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Combined use of nuclear phosphoprotein c-Myc and cellular phosphoprotein p53 for hepatocellular carcinoma detection in high-risk chronic hepatitis C patients

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is a multistage process resulting from various genetic changes. We aimed to determine nuclear phosphoprotein c-Myc and cellular phosphoprotein p53 expression and to evaluate their importance in HCC diagnosis.

Methods: One hundred and twenty chronic hepatitis C (CHC) patients (60 non-HCC CHC patients and 60 HCC patients who had a single small (<5 cm) tumour) were recruited. The gene products of c-Myc and p53 were identified in liver tissues and serum samples using immunostaining, western blot and ELISA.

Results: Immunohistochemical detection of c-Myc and p53 with monospecific antibodies revealed intense and diffuse cytoplasmic staining patterns. Accumulated mutant proteins, released from tumour cells into the extracellular serum, were detected at 62 KDa, for c-Myc, and 53 KDa, for p53, using western blotting. In contrast to alpha feto-protein, there was a significant increase ($p < 0.0001$) in the positivity rate of c-Myc (86.7% vs. 6.7%) and p53 (78.3% vs. 8.3%) in the malignant vs. non-malignant patients. The parallel combination of c-Myc and p53 reach the absolute sensitivity (100%), for more accurate and reliable HCC detection (specificity was 87%).

Conclusion: c-Myc and p53 are potential HCC diagnostic biomarkers, and convenient combinations of them could improve diagnostic accuracy of HCC.

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Introduction

Each year, about 0.5 million cases of hepatocellular carcinoma (HCC) were diagnosed worldwide. The HCC mortality rate is very high, with five-year overall survival rates <10% [1]. Noteworthy, in Egypt HCC constitute 13% of all malignancies and is the second most frequent tumour in men [2]. Most HCC cases occur after a history of cirrhosis related to chronic hepatitis in which there is continuous hepatocytes injury and regeneration [3]. In Egypt, 94% of HCC cases are related to chronic hepatitis C (CHC) infection [2]. Prognosis for HCC patients and favourable clinical outcome depends on medicinal therapies that only helpful for patients diagnosed at an early stage [4,5].

The primary HCC surveillance modality is ultrasound (US); owing to its modest sensitivity, 60%, increased detection of more HCC could be established by computed tomography (CT). Outcomes of these tests depend on the radiologist experience. Moreover, they are associated with high costs, higher false-positive rate and potential harm related to radiation exposure and contrast-related injury [6]. Other limitations exist when imaging obese individuals or cirrhotic patients. In some cases with cirrhosis, the sensitivity can be as low as 35% [7]. However, although alpha-fetoprotein (AFP) is the routine biomarker in HCC detection, it is unsatisfactory because of its diagnostic value [8]. For example, in the absence of HCC, AFP may increase in other chronic liver diseases (CLD) patients [9]. Therefore, more sensitive and specific molecular biomarkers may help in the early diagnosis of HCC in patients with CHC.

Under normal conditions, the expression of *c-Myc*, is regulated by ligand-stimulated receptor signalling. By organizing transcription of its target genes, *c-Myc* over expression or hyper activation share in many aspects of cancer progression, including DNA replication, growth, proliferation, metabolism, angiogenesis, cell adhesion, protein synthesis, metastasis and immune responses [10,11]. The relation between *c-Myc* and hepatocarcinogenesis was reported by the high level of *c-Myc* in HCC related to CLD [12]. *p53* is the most evaluated tumour suppressor gene in HCC [13]. p53 executes the function of pro-apoptosis and DNA reparation, in tumour cells [14]. In HCC, over-expression of p53, including both wild-type and its mutations, shows an increased predictability.

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Due to its role in progression of CHC to HCC, the p53 expression level can be of help for HCC identification and monitoring at an early stage [13].

We aimed to determine whether combining of c-Myc and p53 can improve sensitivity of HCC prediction in high-risk CHC patients without significant loss in specificity.

