with tumor size more than 3 cm ($p = 0.8$).

Table 2: Levels of Bcl-2, P53 and TNF-α in relation to tumor size

Variable	Bcl2	P53 (%)	TNF-α (pg/ml)
Tumor Size			
< 3 cm	26.1 ± 13.0	51.6 ± 21.7	51.6 ± 28.6
≥ 3 cm	19.4 ± 6.2	52.4 ± 27.5	52.5 ± 24.4

3) Levels of expression of Bcl-2, P53 and TNF-α in relation to child Pugh classification

Patients with HCC were classified according to child Pugh classification into two groups, child A and child (B+C). As shown in table (3), the level of Bcl2 in patients with child A was higher than those with child (B+C) and non-significant differences in bcl2 were observed between child A and child (B+C) (p=0.5). The percentage of p53 in patients with child (B+C) was found to be higher than those with child A and non-significant differences in p53 were observed between child A and child (B+C) (p=0.4). Meanwhile, the level of TNF-α in patients with child (B+C) was higher than those with child A and non-significant differences in TNF α were observed between child A and child (B+C) (p =0.4).

Table 3: Levels of Bcl2 in relation to child Pugh classification in HCC patients

Variable	Bcl2	P53 (%)	TNF-α (pg/ml)
Child A	22.9 ± 10.2	41.4 ± 14.5	38.7 ± 13.2
Child (B+C)	22.8 ± 11.7	52.6 ± 24.7	53.0 ± 26.7

DISCUSSION

Occurrence and biological characteristics of tumors are related not only to over-proliferation of carcinoma cells but also to decrease of apoptosis. Investigation of apoptosis helps to disclose the biological characteristics of tumors, and seeks new methods of diagnosis and treatment for tumors. Various proteins or oncogenes and suppressor gene are involved in the process of apoptosis, including p53, Bcl-2, and the Fas/Fas system (Gu et al., 2000).

In the current work, the expressions of p53 and bcl2 were determined by flow cytometric analysis; while, serum level of TNF- in was determined by ELISA in HCC, LC and normal controls. The relationship between the expression of these genes and clinical parameters were studied. The present study, showed that the expression of Bcl2 was significantly increased ($p<0.05$) in patients with HCC (23.7±11.6%) when compared to that of cirrhotic patients or healthy individuals (11.2 ±6.4% and 5.8 ±2.3 %respectively). This data are in agreement with Osman et al. (2007) who

reported that the expression of Bcl-2 protein in HCC patients is higher than that in liver cirrhosis patients.

The significantly higher Bcl-2 values were in concomitant with Hamazaki et al. (1995), Frommel et al. (1999) and Feng et al. (1999) whose stated that bcl-2 expression was significantly increased elevated in HCC patients as compared with normal healthy individuals. Moreover, results indicated a no statistically significant increase in the expression of Bcl-2 at the early stage of HCC as compared to that of late stage HCC patients. Bcl-2 appeared to be high expressed in patients with tumor size less than 3 cm (26.1 ±13.0%) when compared to those having tumor size more than 3 cm (19.4±6.2%). Based on child Pugh the levels of Bcl2 in HCC patients with child A (22.9 ±10.2%) were higher than those with child (B +C) (22.8±10.0%). Guo et al. (2002) reported that there is as no significance between the expression of Bcl2, tumor differentiation and tumor stage of HCC patients. In the study of An et al. (2001) ,Li et al. (1997) and Sundblad and Tamayo, (1995).on gastric carcinoma they found that the expression of bcl-2 reached the top at the early stage of gastric cancer and decreased in the progressive gastric cancer.

Results of this study showed that the expression of P53 was significantly increased ($p<0.05$) in patients with HCC (50.8±23.7%) when compared to that of cirrhotic patients or healthy individuals (45.0±20.2% and 4.9±1.6% respectively). Qiao et al. (1994),Haldar et al. (1994) and Mei et al, (2009) founds that the expression of P53 was significantly increased ($p<0.05$) in patients with HCC when compared to that of healthy individuals .Meanwhile, this finding is go in parallel with Atta et al. (2008) who found that p53 of HCC patients in Egypt were significantly higher ($p = 0.0001$), than both liver cirrhosis patients and healthy control groups.

