

Correlation between Hcv-NS3P and P53, P21^{waf}, MDM2, P21 RAS, and C-ERBB2 in HCV Associated Hepatocellular Carcinoma and Adjacent Pericarcinomatous FOCI

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ABSTRACT

Purpose: The non-structural protein 3 (NS3P) of HCV genome was linked to the neoplastic transformation of normal hepatocytes in chronically infected patients. However, the exact mechanisms involved in this process are unidentified yet especially in the Egyptian population where genotype 4 represents the commonest type.

Patients and Methods: We investigated 32 HCV RT-PCR positive hepatocellular carcinoma (HCC) and 18 adjacent pericarcinomatous (PC) foci for the correlation between HCV- NS3P, p53, p21^{waf}, mdm2, p21 ras and c-erbB2 and DNA content.

Results: NS3P expression was lower in HCC (65.6%) than in PC (94.4%). The expression level of studied genes in HCC was: p53 (56.25%), p21^{waf} (43.7%), mdm2 (59.4%), p21 ras (73.3%) and c-erbB2 (75%). In PC it was 22.2%, 61.1%, 44.4%, 41.2% and 77.8% respectively. NS3P expression showed a significant correlation with the tumor grade, the presence of cirrhosis and chronic active hepatitis (CAH) ($p < 0.05$).

Overexpression of c-erbB2 and absence of p21^{waf} were higher in PC than in carcinomatous foci however, this did not reach a statistically significant level. There was no correlation between NS3P and any of the studied genes in HCC however there was a strong positive correlation between NS3P, c-erbB2 and p21^{waf} ($p < 0.01$) in the PC. There was a significant correlation between absence of p21^{waf} and CAH ($p = 0.01$) as well as between mdm2, c-erbB2 and cirrhosis ($p = 0.025$ and 0.001) in HCC cases.

There was a statistically significant difference in the ploidy status between HCC and PC ($p < 0.05$), however there was no significant relation between the ploidy status and other clinicopathological features.

Conclusion: NS3P may exert its carcinogenic effect at an early stage of HCC possibly through a pathway involving c-erbB2 and p21^{waf} alterations. On the other hand, p53, p21ras and mdm2 alterations are late events in this

genetic cascade and are usually associated with worse differentiation and an aggressive behavior.

Key Words: HCV - HCC - P53 - P21^{waf} - MDM2 - Gerb-B₂.

INTRODUCTION

Hepatitis C virus (HCV) represents a major health problem. It is considered a principle causative agent of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) throughout the world it accounts for 20% of acute hepatitis, 70% of chronic hepatitis, 40% of end stage cirrhosis and 60% of hepatocellular carcinoma (HCC) cases [20]. In Egypt, HCV infection has been reported in 11% to 22% of the population [3]. Subtype 4 was shown to be the commonest HCV type in Egypt being detected in 73.3% of the positive cases either alone or as mixed infection [33]. However, data regarding subtype 4 is still scarce since it is rare in Western countries and USA [34]. HCV has a positive single strand RNA genome encoding a polyprotein which is cleaved co- and post-translationally into, at least 10 different products among which is the non-structural protein 3 (NS3P) [5]. The NS3P is a serine protease that affects normal cellular functions such as cell proliferation and death. The frequent involvement of NS3P in HCV-associated HCC was recently demonstrated but the mechanisms are still unclear [11]. However, Zemel et al., 2001 [36] suggested a direct effect on hepatic transformation via modifying cellular regulatory mechanisms and Feng

et al., 1998 [7] mentioned that, p53 inactivation might be one of the possible pathways through which NS3 induces HCC.

The p53 monitors various cellular stresses and DNA damage through affecting cell cycle arrest and apoptosis [20]. Previous studies showed that, p53 functionally interacts with several cellular proteins, some of them suppress [28] while others enhance its function [14]. Studies on the p53 inactivating mechanisms demonstrate an autoregulatory feedback loop between the mdm-2 gene and p53 since the wild type (WT) p53 can induce the expression of mdm-2 via a p53 binding site in the mdm-2 gene while mdm-2 protein functions as a negative regulator of p53 [24]. Abnormalities of the p53 gene and/or its protein product has been reported in different studies on HCC with frequencies ranging from 23% to 67% [12,22]. However, due to the few available reports, the relationship between mdm-2 gene expression and p53 in HCC is still unclear [24,28].

The p21^{waf} is a down-stream effector of p53. Cells lacking functional p53 express a very low level of p21^{waf} whose promoter contains a p53 binding domain suggesting that, the expression of p21^{waf} depends on normal p53 function [10]. However, p21^{waf} can be induced in p53 null cells indicating that its expression can also be induced in p53 independent pathways [13,27]. Hui et al., 1997 [10] showed that reduced expression of p21^{waf} as a consequence of p53 mutations may contribute to hepatocarcinogenesis.