Material and methods

One hundred and twenty patients with chronic HCV infection, at high risk of HCC, were recruited from January 2014 to July 2015 at the Tropical Medicine Department, Mansoura University Hospitals. All patients were hepatitis C antibodies positive and were divided into two groups: non-HCC CHC patients (n = 60) and HCC patients (n = 60). Liver enzymes, total bilirubin, serum albumin, platelet count and AFP were examined in the enrolled patients. None of patients had given any drugs for antiviral or tumour during or before this study. The selection criteria applied in this study included only HCC patients with size <5 cm, single tumour and absent vascular invasion. According to practice guidelines of the American Association for the Study of Liver Diseases (AASLD) [15], HCC was diagnosed based on AFP ≥ 400 UI^{-1}, detection of liver focal lesion(s) by liver ultrasound, confirmed by computed tomography. Thirty healthy volunteers were enrolled as controls. They were of comparable age with patients. Healthy volunteers were negative for hepatitis viruses. The study protocol confirmed to the ethical guidelines of the 1975 Helsinki Declaration.

Routine needle biopsy liver tissue samples were processed for histopathological diagnosis. After centrifugation of blood samples, at 4,000 rpm for 10 minutes, the serum was stored at −20 °C until used. Immunoperoxidase staining was performed as previously described [16]. To block endogenous peroxidase activity, each paraffin-embedded tissue section (thickness of 3–5 µm) was deparaffinized, hydrated and incubated in methanol and 3% H_2O_2 (v/v) for 20 minutes. The sections were incubated with 3% (v/v) normal goat serum for 30 min. All the primary monospecific rabbit antibodies (ABC Diagnostics, New Damietta, Egypt) for c-Myc and p53 were diluted 1:20 in PBS (pH 7.2). The tissue sections were incubated with the primary antibodies. After washing with PBS for 5 min, sections were incubated with anti-rabbit IgG peroxidase secondary antibody (Sigma, CA, U.S.A.) for two hours at room temperature. After washing, the sections followed by incubation with amino ethyl carbazole substrate (Sigma, CA, U.S.A.) for 30 min. Subsequently, the sections were counterstained with haematoxylin.

For western blot immunolabeling, the protein lysates in serum samples of selected patients were separated SDS-PAGE [17]. Prestained standards for molecular weight (Sigma) were used in parallel. Proteins were transferred onto nitrocellulose blotting membranes. After blocking, the membranes were incubated overnight with rabbit monospecific antibodies against c-Myc or p53, followed by incubation with conjugated (rabbit alkaline phosphatase IgG; Sigma). The blots were then visualized by soaking the membranes in premixed substrate, 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium in 0.1 M Tris buffer, pH 9.6, (Sigma) and stopped by distilled water after colour development within 10 min.

Detection of circulating c-Myc and p53 proteins using ELISA was determined as previously described [18]. Serum was diluted in carbonate/bicarbonate buffer (pH 9.6) and 50 µL coated onto ELISA plates (Costar, Corning Life Sciences, Acton, MA). The coated ELISA plate was incubated overnight at 4 °C with phosphate buffered saline containing 0.1% Tween 20 (PBS-T20), the precoated plates were washed four times. The non-specific sites were blocked using bovine serum albumin (BSA) (0.2%) for one hour at room temperature. After four washes with PBS-T20, the plates were incubated with 50 µL/well of diluted monospecific anti-c-Myc antibody, in case of c-Myc detection, or monospecific anti-p53 antibody, in case of p53 detection and incubated at 37 °C for two hours. After washing and at dilution of 1:500 in PBS-T20 containing 0.2% BSA, anti-rabbit IgG (Sigma, U.S.A.) alkaline phosphatase conjugate was added. After incubation at 37 °C and washing, the nitrophenyl phosphate substrate (Sigma, U.S.A.) was added for half hour at 37 °C.After stopping the reaction with 3 M NaOH, the optical density was reading using microplate spectrophotometer (Metreiteck, Axiom, Burstadt, Germany) at 405 nm.

Statistical analysis was as follows. Data were analyzed using Mann–Whitney or ANOVA test and Fisher's Least Significant Difference was used as *post hoc* test. Positivity rates were compared among malignant, non-malignant and healthy groups using chi square test. The level of significance was determined at <0.05. Receiver operating-characteristic (ROC) curves were used for determining the diagnostic power of biomarkers. From a 2 × 2 contingency table, common performance indicators of the candidate markers were derived. All analyses were performed using SPSS and GraphPad programs.

