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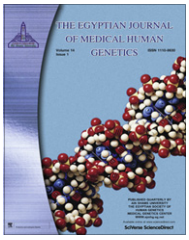

ORIGINAL ARTICLE

Multiple molecular markers MAGE-1, MAGE-3 and AFP mRNAs expression nested PCR assay for sensitive and specific detection of circulating hepatoma cells: Enhanced detection of hepatocellular carcinoma

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Hepatocellular carcinoma;
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α Fetoprotein;
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mRNA

Abstract Hepatocellular Carcinoma is a multifactorial, multistep and complex process. Its prognosis is poor and early diagnosis and monitoring of metastasis of HCC is of utmost importance. Circulating alpha-fetoprotien mRNA has been proposed as a marker of HCC cells disseminated into the circulation but the specificity of this molecular marker and its correlation with the main HCC clinico-pathological parameters remain controversial. In recent years; several different multi-marker assays have been developed for the detection of hepatoma cells in the peripheral blood of patients with HCC.

In this study 58 patients and 15 matched healthy volunteers were included; the patients were divided into three groups; group A: patients with primary HCC (n = 32), group B: patients with cirrhosis with no evidence of HCC (n = 12), group C: patients with metastatic cancer in liver (n = 14). Group D: 15 healthy volunteers age and sex matched. The staging of HCC was carried out according to the Tumor/Node/Metastasis (TNM) classification.

Peripheral blood samples were obtained from all subjects; MAGE-1 and MAGE-3 and AFP mRNAs were detected by nested RT-PCR.

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The positive rates of MAGE-1, MAGE-3 and AFP mRNAs were 18/32 (56.3%), 15/32 (46.9%) and 19/32 (59.4%) respectively in the primary HCC patients.

In the cirrhotic group only 4/12 (33.3%) patients were positive for AFP mRNA, whereas in the metastatic group 5/14 (35.7%) and 4/14 (28.6%) were positive to MAGE-1 and MAGE-3 mRNAs respectively. MAGE-1 and MAGE-3 mRNAs were correlated with TNM clinical stages; tumor number and tumor size ($p < 0.05$).

Our results indicate that a multi-marker nested RT-PCR assay with cancer-specific markers such as MAGE-1 and MAGE-3 in combination with a hepatocyte-specific AFP marker may be a promising diagnostic tool for monitoring hepatocellular carcinoma patients. Nested PCR exhibits higher sensitivity, stronger specificity and lower false-positive occurrence as compared to single RT.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases in the world. Approximately, 560,000 new cases of HCC are diagnosed each year, constituting 6% of all new human cancers [1]. Hepatoma tissues can synthesize many tumor-related proteins, polypeptides and isoenzymes. Hepatoma cell circulating specific biomarkers are useful predictors for early diagnosis of HCC or monitoring metastasis or post operative recurrence of disease [2,3].

Alpha Fetoprotein is a 70 kDa glycoprotein that in humans is encoded by the AFP gene. AFP is produced by the yolk sac and the liver during fetal life [4,5]. Human α-Fetoprotein is a well-known marker for hepatoma cells. Although the serum AFP level could be one of the useful biochemical markers for the diagnosis of HCC, it is not so sensitive. Recently; AFP gene transcripts have been used as targets of the RT-PCR assay in blood from HCC patients but the detection of these gene transcripts do not truly reflect the presence of tumor cells, since non-tumors liver cells also abundantly express AFP mRNA, which could be released into blood because of hepatitis, liver cirrhosis and surgical injuries [6].

Since cancer cells are usually very heterogeneous, different cancers express different markers and even cells from the same tumor may not be identical. Thus, single-marker RT-PCR has its limitations in sensitivity and specificity [7,8].

The melanoma antigen gene family (MAGE) consists of groups of genes: MAGE-A, B and C... These are all localized on the X chromosome at locations Xq28, Xp21.3, and Xq26, respectively. MAGE-A comprises 12 genes (MAGE-1–MAGE-12). The products of the MAGE family are highly specific to cancer cells; thus, they have been extensively studied as important tumor markers for cancer prognosis and immunotherapy. The members of MAGE gene family are highly expressed in human HCC. MAGE-1 and 3 are among these tumor associated antigens that play an extremely important role in tumor growth by rendering high, tumor cell metabolic rate. They encode 46 and 44 kDa proteins, respectively and are expressed in a very limited range of normal tissues (germ cells and placenta) [9,10].

This study was conducted to examine the expression of MAGE-1 and MAGE-3 and AFP gene transcripts in blood specimens obtained from patients with primary HCC and also from HCC-free controls to assess a multi-marker nested RT-PCR assay for the detection of circulating HCC cells and may suggest a diagnostic capability for these markers.