Recently, it was shown that, c-erbB2 overexpression leads to p21^{waf} inactivation through phosphorylation of the Akt protein with subsequent cytoplasmic sequestration [38]. The few available studies show that c-erbB2 overexpression is a frequent finding in HCC that usually correlates with poor prognosis [9,32,33]. Voravud et al., 1998 [32] were able to detect positive immunostaining for c-erbB2 in 86% of the cases and Zekri et al., 2000 [33] reported c-erbB2 overexpression in 78.8% of their cases using ELISA technique.

An important function of HCV proteins is the modulation of various cellular signal transduction pathways. The p21^{ras} gene is a crucial regulator of signal transduction cascades that leads to cell proliferation, differentiation and apoptosis. It is also one of the most commonly

mutated genes in solid tumors where aberrant ras signaling can be induced directly by mutations or indirectly by altering genes that associate with ras or its signaling pathway [4]. The involvement of the ras gene in liver regeneration was documented in rats following partial hepatectomy and CCl₄ injury [30]. In addition, Ray et al., 1996 [33] demonstrated that, HCV core protein cooperates with H-ras to immortalize rat fibroblasts in culture. Haritani et al., 1990 [8] reported a relatively high level of K-ras in nontumor liver tissues indicating the importance of this gene in normal liver function and its latent ability to induce proliferation in response to appropriate stimuli.

Abnormal DNA content is considered an important marker for the biological behavior and prognosis of many solid tumors including HCC [2]. Attallah et al., 1999 [2] demonstrated in a recent study that abnormal DNA content could be recognized not only in HCC but also in cases of liver cirrhosis. However this is still a debatable issue since other studies failed to confirm such finding [29].

In the present study we investigated the correlation between NS3P, DNA ploidy and the expression level of p53, p21^{waf}, p21^{ras}, mdm2 and c-erbB2 in HCV-associated HCC and pericarcinomatous foci (PC). This was done in an attempt to understand the possible pathogenetic mechanisms contributing to the development and progression of HCC in chronic HCV infected patients.

PATIENTS AND METHODS

Patients and tissues: We examined 32 cases of operable primary HCC resected at the National Cancer Institute, Cairo University during the period 1998-2000. The mean age of the patients was 51.6 years (range 10-74) and the male: female ratio was 1.3:1. Eighteen samples of the pericarcinomatous (PC) hepatic tissues showing cirrhosis and/or chronic active hepatitis (CAH).

Tissue samples were freshly obtained at operation and divided into two pieces. One piece was immediately snap frozen and stored at -70°C for molecular detection of HCV and HBV. The other was fixed in neutral buffered formalin and processed for routine histological examination and immunohistochemical (IHC) studies. Unintentional bias was prevented by

coding patient tissue samples so that, molecular and IHC studies were done without knowledge of the patient and tumor characteristics. All samples included in this study were HBV negative by PCR and serum markers. A written consent was taken from the patients prior to enrollment in the study.

Analysis of pathological features:

Macroscopic and microscopic parameters of the tumors and the non-tumorous liver was assessed by 2 independent pathologists and reported as described by Ng et al., 1995 [16] (Table 1).

Viral studies:

1- *Molecular detection of HCV:* RNA extraction, RT and PCR amplification were done according to Zekri et al., 2000 [33]. RNA was assessed for degradation, purity and DNA contamination by spectrophotometry and electrophoresis in an ethidium bromide-stained 1.5% agarose gel. Ten microlitres of each aplicon were analyzed by electrophoresis through a 1.5% ethidium bromide-stained agarose gel. DNA was transferred from the gel onto a nitrocellulose filter with alkaline buffer (4N NaOH), cross-linked by incubation for 2-3 hours at 80°C and the blot was then hybridized with an internal probe.

2- *Molecular detection of HBV:* DNA extraction from fresh tissues and PCR amplification were performed as described by Boom et al., [6].

Immunohistochemistry: Five micron thick sections were placed onto sialinized slides and air-dried overnight at room temperature (RT). After dewaxing in xylene and rehydration through graded alcohol, slides were incubated for 10 min in 3% H₂O₂ to abolish endogenous peroxidase activity. Sections were immersed in citrate buffer (Antigen Retrieval: Biogenex, San Romano, CA), heated for 20 min at 900C in a microwave and cooled for 30 min at RT for antigen retrieval. The primary antibody was added at a working concentration (Table 2) and incubated for 2 hours at RT. After washing, all sections were immunostained with the universal labeled streptavidin-biotin method (Vector) according to the manufacturer's instructions. Positive staining was detected with 0.3% 3,3'-diaminobinazidine tetrahydrochloride in distilled

water and nuclei were counterstained with Meyer's hematoxylin.