Results

Detection of circulating c-Myc and p53 was as follows. The presence of serum c-Myc, at 62 KDa, and p53, at 53 KDa, expression in HCC was confirmed by western blotting (Figure 1(a) and (b)). From all selected samples, 10 samples for each group, for both c-Myc and p53, revealed positivity in HCC patients in contrast to one sample in CHC patients. For screening, circulating c-Myc and p53 proteins were detected in all included patients using ELISA. Positivity for c-Myc was found in 52/60 (86.7%) patients with HCC in contrast to 4/60 (6.7%) non-malignant CHC patients. Positivity for p53

Table 1. The background clinical characteristics of the study groups.

Parameter[a]	Groups			*P* value[b]
	Healthy	CHC	HCC	
Number	30	60	60	—
Male/Female	22/8	42/18	45/15	0.564
Age (years)	44 (39–51)	53.5 (47–60)	56 (50–62)	0.0235
AST (U/L)	21 (19–33)	58 (39.3–83)	80 (52–102)	0.005
ALT (U/L)	25 (12–35)	34 (25–66)	81 (53–104)	0.007
ALP (U /L)	62 (23–81)	108 (91.5–139)	153 (99–180)	<0.0001
Albumin (g/dL)	4.6 (4.3–4.8)	3.5 (3–4)	3.2 (2.7–3.9)	<0.0001
Total bilirubin (μmol/L)	12 (10.3–17.1)	18.8 (12–29.1)	35.9 (20.5–54.7)	<0.0001
Platelets ($\times 10^{-9}$/L)	230 (150–300)	120 (97–160)	97 (66–193)	<0.0001
AFP (U/L)	2 (1–10)	8.7 (3–12)	120 (83–1250)	<0.0001

Note: Continuous variables were expressed as Median (IQR). CHC = Chronic hepatitis C; HCC = Hepatocellular carcinoma.
[a]Reference values: aspartate aminotransferase (AST) up to 40 (U/L); alanine aminotransferase (ALT) up to 45 (U/L); alkaline phosphatase (ALP) 22–92 U/L; total bilirubin 3–25 μmol/L; albumin 38–54 g/L; platelet count 150–400 $\times 10^9$/L; alpha-fetoprotein (AFP) up to 10 (U/L).
[b]$p > 0.05$ is considered not significant, $p < 0.05$ considered significant.

was found in 47 out of 60 (78.3%) patients with HCC in contrast to 5 out of 60 (8.3%) non-malignant CHC patients (Table 2).

The demographic and laboratory features of patients and controls are presented in Table 1. Localization of c-Myc and p53 within HCC liver tissues were as follows. By immunohistochemical stain, the c-Myc protein was detected in all selected HCC tissues, 10 liver specimens. They showed intense and diffuse cytoplasmic immunostaining patterns (Figure 1(c)) in comparison with negative control (Figure 1(d)). Similarly, in contrast to the negative control, extremely strong and diffuse cytoplasmic staining for P53 was observed in HCC tissues (Figure 1(e) and (f)).

Combining c-Myc and p53 indicated potential diagnostic values: sensitivity and specificity for circulating AFP, c-Myc as well as p53 proteins were tested for detection of HCC. Sensitivity of serum c-Myc to detect HCC was the highest, whereas serum AFP revealed the lowest sensitivity while the best sensitivity for HCC was achieved when c-Myc and p53 were combined (Table 3). All of 60 HCC patients were positive for at least one marker apart from AFP (100% sensitivity). Despite this, from 60 CHC patients, 52 were negative to both of c-Myc and p53 (87% specificity). This combination showed an area under curve (AUC) of 0.90 for identifying HCC. Correlation between c-Myc/p53 positivity with AFP was ($r = 0.149$; $p = 0.187$).

