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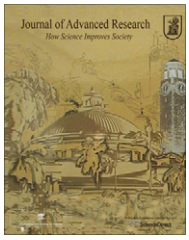

ORIGINAL ARTICLE

Molecular markers as a prognostic system for hepatocellular carcinoma

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KEYWORDS

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Abstract The gene expression profile p16, c-erbB-3 and bcl2 in hepatocellular carcinoma (HCC) patients with and without associated HCV infection, was assessed. Forty-eight subjects were included in the study and divided equally into two groups: HCC with and without HCV associated infection. Adjacent paracancerous tissues were assessed as control samples. Correlations with various clinico-pathological parameters of the tumour were assessed: stage, grade, and tumour size. The c-erbB-3 oncogene was expressed in 83.33% (40/48) of the total HCC sample and in 31.25% (15/48) of the noncancerous lesions. C-erbB-3 was expressed in 87.5% (21/24) of the HCC cases with associated HCV infection and in 79.16% (19/24) of the HCC cases without associated HCV infection. Gene expression of c-erbB-3 was significantly correlated with the clinico-pathological parameters of the tumour. P16 gene expression was found in 12.5% (6/48) of the total HCC sample and in 25% (12/48) of the para-cancerous lesions. P16 was expressed in 12.5% (3/24) of HCC cases with and without associated HCV infection. Gene expression of p16 exhibited significant negative correlation with clinico-pathological parameters of the tumour. Bcl2 gene expression was found in 20.8% (10/48) of the total HCC sample and in the para-cancerous lesions. Bcl2 was expressed in

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20.8% (5/24) of the HCC cases with and without HCV associated infection. Gene expression of bcl2 did not show significant correlations with the clinico-pathological parameters of the tumour. In conclusion, gene expression profiles of p16 and c-erbB-3 could be used as prognostic molecular markers in HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of death from cancer in several regions in the world including Egypt. In spite of enormous efforts to improve clinical treatment, HCC remains a major carcinoma with high mortality. Poor differentiation, larger size, portal invasion and intra-hepatic metastasis are known to shorten disease-free survival with this carcinoma. One of the most prominent parameters in the evaluation of the biological aggressiveness of carcinoma is cell behavior. Growth factor receptors with tyrosine kinase activity are known to contribute greatly to the regulation of cell behavior such as cell growth, proliferation and mortality [1,2]. The type I family of growth factor receptors is the most prominent and is recognized as a proto-oncogene family. The family includes c-erbB-3 [3,4]. When specific ligands bind to a receptor of the family, the receptor is activated by phosphorylation of the tyrosine residue in the molecule [1]. It then forms a dimer with another receptor of this family, causing activation by transphosphorylation, which contributes to a variety of growth signal transductions [5]. These receptors share high sequence identity with each other and are co-expressed in various combinations in neoplasms. Thus far, of the four receptors of the family, the expression of c-erbB-3 has been investigated in various neoplasms, including malignancies of the liver and the biliary tract [6–8].

Although the mechanisms of hepatocellular carcinogenesis are not yet expounded, alterations of some oncogenes, tumour suppressor genes and apoptosis/antiapoptosis signaling, have been reported in hepato-carcinogenesis. Some cell cycle tumour suppressor genes such as p16 have been proved to be involved in hepatocellular carcinogenesis. P16INK4a is a cell cycle tumour suppressor that acts as competitive inhibitor by binding directly to CDK4 and CDK6 and preventing their association with a cyclin, which in turn arrests the cells in late G1 phase of the cycle with pRB in a hypophosphorylated state [9]. On the other hand, hepatitis C virus (HCV) infection was proved to be closely linked to the development of HCC and HCV may be the second important factor in HCC etiology [10–12]. The molecular mechanisms involved in hepato-carcinogenesis of HCV remain poorly understood. Up to now, many authors have believed that HCV cannot directly change the structure of host genes such as the hepatitis B virus by integration because HCV is a RNA virus. Therefore, the effect of HCV on factors controlling the cell cycle, apoptosis and oncogenes, is an important field of study in hepatocarcinogenesis research [13,14].

On the other hand, the bcl-2 gene family is a group of apoptosis-related genes that is studied extensively at present [15]. Accumulated reports show that there is a high-level expression of bcl-2 in many tumour tissues [16]. Primary HCC is a very common malignant tumour in Egypt. There are specific characteristics in the expression of bcl-2 in HCC [17,18]. Yildiz et al. [19] stated that bcl-2 is highly expressed in B and C hepatitis and in hepatocellular carcinomas. The high incidence of bcl-2 activity in the non-neoplastic liver parenchyma of HCC cases suggest that bcl-2 activation may be involved in the development of at least some cases of HCC.