Interpretation of immunoreactivity:

Expression of p53 and mdm2 was assessed quantitatively by counting the total number of positively-stained nuclei/10 high power field (HPF) (X400) in tumors and non tumorous liver tissues, c-erbB2 assessment was based on membranous staining pattern, that of NS3P and p21ras was based on cytoplasmic pattern whereas p21waf assessment was based on the nuclear staining however, the presence of cytoplasmic staining was reported. The immunoreactivity for p53, p21waf, p21ras and mdm2 was classified as follows: negative, <10% positive cells; minimally positive, 10-50% positive cells; moderately positive, >50% but <75% positive cells; markedly positive, >75% positive cells. Both c-erbB2 and NS3P proteins were considered over-expressed when at least 1% of the tumor cells were positively stained [27,31].

Ploidy analysis:

Twenty-seven HCC cases and 18 samples obtained from PC were eligible for determination of the ploidy status using the CAS 200D image analysis system (Bekton Dickinson) with specific filtering technique as previously described [25]. Each nucleus was classified according to its DNA content, size and shape following the deletion of nuclear fragments, touching and overlapping cells using the cut and point and classify modes. The tissue correction mode of the system (DNA Quantitative Analysis, Version 3) was applied to adjust tissue thickness using internal control cells on the same section examined that have normal diploid value, either lymphocytes or normal epithelial cells. This was achieved by adjusting the diploid cells to fall within the range 1-1.1 as compared with the DNA content of normal diploid cells. In each case, 100-200 nuclei were measured for their DNA content.

Interpretation of DNA Histograms was performed according to Shankey et al., 1993 [25]: diploid; when the main peak falls within the range of 0.9-1.1, tetraploid; when it is in the range of 1.8-2.2, aneuploid; tumors with DNA peaks outside these areas, and multiploid; tumors with multiple aneuploid cell populations.

Genotyping of HCV was performed using Inno-LIPA-2 kit according to manufacturer's instructions.

Statistical analysis:

T and Wilcoxin's rank tests as well as X2 test were used for the analysis of categorical data, whereas correlation analysis and ANOVA tests were used for continuous data. Tests were considered significant when *p* values were less than 0.05.

RESULTS*Results of immunostaining:*

The results of IHC are compiled in tables 1,3 and figures 1,2,3. Positive immunostaining for NS3P was detected in 26/32 HCC cases (81.25%) and 17/18 of the PC (94.4%). In all positive cases the intensity and the number of positively-stained cells were higher in morphologically normal hepatic tissues surrounding the tumors, if present, than in the neoplastic foci. The majority of the cases (19/26) showed a diffuse granular cytoplasmic staining whereas 7 cases revealed a heterogeneous staining pattern.

In HCC cases, p53 overexpression was detected in 18/32 cases (56.25%), 5 of them showed marked expression, 6 showed moderate expression and 7 showed minimal expression. mdm-2 overexpression was present in 19/32 cases (59.4%), 5 showed marked expression and 14 showed moderate expression. Out of the 19 positive cases, 13 cases (68.4%) revealed simultaneous p53 overexpression. Overexpression of p21ras was detected in 22/30 cases (73.3%) and 2 cases were inconclusive. Membranous immunostaining for c-erbB2 was detected in 24/32 cases (75%) whereas p21 waf expression was detected in 14/32 cases (43.7%) (Table 3).

In PC, p53 overexpression was detected in 4 cases (22.2%), mdm2 in 6 cases (33.3%), p21ras in 11 cases (61.1%), c-erbB2 in 14 cases (77.8%), p21 waf in 9 (50%) cases (Table 3).

DNA ploidy analysis:

Thirteen HCC cases (48.1%) showed aneuploid DNA pattern, 12 (44.4%) were multiploid with multiple aneuploid peaks and 2 cases were diploid (7.4%). Two out of the 3 grade I tumors (66.7%) were aneuploid and a single case (33.3%) was multiploid whereas, 9/20 (45%) GII tumors were aneuploid and another 9 were multiploid. As regards GIII tumors, 2 cases (50%) were aneuploid and the other 2 were multiploid. Thirteen out of the 18 samples of

PCs (72.2%) showed diploid peaks and 5 were aneuploid (27.8%). All the aneuploid cases revealed features of CAH. The difference between HCC and PC regarding the ploidy status was statistically significant ($p < 0.05$).

