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RAPID COMMUNICATION

Immunohistochemical analysis of p53, cyclinD1, RB1, c-fos and N-ras gene expression in hepatocellular carcinoma in Iran

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Abstract

AIM: To study the effect of some genes especially those involved in cell cycle regulation on hepatocellular carcinoma.

METHODS: Paraffin-embedded tissue samples of 25 patients (18 males and 7 females) with hepatocellular carcinoma were collected from 22 pathology centers in Tehran during 2000-2001, and stained using immunohistochemistry method (avidin-biotin-peroxidase) for detection of p53, cyclinD1, RB1, c-fos and N-ras proteins.

RESULTS: Six (24%), 5 (20%), 12 (48%) and 2 samples (8%) were positive for p53, cyclinD1, C-fos and N-ras expression, respectively. Twenty-two (88%) samples had alterations in the G1 cell-cycle checkpoint protein expression (RB1 or cyclinD1). P53 positive samples showed a higher (9 times) risk of being positive for RB1 protein than p53 negative samples. Loss of expression of RB1 in association with p53 over-expression was observed in 4 (66.7%) of 6 samples. Loss of expression of RB1 was seen in all cyclinD1 positive, 20 (90.9%) N-ras negative, and 11 (50%) C-fos positive samples, respectively. CyclinD1 positive samples showed a higher (2.85 and 4.75 times) risk of being positive for c-fos and N-ras expression than cyclinD1 negative samples.

CONCLUSION: The expression of p53, RB1 and c-fos genes appears to have a key role in the pathogenesis of hepatocellular carcinoma in Iran. Simultaneous overexpression of these genes is significantly associated with their loss of expression during development of hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and the fourth most common cause of cancer-related death in the world[1]. Male predominance is, however, more obvious in populations at high risk of developing this tumor (mean ratio 3.7:1.0) than in those at low or intermediate risk (2.4:1.0)[2].

HCC has a heterogenous geographical distribution. Countries or regions with the highest incidence (50-120 cases per 100 000 population per year) include China, Taiwan, Korea and other Southeast Asian countries, as well as Sub-Saharan Africa. HCC is linked to environmental, dietary, and lifestyle factors, so that its incidence and distribution vary widely among ethnic groups, geographic regions, and the two sexes[3]. Tumor suppressor genes such as RB1 and p53 may play a significant part in hepatocarcinogensis[3]. As a favorable background for neoplastic transformation, cirrhosis is expected to harbor early genetic changes, but very few studies have been conducted thus far to address this issue. Ashida *et al*[4] have reported in both HCC and adjacent cirrhosis a 60% rate of loss of heterozygosity (LOH) at 13q, the site of the RB1 gene. The loss of heterozygosity and abnormalities in structure and function of the p53 gene are also frequently found in HCC patients[5]. A specific p53 mutation is found in more than 50% of HCC patients from India, China and South Africa, where dietary aflatoxins are suspected to be the major liver specific carcinogens[5-11]. However, it occurs less frequently in Western countries[3,5]. Activation of oncogenes of the "ras" family and others has been detected during chemically induced HCC in rodents, but there is little evidence of such activation in human tumors[12]. CyclinD1 over-expression may be an early event in hepatocarcinogenesis and plays a role in tumor differentiation[13]. Yuen *et al*[14] reported that the expression of c-fos is significantly higher in tumor tissue than in non-

tumor tissue. Specific mutations of the p53, cyclinD1, RB1, c-fos, and N-ras genes and their expression in HCC have been reported from several parts of the world, but to the authors' knowledge to date, the expression status of these genes has not been studied in HCC patients in Iran, where the frequency of chronic hepatitis B and C virus infection as well as exposure to dietary aflatoxin is very high[15].

MATERIALS AND METHODS

Sample collection

Formalin-fixed and paraffin-embedded tissue samples of 25 patients (18 males and 7 females) with documented HCC (surgically resected material or biopsy) were provided for analysis. The samples were collected from 22 pathology centers in Tehran during 2000-2001. The study was approved by the Medical Ethics Committee, Ministry of Health, Iran, as conforming to the ethical guidelines of the 1975 Declaration of Helsinki. Hospital records were used to verify age, sex and other demographic items.