2. Subjects and methods

2.1. Subjects

This study had been carried out at Internal Medicine Department; Ain Shams Hospital; it included 58 patients and 15 healthy volunteers as a control. All subjects provided their informed consent to participate in the study. The patients were divided into three groups: Group A; patients with primary HCC ($n = 32$) at variable stages of the disease (proven histopathologically), 18 males and 14 females. Group B; patients with cirrhosis ($n = 12$), 7 patients on top of HBV and 5 patients on top of HCV with no evidence of HCC (proven radiologically), 7 males and 5 females. Group C; patients with metastatic cancer in the liver ($n = 14$), 9 males and 5 females. Group D; 15 healthy volunteers' age and sex matched, proved clinically and by laboratory investigations to be free from diseases. Brief information on each group is given in (Table 1).

Each patient was subjected to full history taking, complete general and abdominal examination, imaging investigation (abdominal ultrasonography and C.T) and laboratory evaluation; analysis of liver functions, including: total bilirubin, Alanine Transaminase (ALT), Aspartate Transaminase (AST), serum albumin and prothrombin time, viral hepatitis markers, including HCV antibody, HBV surface antigen and antibody, HBV core antibody.

Ultrasound guided liver biopsy with written consent was done for group A patients to confirm the diagnosis of HCC by histopathology. The procedure was done by semi-automated true cut needle under local anesthesia (xylocaine). Platelet count above 50,000 and prothrombin time not more than three seconds above normal were mandatory before the procedure. Patients were kept at the hospital 6 h after the procedure for observation of vital data and occurrence of any complications.

According to radiological findings and Tumor/Node/Metastasis classification, the HCC patients were divided into: Stage I: 6 patients (18.8%), stage II: 7 patients (21.9%), stage III: patients 10 (31.2%) and stage IV: 9 patients (28.1%).

2.2. Methods

2.2.1. Blood samples

Fifteen ml of whole blood was withdrawn from the peripheral veins from both patients and healthy volunteers and were collected into two tubes: 10 ml of heparinized blood for

Table 1 Demographic information of enrolled groups.

	Healthy volunteer	HCC	Cirrhosis	Metastatic cancer	F*	P
n	15	32	12	14		
Gender						
Male	10 (66.7%)	18 (56.2%)	7(58.3%)	9 (64.3%)		
Female	5 (33.3%)	14 (43.8%)	5 (41.7%)	5 (35.7%)		
Age						
Mean ± SD	55.73 ± 9.1 (39–72)	56.62 ± 8.42 (41–69)	58.10 ± 7.50 (47–70)	60.28 ± 6.83 (51–71)	0.92	$p > 0.05$

* ANOVA test.

separation of mononuclear cells and another 5 ml in plain tubes for separation of serum to detect AFP.

2.2.2. I. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation using Ficoll-hypaque 1077 (Sigma, USA) at 1200 g for 30 min at 4 °C. The interface cells were removed, washed twice with 25 ml of sterile PBS (pH7.3), pelleted, and resuspended in 1 ml of PBS. The cells were pelleted again at 1200 g for 2 minutes. The cell pellets were kept at −80 °C until RNA extraction.

2.2.3. II. Extraction of total RNA from nuclear cells

Total RNA of nuclear cells was extracted using RNA extraction kit (Qiagen) according to the manufacturer's instruction. All RNA preparation and handling steps took place in a biosafety level 2 laminar flow hood, under RNAse-free conditions. The isolated RNA was resuspended in RNAse-free water and stored at −80 °C until assay. The RNA concentration was assessed by absorbance reading at 260 nm with UV spectrophotometry (Beckman; Du series 650, INC, USA). As positive controls, RNA samples were also prepared from the human tumor cell line HepG2 prepared by cell culture unit in a medical research center; Ain Shams University.

2.2.4. III. Amplification by nested reverse transcriptase polymerase chain reaction (RT-PCR)

Three different PCR reactions were performed with each sample in order to amplify fragments of MAGE-1 and MAGE-3 and AFP mRNAs.

2.3. Reverse transcription (Complementary cDNA synthesis)

Reverse transcription reaction was carried out in a 20 µl reaction mixture by using first strand cDNA synthesis kit (Promega; USA) according to manufacture's instruction.