Correlations:

Statistical analysis revealed significant correlation between the expression level of NS3P and the grade of the tumor ($p = 0.025$) as well as with the presence of CAH ($p = 0.001$) and cirrhosis ($p = 0.001$) in HCC cases. However, there was no significant relationship between neither the intensity nor the pattern of NS3P staining and any of the clinical features of the studied cases. Similarly there was no significant relation between NS3P and the expression level of any of the studied genes.

A positive correlation was present between overexpression of p53 and both mdm2 ($p = 0.0001$) and c-erbB2 ($p = 0.001$) as well as between mdm2 and c-erbB2 ($p = 0.008$). On the other hand, there was a negative correlation between absence of p21 waf expression, p53 ($p = 0.0001$) and c-erbB2 ($p = 0.02$) overexpression.

There was a statistically significant difference between HCC and PC in the expression levels of p53 and mdm2.

Using the Wilcoxin's rank test the degree of expression of the studied genes in relation to PC showed that the highest expression is that of NS3P and c-erbB2 ($p = 0.0001$), p21ras ($p = 0.001$), loss of expression of p21 waf ($p = 0.006$), overexpression of p53 ($p = 0.02$) and mdm2 ($p = 0.04$). There was a significant relationship between overexpression of c-erbB2 ($p = 0.0008$), mdm2 ($p = 0.025$) and the development of HCC in cirrhotic patients as well as between absence of p21 waf expression and CAH-associated HCC ($p = 0.015$) (Table 1).

The expression level of p21 waf was significantly correlated with the presence of intrahepatic and venous metastases ($p = 0.0001$ & 0.001 respectively). Overexpression of p53 was strongly correlated with intrahepatic ($p < 0.001$), venous ($p = 0.02$) and lymph node ($p = 0.001$) metastasis as well as with large tumor size and the degree of differentiation. On the other hand, overexpression of c-erbB2 was correlated to the number of hepatic nodules whereas overexpression of p21ras was significantly associated with

the number of hepatic nodules, large tumor size and lymph node metastasis ($p < 0.05$) (Table 1).

Statistical analysis did not reveal any significant relation between the ploidy status of the

examined tumors and any of the investigated parameters including the expression level of NS3P, p53, p21 waf, p21ras, mdm2 and c-erbB2 or any of the clinicopathological features of the patients.

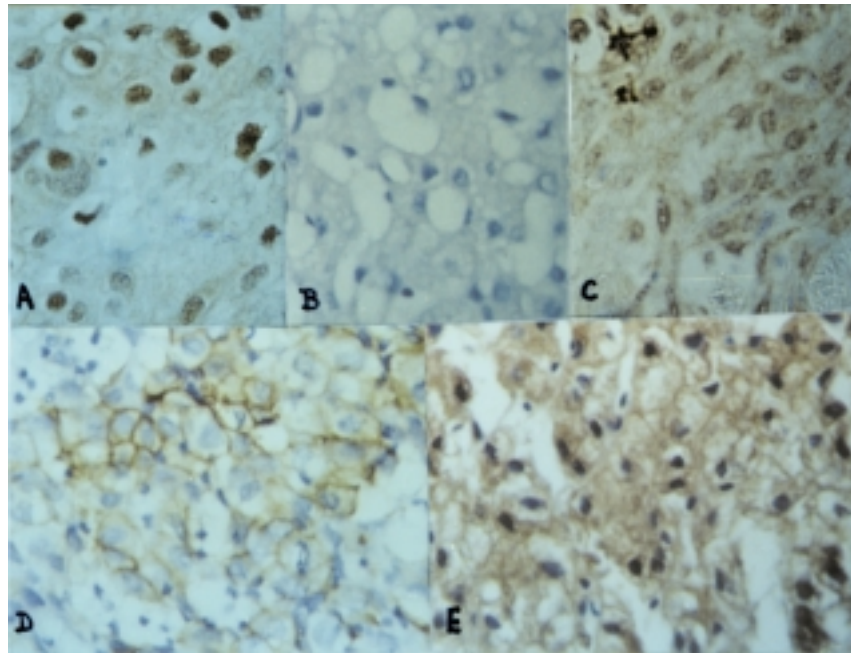


Fig. (1): Cases of HCC showing overexpression of p53 (a), mdm2 (c), c-erbB2 (d), p21ras (e) and absence of nuclear p21waf expression (b).