Tissue preparation

These samples were sectioned and stained with hematoxylin and eosin (HE). Diagnosis of HCC was confirmed and the grade of tumor was determined according to the criteria proposed by the World Health Organization by the collaborating pathologist in Research Center for Gastroenterology and Liver Disease.

HCC was considered to be adequate for immunohistochemical study only if the block was of adequate size (surface area of section > 4 cm^2 and > 10% of the surface area of the block was occupied by the tumor).

Immunostaining

The technique was based on avidin-biotin-peroxidase method using 10% formaldehyde-fixed and paraffin-embedded sections. The selected paraffin blocks were cut into 5 μm-thick sections. The sections were applied to precoated glass slides to avoid becoming detached, then dried at 37℃ overnight followed by drying at 56℃ for 60 min, deparaffinized with xylene and rehydrated through graded concentrations of alcohol. Antigen retrieval was performed by 3 × 5-min cycles of microwave oven heating (750W) at 100℃ in 0.01 mol/L citrate buffer at pH 6. After washing and rinsing with Tris-buffered saline (TBS, 0.05 M, pH 7.2-7.6), endogenous-peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature and then rinsed with distilled water. Subsequently, sections were treated for 10 min with 10% bovine serum albumin (BSA) at room temperature for blocking nonspecific background staining. Afterward, primary monoclonal mouse antihuman-p53 protein, clone DO-7, isotype IgG2bkappa (DAKO, Lot 108) at dilution 1:100, primary monoclonal mouse antihuman-RB1 gene product, clone RB1, isotype IgG1kappa (DAKO, Lot 019) at dilution 1:50, primary monoclonal mouse antihuman-cyclinD1, clone DCS-6, isotype IgG2a kappa (DAKO, Lot 012) at dilution 1:50, primary monoclonal mouse antihuman-c-fos, clone D-1, isotype IgG2b (Santa Cruz Biotechnology Inc., Lot J 251) at dilution 1:100 and primary monoclonal mouse antihuman-N-ras, clone F155, isotype IgG1 (Santa Cruz Biotechnology Inc., Lot I 251) at dilution 1:100 were added and incubated in a moist chamber overnight at 4℃. The sections were again washed three times in TBS for 5 min using the DAKO LSAB2 system (Universal, HRP, Lot 10106). Goat anti-mouse and anti-rabbit biotinylated IgG (diluted in PBS containing carrier protein and 0.015 mol/L sodium azide) and preincubated streptavidin conjugated to horseradish peroxidase (diluted in PBS containing carrier protein and anti-microbial agents) were added for 30 min at room temperature. The sections were washed in TBS as before and then developed in prepared 3-amino-9-ethylcarbazole substrate chromogen (AEC/H_2O_2) for p53 and RB1 and in prepared 3,3'-diaminobenzidine chromogen solution (DAB/H_2O_2) for cyclinD1, c-fos and N-ras for 10 min at room temperature. The sections were then washed in water, counterstained with Mayer's hematoxylin for 2-5 min at room temperature, dehydrated, cleared with 37 mmol/L ammonia water, rinsed in a bath of distilled water for 2-5 min, finally mounted and coverslipped with Faramount aqueous-based mounting medium (DAKO, Lot 00029). A section of the same tumor incubated in BSA instead of the primary antibody was included as the negative control. We used one standard p53 positive section of human SCC (DAKO, Lot 071-1), one known RB1 negative retinoblastoma section, one known cyclin D1 positive breast tumor section, one known c-fos positive astrocytoma section and one known N-ras positive lymphoma section as a positive control for each staining.

Assessment of immunostaining

Staining of p53, RB1, cyclinD1, c-fos and N-ras genes was examined at high power fields (× 400) under a standard light microscope. Nuclear staining was regarded as positive if there was homogeneous staining or > 10% of the cancer cells were heterogeneously stained.

Statistical analysis

The results were expressed as frequency for gene expression changes and odds ratio for association between expression changes of these five genes. All statistical tests were performed with the Program Statistical Package for the Social Sciences (SPSS version 11, Chicago, IL).