The present study was conducted to evaluate the gene expression profile of p16, c-erbB-3 and bcl2 in HCC patients with and without HCV associated infection. Correlations with various clinic-pathological parameters of the tumour were assessed to find whether the expression profile of the studied genes could be used as prognostic markers in HCC patients.

Material and methods

Tissue specimens

Ten percentage buffered formalin-fixed paraffin-embedded blocks of HCC were prepared from 48 patients who had undergone surgery for HCC during the period from January 2009 to February 2010. Informed consent was obtained from each patient. The clinico-pathological characteristics of the patients are shown in Table 1. HCC was ranked using the CLIP staging system. The survival rate at 12 months follow up was 70% for patients with a CLIP score of less than three, and 38.8% for patients with a CLIP score of more than three.

Table 1 Clinico-pathological characteristics of the patients.

Age (years) 62.3 ± 7.5	Number of subjects
Gender	
Male	38
Female	10
HCV	
+ve by PCR	24
−ve by PCR	24
Tumour stage	
< III	30
⩾ III	18
Tumour size	
⩾ 5 cm	20
< 5 cm	28
Intrahepatic metastasis	
With	17
Without	31
Carcinoma differentiation	
Poor	16
Moderate or well	32

Table 2 The oligonucleotide primers sequence of the studied genes.

Primer sequence	Annealing temperature (°C)	Product size (bp)
c-erbB-3		
Forwardprimer: 5′-GCCTGGACTTGGAAGGCACCTG -3′	58.5	348
Reverseprimer: 3′-GAGCCACAGAGACCGCGTGA-5′		
GenBank® Accession Number: GenBank: Z23134.1		
P16 (CDKN2A)		
Forwardprimer: 5′-TCTCCGTTGGCCGGAGGTCA-3′	59.5	260
Reverseprimer: 3'-TGCGCAGGTACCCTGCAACG-5'		
GenBank® Accession Number: NM_078487.2		
Bcl2		
Forward primer: 5′-CGTCAACCGGGAGATGTCGCC-3′	59	220
Reverse primer: 3′-TGATTTTATTTCGCCGGCTCCACAG-5′		
GenBank® Accession Number: NM_000657.2		
Beta actin		
Forward primer: 5′-CGCGGCGGCGCCCTATAAA-3′	59.5	397
Reverse primer: 3′-ACCGTGCGATCCCCATTGGC-5′		
GenBank® Accession Number: M10277.1		

Gene expression profile of p16, c-erb-B3 and bcl2 in HCC samples

Total RNA was extracted using RNeasy Purification Reagent (Promega, Madison, WI, USA), and then a sample (1 μg) was reverse-transcribed with M-MLV (Moloney–Murine Leukemia virus) reverse transcriptase (RT) for 30 min at 42 °C in the presence of oligo-dT primer. Polymerase chain reaction (PCR) was performed using the primers specified in Table 2. PCR was performed for 45 cycles, with each cycle consisting of denaturation at 95 °C for 30 s, annealing at 58.5–59.5 °C as specified for each primer pair for 30 s and elongation at 72 °C for one min, with an additional 10 min incubation at 72 °C after completion of the last cycle. To exclude the possibility of contaminating genomic DNA, PCRs were also run without RT. Beta actin gene expression was assessed as a positive control housekeeping gene. The PCR product was separated by electrophoresis through a 1% agarose gel, stained, and photographed under ultraviolet light.

Semi quantification of PCR products by gel documentation

RT-PCR of the gene products was semi-quantified using a densitometry gel documentation system (BioDocAnalyze, Biometra, Goettingen, Germany) according to the manufacturer's specification of the software. The amounts of PCR products were evaluated according to the relative intensity of the studied genes and beta actin bands by using the computed densitometry assay of the Biometra BioDoc Analyze System.

Statistical analyses

Values were expressed as mean ± S.D. The chi-squared test and the Kruskal–Wallis test, followed by Dunn's test of multiple comparisons, were employed for analyses of the relationship between the expression of the genes and various clinicopathological parameters. A p value less than 0.05 was considered to be statistically significant. All the statistical analyses were performed using SPSS version 10 software (SPSS, Chicago, IL, USA).