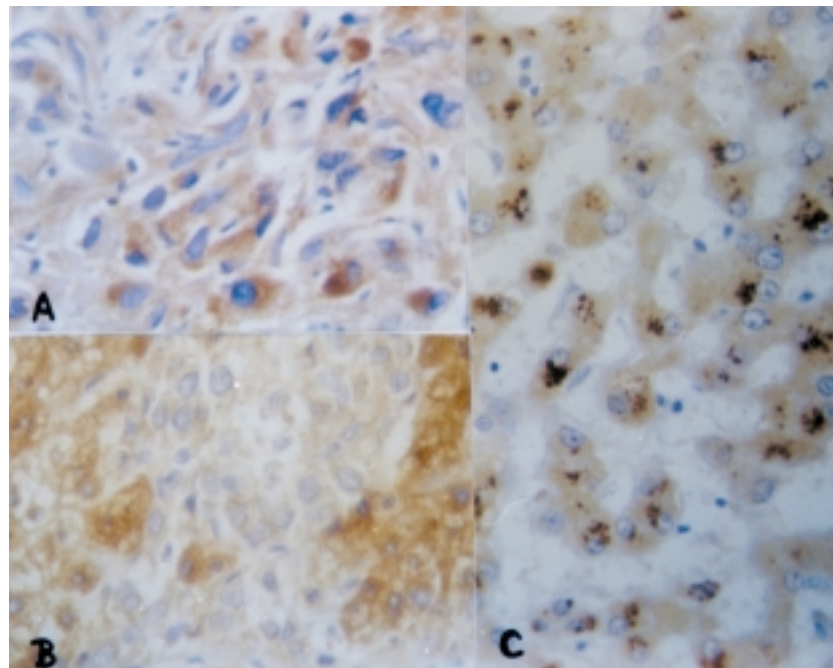


Fig. (2): Positive immunostaining for NS3P showing diffuse cytoplasmic staining pattern in a sample obtained from adjacent pericarcinomatous tissue (a), HCC (b) and a strong granular staining pattern in normal hepatic tissue (c).

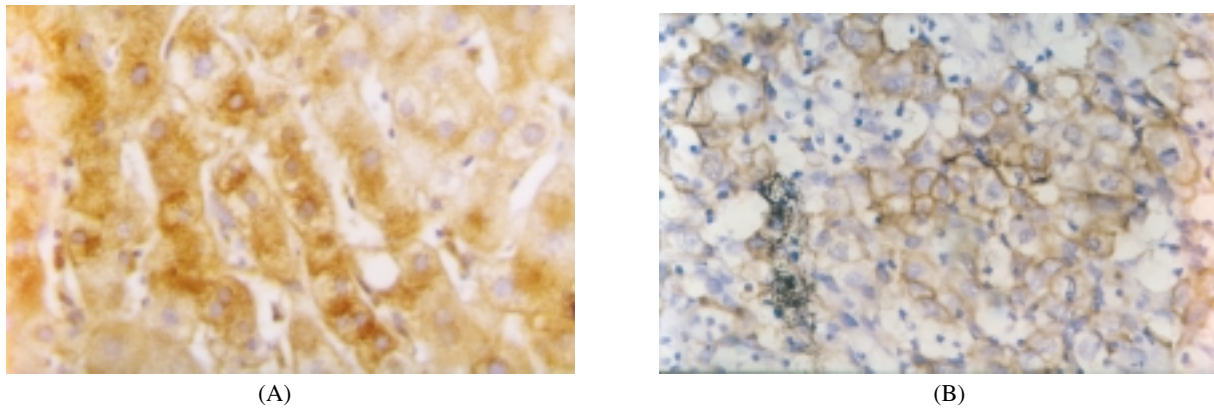


Fig. (3): Positive immunostaining for p21waf showing cytoplasmic sequestration of the protein (a) in a case of HCC with c-erbB2 overexpression (b).

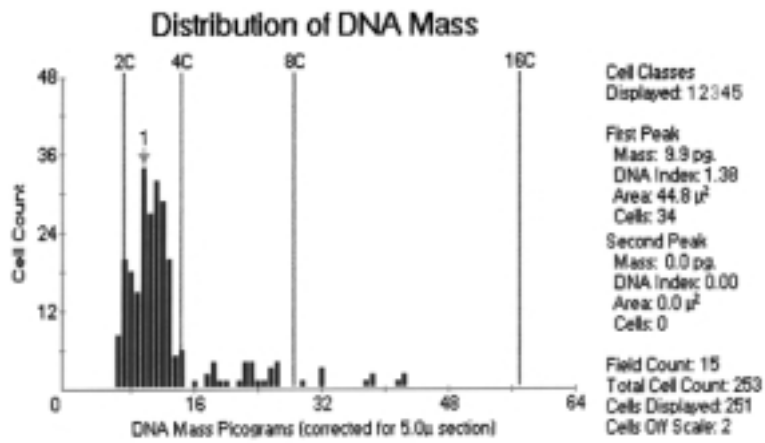


Fig. (4-A)