RESULTS

The mean ± SD age of our patients was 60.56 ± 12.52 years and the highest frequency (44%) was seen in the sixth decade of life. Male to female ratio was 2.57 (18 males and 7 females) and mean ± SD age of patients in each sex was 62.72 ± 10.43 years and 55 ± 16.4 years, respectively.

Histopathology

All the 25 samples were well differentiated (grade Ⅰ).

Accumulation of p53, RB1, cyclinD1, c-fos and N-ras proteins

Intense immunostaining of p53, RB1, cyclinD1, c-fos and

Table 1 Expression of p53 gene in relation to the expression of RB1, cyclinD1, c-fos and N-ras genes, *n* (%)

Gene expression	RB1		Cyclin-D1		c-fos		N-ras	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
P53 positive	2 (33.3)	4 (66.7)	2 (33.3)	4 (66.7)	4 (66.7)	2 (33.3)	1 (16.75)	5 (83.3)
P53 negative	1 (5.3)	18 (94.7)	3 (15.8)	16 (84.2)	8 (42.1)	11 (57.9)	1 (5.3)	18 (94.7)
	OR: 0.1, 95% CI: 0.0-1.5		OR: 2.6, 95% CI: 0.3-21.7		OR: 2.7, 95% CI: 0.4-18.8		OR: 3.6, 95% CI: 0.1-68.3	

Table 2 RB1 gene expression in relation to the expression of c-fos and N-ras genes, *n* (%)

Gene expression	c-fos		N-ras	
	Positive	Negative	Positive	Negative
RB1 positive	1 (33.3)	2 (66.7)	0	3 (100)
RB1 negative	11 (50)	11 (50)	2 (9.1)	20 (90.9)
	OR: 0.5, 95% CI: 0.0-6.3		OR: 1.1, 95% CI : 0.9-1.2	

Table 3 G1 checkpoint protein expression in relation to p53, *n* (%)

Expression pattern of G1 checkpoint proteins	P53 (+)	P53 (-)	*P*
Rb+/cyclinD1+	Zero	Zero	
Rb-/cyclinD1+	2 (40)	3 (60)	
Rb-/cyclinD1-	2 (11.8)	15 (88.2)	0.02
Subtotal	4 (66.6)	18 (94.7)	
Rb+/cyclin D1-	2 (66.7)	1 (33.3)	
Total	6 (24)	19 (76)	

N-ras proteins was observed in the cell nuclei of tissues. Overall, six (24%) samples showed nuclear accumulation of p53 protein in varying proportions of tumor cells, the rest of the samples (76%) were negative. Twenty-two (88%) samples showed complete loss of RB1 protein expression in the primary tumor, the rest of the tumors (12%) displayed variable proportions of RB1 protein positive tumor cells. The intensity and subcellular location of the staining in the tumor were similar to those observed in the normal epithelia. We detected high levels of cyclinD1 protein in 5 (20%) samples while 20 (80%) samples were negative for cyclinD1 expression. The frequency of c-fos and N-ras positive staining was 48% (12 samples) and 8% (2 samples), respectively. The expression of p53 gene in relation to the expression of RB1, cyclinD1, c-fos and N-ras genes is depicted in Table 1. The RB1 gene expression in relation to the expression of c-fos and N-ras genes is shown in Table 2. Of those samples positive for the c-fos gene, 1 (8.3%) was N-ras positive and 11 (91.7%) were N-ras negative. On the other hand, among the c-fos negative samples, 1 (7.7%) was N-ras positive and 12 (92.3%) were N-ras negative.

When overexpression of p53 was seen, loss of expression of RB1 was found in 4 (66.7%) samples. Loss of expression of RB1 was observed in all those with positive cyclinD1 (5 samples), while expression of RB1 was found in 17 (85%) with negative cyclinD1, and in 3 (15%) samples with positive RB1. CyclinD1 positive samples showed a higher risk of being positive for C-fos and N-ras (2.85 and 4.75 times, respectively) than cyclinD1 negative samples. Finally, loss of expression of RB1 was detected in 2 samples with overexpression of N-ras. On the other hand, among the samples with loss of expression of RB1, overexpression of c-fos was found in 11 (50%).