2.4. Nested PCR for MAGE-1 and 3 mRNAs and AFP mRNA

A total amount of 50µl reaction solution contained 5 µl 10X PCR buffer, 0.2 mmol/L of each dNTPs, 30 pmol of each external primers, 2.5U Taq DNA polymerase (Promega; USA) and cDNA. Thermocycling in either an MJ Research PTC 200 (MJ Research, Inc., Boston, Mass.) or Perkin-Elmer 9600 (Perkin-Elmer, Cambridge, United Kingdom) according to the following cycle profile using different primers for MAGE-1 and MAGE-3 and AFP (Table 2) for MAGE-1; denaturation at 94 °C for 6 min, annealing at 60 °C for 30 s and extension at 72 °C for2 min for the first cycle followed by (denaturation 94 °C for 40 s, annealing 60 °C for 30 s and extension 72 °C for 30 s) thermal cycles were repeated 35 times; terminal extension at 72 °C for 2 min. For AFP; initial denaturation at 94 °C for 2 min followed by the following sequence: (94 °C for 1.5 min., 57 °C for 1.5 min. and 72 °C for 2.5 min) plus a terminal extension at 72 °C for 10 min.; thermal cycles were repeated 35 times and for MAGE-3 denaturation at 94 °C for 4 min. Followed by (94 °C for 45 s and 72 °C for 3 min) for 35 cycles; terminal extension at 72 °C for 2 min.

A sample of 10 µl of the first amplification product was further amplified using the inner pair of the primer. PCR procedure was; (94 °C for 45 s, 58 °C for 45 s, and 72 °C for 45 s) for MAGE-1 and (94 °C for 40 s, 58 °C for 30 s, and 72 °C for 30 s) for AFP and (94 °C for 45 s, 69 °C for 45 s, and 72 °C for 45 s) for MAGE3; the thermal cycles were repeated 32 times as well as followed by final extension at 72 °C for 2 min.

To verify the successful preparation of mRNA, samples were detected for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Reaction tubes containing cDNA from Hep G2 cells as positive controls and no template control sample without cDNA addition were included as negative controls for each PCR reaction.

2.4.1. Immunoassay for detection of AFP

For the analysis of AFP; we used a commercially available kit. The assay was performed according to the manufacturer's recommendation using Elecsys 2010 (roche Diagnostics Gmbh, D-68298 Mannheim).

2.4.2. Gel electrophoresis

The final amplification product was electrophoresed on 2% agarose gel and stained with ethidium bromide for the specific band of bp.

2.5. Statistical analysis

The results were analyzed using the Statistical Package of Social Sciences (SPSS) computer software program, version 16.0 (Chicago, IL, USA). Quantitative data were presented as mean ± SD for normally distributed data and as medians and percentiles for skewed data. Qualitative data were presented in the form of frequencies and percentages. For normally distributed parameters, differences among groups were

Table 2 Sequences of oligonucleotide primers used for PCR reaction [7,11,12].

Primers	Sequence of primers (5′–3′)	(bp)
AFP primer7		
Outer-sense	ACTGAATCCACAACACTGCATAG	
Outer-antisense	TGCAGTCAATGCATCTTCACCA	176
Inner-sense	TGGAATAGCTTCCATATTGGATTC	
Inner-antisense	AAGTGGCTTCTTGAACAAACTGG	101
MAGE-1 primer7		
Outer-sense	GTAGAGTTCGGCCGAAGGAAC	149
Outer-antisense	CAGGAGCTGGGCAATGAAGAC	
Inner-sense	TAGAGTTCGGCCGAAGGAAC	143
Inner-antisense	CTGGGCAATGAAGACCCACA	
MAGE-3 primer11	TGG AGG ACC AGA GGCCCC C	725
Outer-sense	GGA CGA TTA TCA GGAGGC CTG C	
Outer-antisense	CGG AGG AGC ACT GAAGGA GAA G	371
Inner-sense	CCT CCT CTT CTT GGTTGC TGG	
Inner-antisense		
GAPDH primer12		
Sense	CTA CTG GCG CTG CCA AGG CTG T	390
Anti sense	GCC ATG AGG TCC ACC ACC CTG T	

tested by one-way analysis of variance (ANOVA). For Qualitative data, differences among groups were tested using Pearson's chi-square test (X^2) and Fisher's exact test. A p value less than 0.05 was considered statistically significant.

2.6. Results

2.6.1. Interpretation of the results

According to the design of primer pairs, the PCR products of (149 and 143); (725 and 371) and (176 and 101) base pairs were amplified from MAGE-1 and MAGE-3 and AFP cDNA respectively (Figs. 1–3); while GAPDH band amplification was visualized corresponding to 390 bp. The sizes of PCR products were estimated by comparison with DNA molecular mass markers (Bio-Rad; EZ Load).