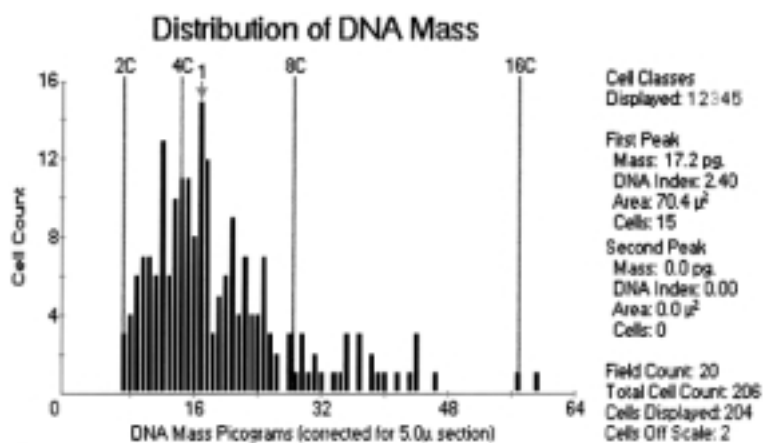


Fig. (4-B)

Fig. (4): Image cytometry histogram showing aneuploid DNA pattern (a), and multiploid pattern with multiple aneuploid peaks (b).

Table 1: Genes Expression In Relation To Pathological Features of HCC Cases

Parameters	No. (32)	P53 (18/32)	P21waf (16/32)	Mdm2 (19/32)	P21 ras (22/30)	c-erbB2 (24/32)	NS3 (26/32)
<i>No. of tumor nodules</i>							
Single	12	10	9	8	5	6	12
>2	20	8	7	11	17	18	14
<i>Microsatellites</i>							
Absent	11	5	2	9	10	11	11
Present	21	13	14	10	12	13	15
<i>Cirrhosis</i>							
Absent	7	1	7	5	7	3	5
Present	25	17	9	14	15	21	21
<i>T. Size</i>							
<5 cm	16	5	9	10	7	13	16
>5 cm	16	13	7	9	15	11	10
<i>Differentiation</i>							
I/II	10	4	7	8	10	10	8
III/IV	22	14	9	11	12	14	18
<i>Eggle's Class.</i>							
Massive	12	9	7	10	12	12	11
Nodular	20	9	9	9	10	12	15
<i>Encapsulation</i>							
Absent	17	7	8	9	9	14	11
Present	15	11	8	10	13	10	15
<i>Direct Invas.</i>							
Absent	20	9	11	12	11	14	15
Present	12	9	5	7	11	10	11
<i>Res. Margin</i>							
Neg.	20	10	7	10	13	11	14
Pos.	12	8	7	9	9	13	12
<i>Venous perm.</i>							
Absent	13	5	4	11	11	14	13
Present	19	13	12	8	11	10	13
<i>Inflamm. Infilt.</i>							
Mild	14	9	5	9	12	12	8
Moderate/severe	18	9	11	10	10	12	18
<i>L. Node Involvement</i>							
Negative	20	3	7	7	19	12	12
Positive	12	15	9	12	3	12	14

Table 2: Summary of Specific Monoclonals and Immunohistochemistry Conditions

Protein	Antibody	Dilution and incubation	Antigen pretreatment	Positive control	Staining pattern
NS3P	Biogenex Monoclonal MMM33	1:50, 2h	Microwave	Human liver infected with HCV	Cytoplasmic
P53	Biogenex Monoclonal DO7	1:50, 2h	Microwave	Breast cancer	Nuclear
P21waf	Oncogene Monoclonal	1:50, 2h	Microwave	Epidermis	Nuclear
Mdm2	Oncogene Monoclonal IF2	1:20, 2h	Microwave	sarcoma	Nuclear & Cytoplasmic
P21ras	Oncogene	1:25, 2h	Microwave	Colon cancer	Cytoplasmic
c-erbB2	Oncogene Monoclonal CB11	1:20, 1h	None	Breast cancer	Membranous

Table (3): Expression Level of the Studied Proteins In hepatocellular carcinoma and pericarcinomatous foci.