Moreover, results indicated a no statistically significant increase in the expression of p53 at the early stage of HCC as compared to that of late stage HCC patients. P53 appeared to be low expressed in patients with tumor size less than 3 cm (51.6 ±21.7%) compared to those tumor size more than 3 cm (52.4±27.5%). Based on child Pugh the levels of p53 in HCC patients with child A (41.4±14.5%) were lower than those with child (B +C) (52.6±24.7%). Xiao et al. (2005) in the late stage of HCC, p53 was significantly high expressed. In the study of Ikeda *et al.* (1998) and An et al. (2001), on gastric cancer observed that the *p*53 expression renders an increasing tendency during the formation of gastric cancer. Qiao et al. (1994) and Sheen et al. (2003) showed no statistically significant difference between p53 and Child- Pugh class A or B, and size of the HCC. Atta et al. (2008) found that p53 Expression in Egyptian HCC patients did not correlate with tumor size and tumor grade.

Results of this study showed that the expression of TNF-α (pg/ml) was significantly increased ($p<0.05$) in patients with HCC (51.3 ±25.6) when compared to that of cirrhotic patients or healthy individuals (10.4 ± 3.8 and 3.3 ± 1.2 respectively). Mervat et al. (2005) and Huseyin et al. (2006), found that Serum TNF-α, levels were significantly higher in cirrhosis and HCC groups compared to the control group ($P < .05$).

Moreover, results indicated a no statistically significant increase in the expression of TNF-α (pg/ml) at the early stage of HCC as compared to that of late stage HCC patients. TNF-α (pg/ml) appeared to be low expressed in patients with tumor size less than 3 cm (51.6 ±28.6) compared to those tumor size more than 3 cm (52.5±29.4). Based on child Pugh the levels of TNF-α (pg/ml) in HCC patient with child A (38.7±13.2) were lower than those with child (B +C) (53.0±26.7). **Iz et al.** (2009) suggested that the high expression of TNF-alpha gene appears to be associated with an increased risk for the development of HCC

in Turkish population. Mervat, et al. (2005) .The increased levels of TNF-α in the HCC patients more than that in Benign (SHF). Larrea et al. (1996) have shown that hepatitis infection is associated with increased transcriptional expression of the TNF-α gene in the liver with high serum levels of TNF-α.

Conclusion: From the results we can be discussed that .The ability to measure bcl-2 protein could be useful as a prognostic marker of cancer patients and determine the early and the late stage of cancer. It plays a significant role in diagnosis, monitoring therapy, and evaluating malignant progression in HCC patientsa also, p53 play important roles in hepatocarcinogenesis. Apoptosis-related genes p53, bcl-2, are related to HCC occurrence, and their expression renders an increasing tendency during the formation of HCC. In HCC, p53 is not noticeably related to cell apoptosis, and its high expression may relate to the proliferation of liver cells. Therefore p53 status and the expression of Bcl-2 by tumor cells might be good indicators of sensitivity to chemotherapy for patients with HCC. The results suggested that the high expression of TNF- α gene appears to be associated with an increased risk for the development of HCC in Egyptian population.

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دراسة التعبير الجيني لجينات p53 وBcl2 وTNF-α في مرضي سرطان الخلايا الكبدية في مصر.

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الهدف من هذا العمل هو دراسة بعض الجينات ، وقياس تأثيرها علي سرطان الكبد. أجريت هذه الدراسة علي مرضي التليف الكبدي و سرطان الكبد و تم اختيار المرضي من (معهد الكبد القومي بشبين الكوم)في الفترة من سبتمبر ٢٠٠٨ الي مايو ٢٠٠٩ -.شملت الدراسة ثلاثة مجموعات على النحو التالي-:

المجموعة الأولي: وهي المجموعة الضابطة من الأصحاء وشملت ٢٠

المجموعة الثانية : مجموعة التليف الكبدي وشملت ٣٨

المجموعة الثالثة : مجموعة سرطان الكبد و شملت

-خضعت جميع الحالات لإستعراض التاريخ المرضي وتم تقسيم مرضى سرطان الكبد إلى أ,ب,س تبعا لتقسيم خاص معروف بأسم تقسيم تشيلد كما تم تقسيم مرضي سرطان الكبد أيضا تبعا لحجم الورم .