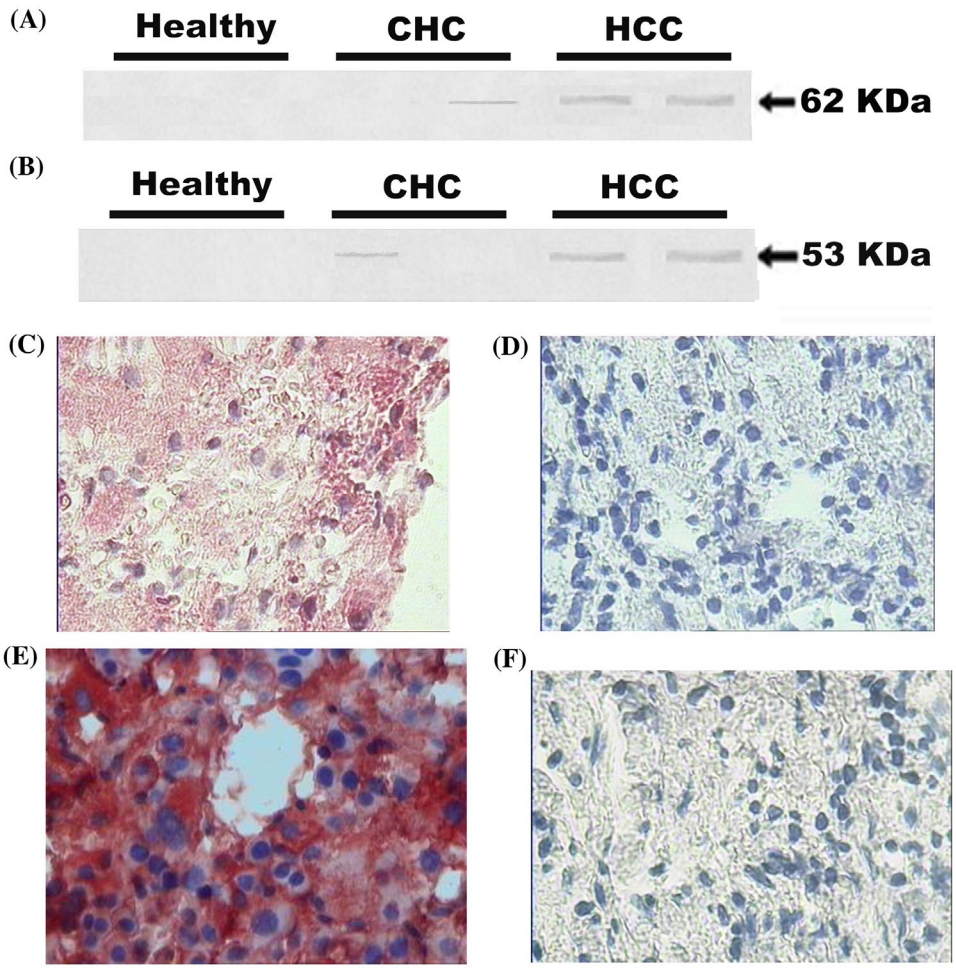

Figure 1. c-Myc and p53 in HCC. (A, B) Representative western blot analysis demonstrates (A) c-Myc and (B) p53 overexpression in HCC tumours compared to CHC samples. (C-F) Representative Immunohistochemical staining showing the expression level and cellular localization of (C) c-Myc and (E) p53 in HCC tissues. The control HCC tissue stained without anti-c-Myc antibody (D) or anti-p53 antibody (F) showing no immunostaining reaction (Original magnification × 400).

Table 2. c-Myc and P53 status in study groups.

	c-Myc	P53	AFP
Groups	No positive (%)	No positive (%)	No positive (%)
CHC ($n = 60$)	4 (6.7%)	5 (8.3%)	0
HCC ($n = 60$)	52 (86.7%)	47 (78.3%)	15 (25%)
χ^2	56.6	39.6	11.4
P value	<0.0001	<0.0001	0.002

Abbreviations: CHC: Chronic hepatitis C; HCC: Hepatocellular carcinoma; AFP: alpha-fetoprotein. $p > 0.05$ is considered not significant, $p < 0.05$ considered significant.

Table 3. Combined sensitivity and specificity for c-Myc and P53 in HCC prediction.

Parameter	AUC	Sensitivity	Specificity	NPV	PPV	Accuracy
AFP	0.625	25	100	57	100	62
c-Myc	0.850	87	93	88	93	90
P53	0.775	78	91	81	90	85
c-Myc+AFP	0.850	88	82	88	83	85
P53+AFP	0.838	87	82	86	83	84
c-Myc+P53	0.900	100	87	100	88.2	93

Abbreviations: AUC: area under receiver-operating characteristic curve; AFP: alpha-fetoprotein; PPV: positive predictive value; NPV: negative predictive value.