Protein	Hepatocellular carcinoma (HCC)	Pericarcinomatous foci (PC)
NS3	26 (81.25%)	17 (94.4%)
P53	18 (56.25%)	4 (22.2%)
P21 waf	14 (43.7%)	9 (50%)
Mdm2	19 (59.4%)	6 (33.3%)
P21ras	22 (73.3%)*	11 (61.1%)
c-erbB2	24 (75%)	13 (72.2%)

* number of informative cases 30

DISCUSSION

The mechanism of HCV-induced HCC is still unknown, however in vitro studies suggest a direct effect of HCV proteins on liver carcinogenesis [23]. The NS3P-transfected NIH3T3 cells grow rapidly, proliferate serum-independently, lose contact inhibition and induce significant tumor formation in nude mice [18]. Furthermore, sequence analysis showed unique changes at the vicinity of the catalytic sites of the NS3P clones isolated from HCC tissues but not from the non-tumorous hepatic tissues [35]. The high incidence of NS3P expression reported in the present study supports previously-published data and denotes the importance of this protein in the development of HCC in chronic HCV patients. This incidence is comparable to Feng et al., 1998 [7] who detected NS3P expression in 62% of HCCs and 83% of PCs. In the present study, we did not find a correlation between NS3P and any of the studied genes in HCC however, there was a strong correlation between this viral protein, p21 waf and c-erbB2 in PCs.

Our results regarding c-erbB2 overexpression are consistent with those of Zekri et al., 2000 [33] who showed that c-erbB2 is highly upregulated in HCV-associated HCC and CAH cases with no significant difference in the expression level between the two groups. This indicates that, alterations affecting c-erbB2 start to manifest at an early stage of hepatocytes transformation. The authors proposed that, HCV infection may induce c-erbB2 overexpression which stimulates signal transduction enhancing the proliferative activity and liability for random mutations and possible malignant transformation of hepatocytes.

The correlation between HCV infection and down regulation of p21 waf was detected in previous studies [11,27]. Kwun et al., 2001 [11] demonstrated that, NS3P specifically repress the promoter activity of p21 waf in a dose-dependent manner especially when combined with HCV core protein. This repression was completely lost when the p53 binding sites on p21 waf promoter were removed indicating that, this function is achieved via modulating the activity of p53. However, there are several other mechanisms for p21 waf regulation [22,27].

Zhou et al., 2001 [38] demonstrated that, c-erbB2 overexpression activates the phosphorylation of phosphatidylinositol-3-kinase (PI-3K)/Akt which associates with p21 waf resulting in cytoplasmic sequestration of the latter protein. Since the cell-growth-inhibiting activity of p21 waf is strongly correlated with its nuclear localization, cytoplasmic sequestration will block its function leading to cellular proliferation [26]. In addition, cytoplasmic p21 waf forms a complex with apoptosis-signal-regulating kinase 1 (ASK1) that inhibits the stress-induced mitogen-activated protein (MAP) kinase cascade with subsequent loss of apoptosis [1]. Our results provide support for this theory, since cytoplasmic immunostaining for p21 waf was recognized in 80% of the cases which overexpressed c-erbB2 (Fig. 3). The results of the present study show higher expression of p21 waf in PCs than in HCCs. The same finding was also reported by Zhu et al., [39] who showed that the expression of p21 waf was lower in HCC than in paratumoral liver tissues. However, in the present work p53 overexpression was detected in 63% of cases with absent nuclear p21 waf indicating that, the p53-dependent pathway is still considered the main pathway for p21 waf regulation [10,22]. Inactivation of p53 was previously reported in HCC and benign hepatic lesions [7,12,20]. Our results regarding the expression level of p53 in the studied groups are in agreement with Livni et al., 1995 [12] and Feng et al., 1998 [7] who reported p53 overexpression in 67% of HCC and the nontumorous tissues within the adjacent regenerative nodules but not in the normal hepatic tissues. However, the expression of p53 was higher in HCC than in PC and was significantly associated with the degree of tumor differentiation. They also reported a significant correlation between p53 overexpression and NS3P in

PC however, we did not find such a correlation neither in HCC nor in PC. This could be partially attributed to the difference in the HCV-genotype between our series and theirs since the predominant subtype in our cases was type 4 but in their study it was type 1.

The present study provides evidence that, complexing with mdm2 protein is a highly suggested mechanism for p53 inactivation. Although amplification of the mdm2 gene was shown to be a rare event in hepatic tumors, the high expression level of mdm2 reported in the present study in HCC and PC (59.4% and 44.4% respectively) indicates that it is a possible candidate for HCV-induced hepatocarcinogenesis. Schlott et al., 1999 [24] were able to detect mdm2 overexpression in HCC case and those with focal nodular hyperplasia, however, point mutations were only found in HCC cases only. They also demonstrated that, the c-terminal RING domain is involved in repression of cyclin A gene which controls the transition from G1 to S phase.