-تم سحب العينات وفصل مصل الدم وإجراء التحاليل الروتينية على جميع العينات وكذلك تحاليل وظائف الكبد وتحليل فيروس س وتم أيضا تقديركمية AFP في مصل الدم لجميع العينات كما تم استخدام تقنية ELISA في تقدير كمية TNFα في مصل الدم لجميع الحالات .كما تم اجراء تحليل Flowcytometry لتقديركمية Bcl2جين وكذلك كمية p53.

- لوحظ وجود علاقة إيجابية ذات دلالة إحصائية بين المجموعة الظابطة ومجموعةالتليف الكبدي ومجموعة سرطان الكبد في تركيزات Bcl2. كما ان Bcl2 يمكن ان يرتفع تركيزه في المراحل المبكره من السرطان مما يؤدي الي زيادة نمو الخلايا السرطانية بينما يقل تركيزه في المراحل المتقدمة للمرض وهذا يتضح من زيادة نسبة Bcl2 في المرضي الذين يعانون من ورم حجمه اقل من٣سم بالمقارنة بنسبة Bcl2 في المرضي الذين يعانون من ورم حجمه اعلي من ٣سم . وكذالك زيادة نسبة Bcl2 في المرضي الذين يندرجون تحت التقسيم تشيلد(ب+س) بالمقارنة بنسبة Bcl2 في المرضي الذين يندرجون تحت التقسيم تشيلد (أ). وهذا يدل علي زيادة حدوث لية الموت المبرمج للخلايا في المراحل المتقدمة من المرض ونقص حدوث هذه العملية في المراحل الاولي للمرض .و لوحظ وجود علاقة ايجابية ذات دلالة إحصائية بين المجموعة الظابطة ومجموعةالتليف الكبدي ومجموعة سرطان الكبد في تركيزات p53.

- لوحظ زيادة نسبة p53 تدريجيا مع تطور المرض وهذا يتضح من زيادة نسبة p53 في المرضي الذين يعانون من ورم حجمه اعلي من٣سم بالمقارنة بنسبة p53 في المرضي الذين يعانون من ورم حجمه اقل من ٣سم .وكذالك زيادة نسبة p53 في المرضي الذين يندرجون تحت التقسيم تشيلد(ب+س) بالمقارنة بنسبة p53 في المرضي الذين يندرجون تحت التقسيم تشيلد (أ).

- لوحظ وجود علاقة إيجابية ذات دلالة إحصائية بين المجموعة الظابطة ومجموعةالتليف الكبدي ومجموعة سرطان الكبد في تركيزاتTNF α لوحظ زيادة نسبة TNF α تدريجيا مع تطور المرض وهذا يتضح من زيادة نسبة TNF α في المرضي الذين يعانون من ورم حجمه اعلي من٣سم بالمقارنة بنسبة TNF α في المرضي الذين يعانون من ورم حجمه اقل من ٣سم . وكذالك زيادة نسبة TNF α في المرضي الذين يندرجون تحت التقسيم تشيلد(ب+س) بالمقارنة بنسبة TNF α في المرضي الذين يندرجون تحت التقسيم تشيلد (أ).

من خلال هذه الدراسة يمكن ان نستنتج الاتي:-

١-يمكن استخدام bcl2 كمؤشر لمعرفة حدوث مرض السرطان كما يمكن من خلاله معرفة المراحل المختلفة لحدوث المرض .

٢-ايضا يمكن استخدام كلا من TNF α و p53 جين في المساعدة علي تشخيص مرض السرطان.