Discussion

Infection with HCV causes hepatic fibrosis and subsequent cirrhosis. Among patients with HCV-induced cirrhosis. Among patients with HCV-induced cirrhosis, 2–5% develops HCC, with a poor survival rate of 7%, due to late-stage diagnosis [19]. As AFP is often non-specifically elevated in non-malignant hepatic diseases, and has poor sensitivity for HCC [20], there is an urgent need to develop sensitive simple diagnostic tools that can assist early diagnosis of HCC. Against this background, we evaluated nuclear phosphoprotein c-Myc and cellular phosphoprotein p53 in seeking a newly potential tumour marker with relative higher sensitivity and specificity for HCC.

c-myc overexpression in chronic liver disease has a role in the predisposition to liver carcinogenesis [21]. In HCC, *c-myc* overexpression is caused by amplification of gene [22]. Abdou et al. showed that no case of chronic hepatitis or cirrhotic tissues was positive for p53, whereas many of HCC cases were positive for p53 expression [23]. Here, by immunohistochemical staining, intense and diffuse cytoplasmic staining for c-Myc and p53 was observed in HCC tissues. Similar results were obtained by Niu et al. [24] who reported in HCC tissues that the positive expression of c-Myc and p53 was markedly higher than that in pericarcinomatous tissues, liver cirrhosis and normal hepatic tissues. This elevated expression rate was associated with the histological differentiation [24].

Results of immunohistochemical staining are reliable because the specificity of c-Myc and p53 antibodies confirmed by immunoblotting at 62 and 53 KDa, respectively [25]. Ben-Mahrez et al., reported that the anti-human *c-myc* specifically reacted with a 60 KDa protein [26]. In malignant cells, the accumulating mutant proteins can be released into serum [27]. There are direct links between expression of *p53* and *c-myc* in malignant tissue and its levels in serum [27,28]. Thus, these proteins, easily detected by ELISA, can be determined to a large degree in different laboratories [27]. In contrast to AFP, our results revealed a marked rise in positivity rate of serum c-Myc and p53 in malignant compared to non-malignant CHC patients while no healthy serum samples showed positive c-Myc or p53. Furthermore, our study shows that the combination of c-Myc and p53 reach absolute sensitivity (100%) for more accurate and reliable HCC detection, rather than use of only one marker. The specificity was 87%. Indeed, this combined use yielded findings that are comparable with other similar combinations of biomarkers in the literature. For example, parallel combination of p53 and heat shock protein 70 revealed sensitivity and specificity for HCC diagnosis of 95.5% and 85.5%, respectively [13].The combination of glypican-3 and AFP yield sensitivity between 67 and 78% [29,30]. AFP-L3 and p53 yield 95% and specificity [31].

Liver tumour genesis is a process with several stages in which sequential accumulation of various epigenetic and genetic alterations precursor lesions progress into early HCC [32,33]. In hepatocytes, c-Myc overexpression leads to development of HCC [12,32]. Another strong association of c-Myc with HCC is its frequent amplification in hepatic malignant tissues [12,34].There were positive correlations between c-Myc and p53 overexpression, thus alterations of these genes cooperated in the progression of HCC [34]. In the liver, overexpression of wild-type p53 and its mutations shows an increased predictability in HCC [35]. Aberrations of *p53* gene represent a relevant factor in hepatocarcinogenesis. These gene mutations result in rise p53 levels in liver cancer and its protein has role in HCC and independent of the AFP level [36].

Due to its role in CHC progression to HCC, the p53 expression level in the process of chronic liver diseases can be of help for monitoring and detecting HCC at an early stage [37]. In conclusion, c-Myc and p53 in a proper combination could improve HCC diagnostic accuracy and may be a more sensitive and accurate alternative to monitor the progression of chronic liver disease. This work represents an advance in biomedical science because it sheds light on the combined use of nuclear phosphoprotein, c-Myc and cellular phosphoprotein, p53, for improving HCC diagnostic accuracy.

Summary table

What is known about this subject:
- Annually, more than half a million people are diagnosed with HCC and the mortality rate is high.
- AFP is the most widely used HCC serum marker but it is unsatisfactory because of its high false positive and false negative ratio.
- More sensitive and specific novel molecular markers may aid in the diagnosis of HCC.

What this paper adds:
- Immunohistochemical detection of c-Myc and P53 revealed intense and diffuse staining patterns in liver tissues from HCC patients.
- There is a marked increase in the positivity rate of circulating c-Myc and P53 in HCC over patients with non-malignant diseases.
- The parallel combination of c-Myc and P53 reach the absolute sensitivity for more accurate and reliable HCC detection.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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