The correlation between mdm2 overexpression and the presence of cirrhosis denotes an important role for this protein in the early stages of hepatocyte transformation in chronic HCV patients. Furthermore, The correlation between mdm2, p53 and c-erbB2 in HCC arising on top of cirrhosis suggests the presence of a dynamic interplay between these genes in HCV-associated HCC.

The p21ras is a highly possible target for HCV since it represents the second most commonly affected gene in HCC cases (73.3%) investigated in the present work. To our knowledge, the results of the present study in this context are novel, since there are only few reports available regarding p21ras status in HCC, the majority of them were performed on cell lines or animal models [4,8,23,30]. Our results clearly show that, p21ras alterations are not only a frequent event in HCC but they are also of a predictive prognostic value since p21ras overexpression was associated with large-sized tumors, the presence of multiple intrahepatic nodules and the presence of nodal metastasis. Similarly, c-erbB2 overexpression was strongly correlated with the presence of multiple intrahepatic nodules and cirrhosis. Whereas absence of p21 waf and p53 overexpression were highly correlated with the presence of intrahepatic and venous metastases. Our results in this regard are

in agreement with those of Qin et al., 1998 [22] and Zhu et al., [39] who mentioned that p21 waf was lower in tumors with intrahepatic metastases than in those without metastases and that, absence of p21 waf expression was highly correlated with p53 mutations. Furthermore Qin et al., 1998 [22] demonstrated that a high protein expression is significantly associated with solitary nodules. Our results are also comparable with Naka et al., 1998 [15] and Qin et al., 1998 [22] who showed that, cases exhibiting aberrant p53 protein and reduced p21 waf expression are those with multiple nodules, advanced stage, high grade and large-sized tumors. Also, Heinze et al., 1999 [9] proved in their study that, patients with overexpressed p53 and c-erbB2 usually have reduced survival time.

Our data regarding the absence of any significant correlation between the ploidy status of tumors and the clinicopathological features of patients are in agreement with Terris et al., 1997 [29] and Ng 1998 [17] who showed that assessment of the ploidy status of HCC cases does not provide additional prognostic information. However, we found a significant difference in the DNA content between HCC and PC foci denoting the importance of DNA content assessment in monitoring CAH patients. Our results in this regard are in agreement with Zepa et al., 1998 [37] and Attallah et al., 1999 [2] who mentioned aneuploidy as an important marker in cirrhotic livers that could be used to monitor the development of carcinoma. They also mentioned that abnormal DNA content is an independent prognostic parameter in predicting survival in HCC patients. These controversy in results could be attributed to the difference in the etiological factor in different studies (HCV, HBV or aflatoxin B1 associated HCC) or to the difference in the way of measuring the DNA content (flowcytoetry vs image analysis). Another explanation was provided by Oriyama et al., 1998 [19] who mentioned the presence marked intratumoral heterogeneity of DNA ploidy status. This heterogeneity may develop along with changes in the growth pattern and dedifferentiation or by confluence of nodules originating from different tumor cell clones.

The presence of a percentage of patients whose PC samples revealed aneuploid populations raises the question whether those patients constitutes a high risk subset that is more prone to progress into HCC. The answer for this ques-

tion could be obtained from a larger follow-up study of CAH and cirrhotic patients who will be monitored by frequent DNA content analysis and correlation with other features of disease progression.

We conclude that, NS3P may exert its hepatocarcinogenic effect in an early stage of hepatic transformation being higher in PC than in carcinomatous foci. At first, there are alterations affecting the expression of c-erbB2 and p21^{waf} which provide a proliferative advantage to the chronically infected cells through promoting cell growth and loss of apoptosis. This enhanced proliferation and prolonged survival will allow for the acquisition of additional mutations in other genes e.g. p53, p21^{ras} and mdm2...etc. Accumulation of genetic aberrations will finally lead to the malignant phenotype. The correlation between c-erbB2, p53 and mdm2 and their strong association with HCC arising on top of cirrhosis suggests a dynamic interplay between these genes especially in HCV-associated HCC which is often preceded by cirrhosis. Furthermore, the significant association reported in the present study between some gene products and the clinicopathological features of patients is of utmost importance since they add some new, biological prognostic factors to the list of the existing prognostic parameters. These new, individually-based markers may help in improving the dismal outcome of HCC patients by accurately categorizing high- and low-risk patients and by patronizing therapy on an individual basis. They can also help in monitoring chronic hepatitis patients and identifying certain groups who are ore prone to develop HCC.

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