Results

In the present study, the c-erbB-3 oncogene was highly expressed in all HCC samples as compared to adjacent paracancerous lesions (Fig. 1); the p16 gene was under-expressed in all

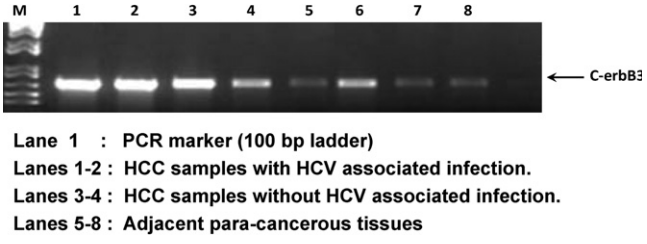

Fig. 1 PCR product of c-erbB-3 gene (348 bp).

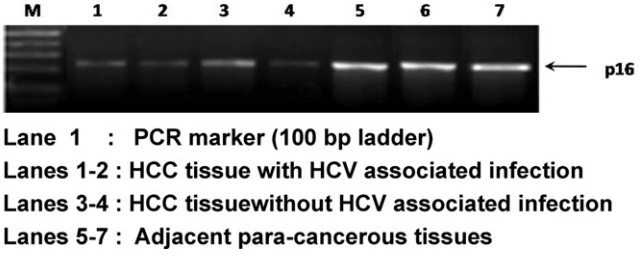

Fig. 2 PCR product of p16 gene (260 bp).

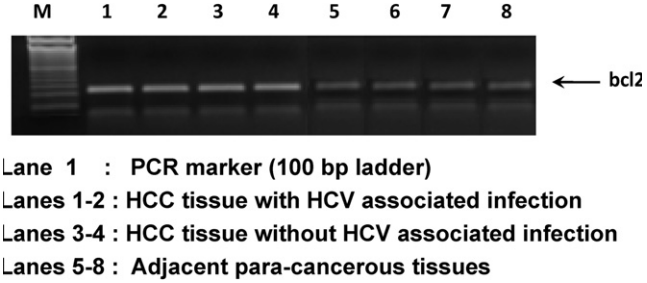

Fig. 3 PCR product of bcl2 gene (220 bp).

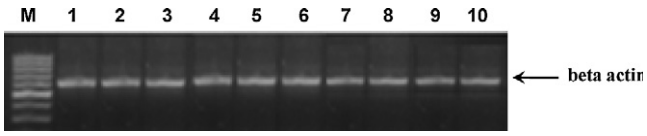

Fig. 4 PCR product of beta actin gene (397 bp).

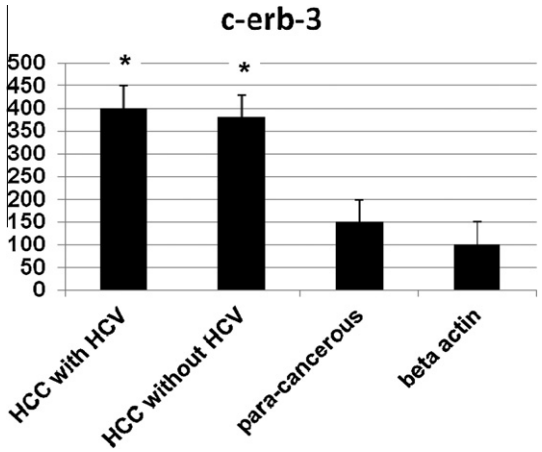

Fig. 5 c-erb-B3 gene gel documentation by semiquantitative RT-PCR density ratios. The gene was calculated in relation to the internal standard beta-*actin* and expressed as means +SEM. (* Significant difference with beta actin). *p value <0.001.

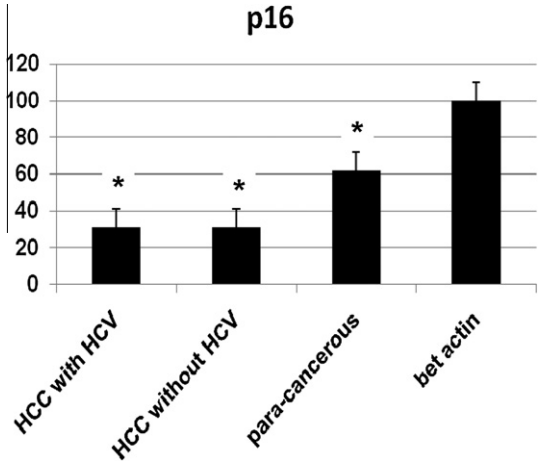

Fig. 6 p16 gene documentation by semiquantitative RT-PCR density ratios. *p value < 0.01.