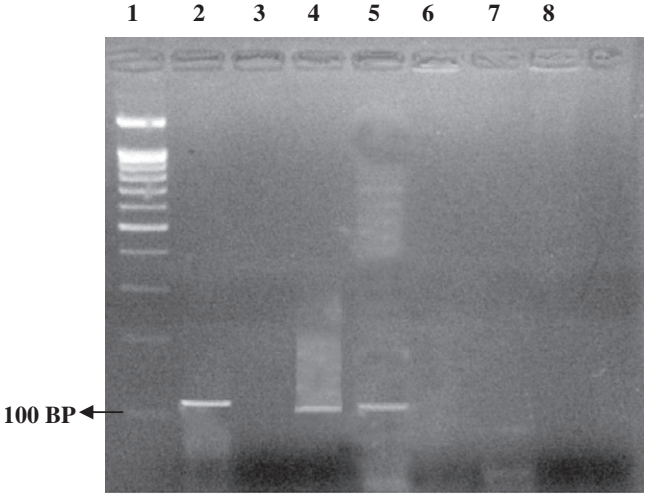

Figure 1 Representative pictures: Electrophoretic separation of PCR amplified product; detection of gene transcripts in blood specimens from HCC patients: Lane 1: DNA molecular size marker, Lane 2: Positive control, Lane 3: Negative control, Lane 4&5: Cases showing AFP positive gene transcript, Lane 6: Negative case.

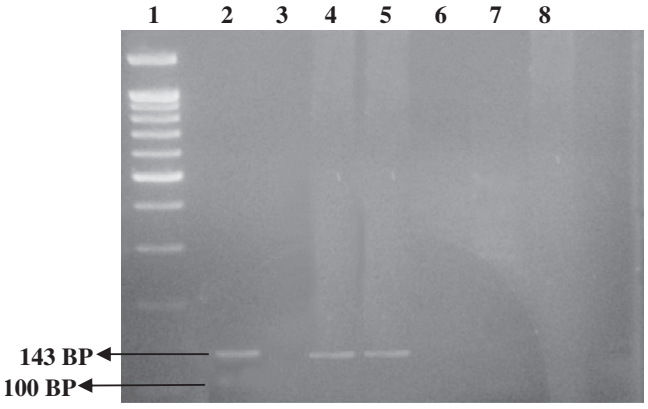

Figure 2 Representative pictures: Electrophoretic separation of PCR amplified product; Detection of gene transcripts in blood specimens from HCC patients: Lane 1: DNA molecular size marker, Lane 2: Positive control, Lane 3: Negative control, Lanes 4 and 5: Cases showing MAGE1 positive gene transcript, Lane 6: Negative case, Lane 7: Negative case.

Expression of MAGE-1, MAGE-3 and AFPmRNAs in peripheral blood in patients and controls (Fig. 4).

MAGE-1 and MAGE-3 mRNAs were detected in 18/32 (56.3%), 15/32 (46.9%) in primary HCC patients and none of the cirrhotic group patients expresses MAGE-1 and MAGE-3 mRNAs 0/12 (0%); and 5/14 (35.7%); 4/14 (28.6%) in patients with metastatic cancer in the liver. The positive rate for MAGE-1 and MAGE-3 mRNAs in blood was higher in primary HCC patients; compared to metastatic group. Neither of the normal healthy volunteers and cirrhotic group gave detectable signals for MAGE-1 and MAGE-3 mRNAs.

The frequencies of the positive rate MAGE-1 and MAGE-3mRNAs in blood were associated with the progression of HCC as indicated by clinical parameters such as TNM tumor stage, the positive rate MAGE-1 and MAGE-3mRNAs in

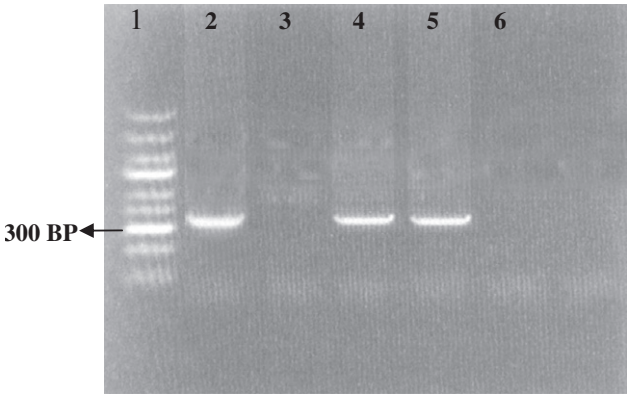

Figure 3 Representative pictures: Electrophoretic separation of PCR amplified product; Detection of gene transcripts in blood specimens from HCC patients: Lane 1: DNA molecular size marker, Lane 2: Positive control, Lane 3: Negative control, Lanes 4 and 5: Cases showing MAGE3 positive gene transcript, Lane 6: Negative case.