Overall, 22/25 (88%) samples had alterations in the G1 cell-cycle checkpoint proteins, as assessed by means of cyclinD1 and RB1 expression (Table 3). These occurred in 4 (66.6%) of 6 p53-positive samples and in 18 (94.7%) of 19 p53-negative samples. P53-negative samples showed absence of the RB1 protein more frequently. P53 positive samples showed a higher (9 times) risk of being positive for RB1 than p53 negative samples, being 3.6, 2.75, and 2.66 for N-ras, c-fos, and cyclinD1, respectively. In samples with cyclinD1 positive staining, the risk of being positive for N-ras was 4.75 times higher in samples with cyclinD1 positive staining than in samples with negative cyclinD1 staining for this protein.

DISCUSSION

Several oncogenic pathways have been implicated in malignant transformation of liver cells. Inactivation of the p53 tumor suppressor gene by mutations and allelic deletions in about 30% of HCC cases has been associated predominantly with exposure to aflatoxin B1 and hepatitis B virus infection[16]. Activation of cyclinD1, c-fos and N-ras and disruption of the RB1 pathway are also commonly involved in liver tumorigenesis. New major challenges include the identification of candidate genes located in frequently altered chromosomal regions and oncogenic pathways driven by different risk factors. Deranged expression of cell cycle modulators has been reported to contribute to the development and progression of HCC[17]. In human HCC, high frequencies of aberration have been detected in the p53 and RB1 genes[14]. Mutations of the p53 tumor suppressor gene have been reported to occur with varying frequency in different geographic regions, which might be a different etiology for HCC[18]. In our study, nuclear accumulation of p53 protein was seen in 24% of samples. Mutations of this gene have been identified in 30%-50% of HCC patients in some geographic areas[19]. An *et al*[20] reported that there is histological heterogeneity in established HCC, which is accompanied with increased proliferative activity and p53 overexpression. Overexpression of p53 has identified in 37.5% of Japanese HCC patients and 62.5% of Indonesian HCC patients[18]. Recently, Ming *et al*[21] also showed that the frequency of mutation of p53 gene is much higher in high prevalent HCC area than in the low-risk HCC area in China. More than 95% cancer specimens