HCC samples as compared to adjacent paracancerous lesions (Fig. 2); whereas the bcl-2 gene showed equal expression in HCC samples as compared to paracancerous tissues (Fig. 3). Beta actin gene was expressed in all samples (Fig. 4).

Moreover, c-erbB3 was over-expressed in HCC samples with HCV associated infection as compared to HCC without HCV associated infection (Fig. 5); whereas p16 and bcl-2 genes were equally expressed in HCC samples with or without HCV associated infection (Figs. 6 and 7).

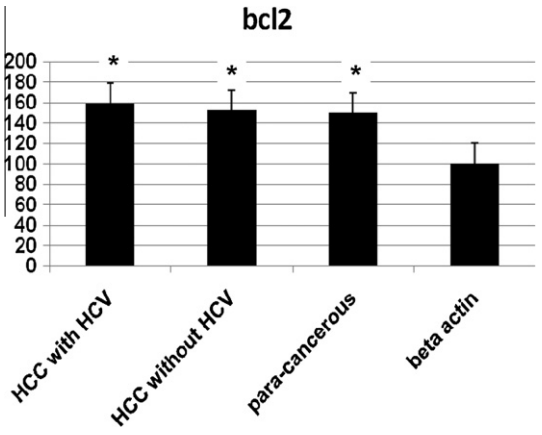

Fig. 7 bcl2 gene documentation by semiquantitative RT-PCR density ratios. *p value < 0.05.

Furthermore, gene expression of c-erbB-3 showed significant positive correlation with the clinic-pathological parameters of the tumour; p16 gene expression exhibited significant negative correlation with clinico-pathological parameters of the tumour; whereas gene expression of bcl2 did not show any significant correlation with the clinico-pathological parameters of the tumour (Table 3).

Discussion

In the present study, the c-erbB-3 oncogene was highly expressed in all HCC cases as compared to adjacent para-cancerous lesions. Moreover, c-erbB3 was over-expressed in HCC samples with HCV associated infection as compared to HCC cases without HCV associated infection. Gene expression of c-erbB-3 was significantly correlated with the clinic-pathological parameters of the tumour. Similar studies have been performed on a few other carcinomas. For example, Sanidas et al. [20] demonstrated that the c-erbB-3 protein was always expressed in both gastric carcinoma and the adjacent mucosa, but the expression level was usually higher in the carcinoma. Travis et al. [21] observed that breast carcinoma expressed c-erbB-3 more intensely and diffusely than the adjacent normal glands, which were usually weakly or moderately positive for this protein. Haugen et al. [22] showed that normal follicles of the thyroid were all negative for c-erbB-3, whereas all types of thyroid carcinoma expressed this protein with very high incidence. The results of these studies, including ours, are similar in that they show c-erbB-3 expression to be more diffuse and/or more intense in the carcinoma nest than in normal or benign lesions [23,24]. Our study also showed that c-erbB-3 expression in HCC was significantly related to some important markers of carcinoma progression, which are also predictors of recurrence, such as stage, tumour size, intrahepatic metastasis and carcinoma differentiation. Furthermore, c-erbB-3 itself, to some extent, affects disease-free survival, as reported in several studies [25,26].

To our knowledge there is no previous study conducted to evaluate the role of c-erb-B3 in liver tissue with hepatitis C virus, nor in HCV-associated HCC. However, El Bassuoni et al. [27] reported that the elevated expression of another member of the c-erbB family of oncogenes (C-erbB-2) in HCV-related chronic liver disease may reflect pre-neoplastic li-

Table 3 Correlations between the expression of erbB-3, p16, bcl2 genes and various clinic-pathological features of HCC subjects.

	Number of subjects	Correlations with c-erb-B3	Correlations with p16	Correlations with bcl2
Tumour stage				
⩾III	18	$p < 0.001$	$p \leqslant -0.05$	NS
<III	30			
Tumour size				
⩾5 cm	20	N.S.	$p \leqslant -0.01$	NS
<5 cm	28			
Intrahepatic metastasis				
With	17	$p < 0.01$	$p \leqslant -0.05$	NS
Without	31			
Carcinoma differentiation				
Poor	16	$p < 0.001$	$p \leqslant -0.01$	NS
Moderate or well	32			

ver cell proliferation, cellular necrosis associated with chronic liver disease or HCV carcinogens that enhance malignant transformation.