exhibit strong intranuclear accumulation of p53 protein, which can be detected by immunohistology. However, Biersing *et al*[22] and Vesey[23] have found little or no point mutations of p53 gene in human hepatocarcinoma in Swedish and Australian patients. Therefore overexpression of p53 protein in hepatocarcinoma specimens can be used as the mutant p53 biopathological marker in tumor tissues. Qin *et al*[8] reported that accumulation of p53 is a valuable marker for predicting the prognosis of HCC patients. Lin GY *et al*[7] reported that inactivation of the tumor suppressor genes p53 and RB1 has been demonstrated in different forms, and implies the pathogenesis of human malignant diseases. The study of Kondoh *et al*[24] supports the idea that deletion or inactivation of tumor suppressors including RB1, p53 and other candidate genes seems to be common events in HCC development. Abnormalities of the RB1 tumor suppressor gene have been found in 20%-25% of HCCs, including 80%-86% of HCCs with p53 mutations[19]. Nishida *et al*[25] reported that RB1 protein is positive in 85.6% of HCC cases but is not related to any clinicopathological parameters. Positive immunostaining for RB1 and mutant p53 protein is detectable in 58% and 37% of HCCs, respectively[26]. Loss of expression of RB1 in HCC has been reported in several studies[21,27]. In this study, loss of expression of RB1 gene was found in 88% of samples. The proto-oncogene c-fos is involved in cell cycle progression and cellular proliferation[14]. Abuthnot *et al*[28] reported that c-myc and c-fos mRNA, as well as their protein products, are increased in human liver cancers. Wang *et al*[29] have also found an apparently higher expression of N-ras and c-fos in human hepatoma than in its adjacent liver tissue. Recently, Feng *et al*[30,31] reported that the positive rates and signal intensity of c-fos and some other proteins in HCC are significantly higher than those in pericarcinomatous tissues. Yuen *et al*[14] found that the expression of c-fos was significantly higher in tumor tissue than in nontumor tissue (91% *vs* 0%, $P < 0.0001$). C-fos primarily induces cyclinD1 up-regulation by a mediator called MAPK/ERK[32]. In our study, the expression of c-fos gene was detected in 48% of patients with documented HCC. There was no significant relationship between c-fos and Cyclin-D1 expressions. Aflatoxin B1 may evoke an intense and prolonged expression of c-fos, including persistent signals for regeneration, which in turn may activate the replication of immature cells[33]. CyclinD1 is frequently overexpressed in a variety of cancers, including HCC, as a result of gene amplification. Overexpression of cyclinD1 protein, through gene amplification, correlates with poor prognosis of several cancers, but its role in HCC is the subject of controversy. Increased expression of cyclinD1 may play an important role in the development of HCC owing to the perturbation of normal control of the cell cycle[34]. On the other hand, Azechi *et al*[35] reported that cyclinD1 is a known oncogene and a key regulator of cell cycle progression. Amplification of the cyclinD1 gene and its overexpression are associated with aggressive forms of HCC. Overexpression of cyclinD1 is sufficient to initiate hepatocellular carcinogenesis. Choi *et al*[17] and Deane *et al*[36] have found a positive relationship between cyclinD1 overexpression and advanced tumor stage and aberrant p53 expression in HCC ($P < 0.05$). Joo *et al*[13] reported that cyclinD1 overexpression may confer additional growth advantages to the tumor in addition to protein RB1 inactivation in HCC. On the contrary, Sato *et al*[37] have found no significant relationship between the expressions of cyclinD1 and p53. Ito *et al*[38] conducted a simultaneous immunohistochemical study with p53 and cyclinD1 antibody in the same series of HCC and revealed that 88% of the patients positive for cyclinD1 also expressed p53 and 91% of the patients negative for p53 did not express cyclinD1, suggesting that cyclinD1 is expressed later than the alteration of p53 in the progression of human HCC. In our study, cyclinD1 was positively related to aberrant p53 expression. In HCC, N-ras was first proved as one of the transforming genes[39], which belongs to the G protein family. When it is converted to an active oncogene by point mutation, chromosome rearrangement or gene amplification, the signal transmission of cell membranes may change, which drives cell division, leading to abnormal differentiation and formation of neoplasm. Cerruti[40] and Tada[41] reported that the mutagenesis of a proto-oncogene from "ras" family and p53 tumor suppressor gene might be the most important event in HCC. Tabor[19] reported that overexpression of oncogenes N-ras and c-fos has been found in high percentages of HCC patients. Imai *et al*[42] and Tamano *et al*[43] found that mutations of ras oncogene may be the early events, and the expression in tumor or non-tumor tissues can be detected with different rates. Luo *et al*[26] reported that N-ras and p53 genes might be involved in the carcinogenesis and development of HCC. They also showed that mutation of the tumor suppressor gene p53 can convert ras gene into oncogene. In their study, 38% of HCCs with N-ras gene mutation did not express p53 protein, indicating that some other genes or factors may participate in the carcinogenesis and development of HCC. In our study, 83% of p53 positive samples did not show N-ras mutation. Chao *et al*[9] suggested that activation of the ras gene might not be a major event in aflatoxin-related human hepatocarcinogenesis. This hypothesis is supported by another study[44] conducted in southern Africa on Blacks, where dietary exposure to aflatoxin is a risk factor.

In conclusion, as in other parts of the world, the change in expression pattern of these genes especially p53, RB1 and c-fos, appears to have a key role in the pathogenesis of HCC in Iran. There is likely a relation between the simultaneous changes in these genes during development of HCC. This research might shed some light on the carcinogenic role of the expression of p53, RB1, cyclinD1, c-fos and N-ras genes. Besides, in order to understand the exact role of these changes in development of HCC, further studies with a larger number of samples are essential.

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