As regards p16, the gene exhibited under-expression in all HCC cases as compared to adjacent para-cancerous lesions. Gene expression of p16 was equally expressed in HCC cases with and without associated HCV infection. Moreover, gene expression of p16 exhibited significant negative correlation with clinico-pathological parameters of the tumour. Jain et al. [24] stated that loss of p16 protein could result from inactivation of p16 by promoter hypermethylation, homozygous deletions, and point mutations, and was noted in both early and late stages of HCC [28]. Moreover, Hayashi et al. [29] stated that hypermethylation of p16 was one of the most important alterations in HCV-associated HCC and that HCV could play a role in hepato-carcinogenesis. Furthermore, Vivekanandan and Torbenson [30] stated that epigenetic instability, manifesting as methylation of important tumour suppressor gene promoters, are associated with hepatocellular carcinomas that arise in the setting of viral induced cirrhosis.

The frequently deleted chromosome regions by loss of heterozygosity (LOH) in HCCs contain many tumour suppressor genes and some oncogenes, (p53, Rb, p16, PTEN, DLC1, and IGF2R) [14,31]. LOH at chromosome 1p is usually seen in early, small or well-differentiated HCC [32], whereas LOH at chromosomes16p and 17p is more frequently associated with HCCs in advanced stages, aggressive tumours, and poor prognosis [33]. By comparative genomic hybridization (CGH), chromosome 8p, 17p and 19p are associated with HCC metastases [32].

On the other hand, several studies proved that loss of p16 gene expression was correlated with the cellular differentiation of malignant tumours and an advanced grade of malignancy [34,35]. The highly significant correlation between p16 and an advanced pathological grade of HCC was also confirmed in our study. Interestingly, the correlation of loss of p16 protein expression with the cellular differentiation of gastric cancer has been also reported [36]. Park et al. [37] also noted that inactivation of p16 exon 1 by DNA hypermethylation occurred during the progression of tumour cells to poorly differentiated HCC, which was induced by diethylnitrosamine plus thioacetamide in Fischer 344 rats. These studies suggested that the aberrant alteration of mRNA expression and methylation of p16 gene might be not only involved in HCC carcinogenesis but also associated with its progress.

As regards bcl2 gene expression, the gene was expressed to an equal extent in all HCC cases and in all para-cancerous lesions. Bcl2 was equally expressed in HCC cases with and without associated HCV infection. Gene expression of bcl2 did not show significant positive correlation to the clinic-pathological parameters of the tumour.

Yang et al. [38] stated that the expression of bcl-2 protein in most tumour tissues is stronger than that in the tissues of origin. Nevertheless, most studies have demonstrated that HCC tissues do not express or have only a low positive rate of bcl-2 protein. Moreover, sometimes the positive rate of bcl-2 in HCC tissues was lower than that in the non-tumour liver tissues immediately adjacent to HCC tissues [39]. The mechanism of this phenomenon is still unclear. There may be specific characteristics of the regulation of bcl-2 in HCC. As regards HCC cases with hepatitis C virus, results of the present study coincided with the study of Tsamandas et al. [39] who found bcl2 expression in liver biopsies with hepatitis B or C viruses. Moreover, Yildiz et al. [19] studied bcl-2 gene expression in B and C hepatitis and hepatocellular carcinomas. The authors stated that no causative relation between bcl-2 positivity and HCC could be implied; however the high incidence of bcl-2 activity in the non-neoplastic liver parenchyma of the HCC cases suggests that bcl-2 activation may be involved in the development of at least some cases of HCC. Case control and/or prospective studies are needed to show whether bcl-2 positivity in a chronic hepatitis case has a predictive value for the development of HCC.

In conclusion, the c-erbB-3 gene was over-expressed in HCC cases; whereas the p16 gene was under-expressed in HCC cases. C-erbB-3 and p16 genes showed significant correlations with the clinico-pathological parameters of the subjects; whereas bcl2 did not show any correlation with the clinico-pathological parameters. Gene expression profile of p16 and c-erb-B3 could be used as prognostic molecular markers in HCC.

Acknowledgements

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Pathology Department, Faculty of Medicine, Benha University.

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