TNM stages III–IV was significantly higher than in stages I–II ($p < 0.05$) (Table 3) and also the positive rate was correlated with tumor number and tumor size ($p < 0.05$) (Table 4), These results indicate that the incidence of the expression of cancer specific molecular markers MAGE-1 and MAGE-3mRNAs increased in advanced stages of disease and correlated with clinical severity.

Concerning AFPmRNA, it was positive in 19/32(59.4%) in primary HCC patients compared to metastatic cancer in liver group 0/14 (0%); 4/12 (33.3%) in patients with cirrhosis of the liver. The positive rate for AFPmRNA in blood was significantly higher in primary HCC patients than in patients with chronic liver disease ($p < 0.05$). Neither of the normal healthy volunteers and metastatic groups showed detectable signals for AFP mRNA.

The correlation of AFPmRNA molecular marker with the main clinico-pathological parameters remains unclear; the frequency of positive cases of AFPmRNA in TNM stages III–IV was significantly higher than in stages I–II but no correlation was found between tumor size or tumor number and the positive rate of AFPmRNA (Tables 3 and 4).

The detection of MAGE-1mRNA in peripheral blood is correlated with that of AFPmRNA and MAGE-3mRNA and the detectable rate of MAGE-1 mRNA in AFPmRNA positive patients was significantly different from that in AFPmRNA negative patients ($p < 0.05$). Also the detectable rate of MAGE-3 mRNA in AFPmRNA positive patients was significantly different from that in AFPmRNA negative patients ($p < 0.05$) (Table 5).

Concerning AFP in different groups (Table 6); in this work, AFP was evaluated at a cut off value of 400 ng/μl (0.400 IU/ml) although HCC patients with a high level of serum AFP exhibited detectable concentrations of MAGE-1and 3 mRNAs and AFPmRNA more often than those with a low serum AFP level, there is no significant difference (Table 7).

Concerning multimarker expression of MAGE1 and MAGE3 and AFP mRNAs; the result indicates that 24/32 (75%) were positive for at least one marker and 10/32 (31.3%) positive for the three molecular markers which strongly indicate hamatogenous spread.

3. Discussion

HCC is the fifth most common cancer worldwide and represents the third cause of cancer deaths after lung and breast

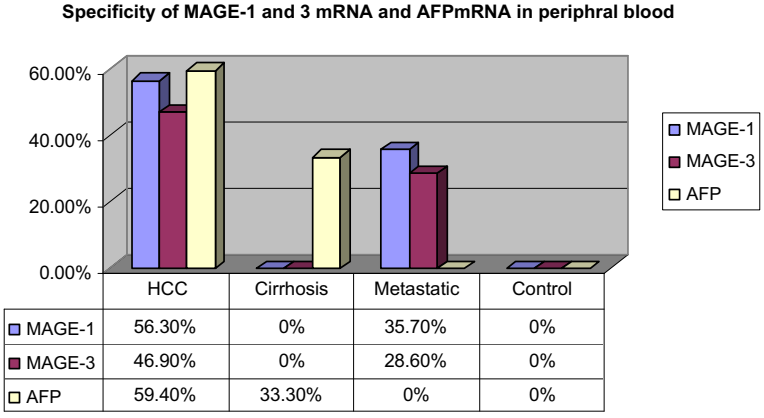

Figure 4 Expression of MAGE-1; MAGE-3 mRNAs and AFPmRNA in different group.

Table 3 Expression of MAGE-1; MAGE-3 and AFPmRNAs in primary HCC patients group in relation to TMN staging.

	HCC				*	
	I	II	III	IV	x^2	*P*
n	*6*	*7*	*10*	*9*		
MAGE-1 mRNA	0 (0%)	2 (28.6%)	7 (70%)	9 (100%)	14.0	0.001
MAGE-3 mRNA	0 (0%)	3 (42.9%)	4 (40%)	8 (88.9%)	5.0	0.03
AFP mRNA	0 (0%)	4 (57.1%)	6 (60%)	9 (100%)	4.8	0.04

* Comparison between TNM I–II and TNM III–IV (Sig. $p < 0.05$).

Table 4 Expression of MAGE-1, MAGE-3 and AFP mRNAs in primary HCC patients in relation to Gender, tumor number and size.

	n	MAGE-1	p^*	MAGE-3	p^*	AFP	p^*
Gender							
Male	18	8 (44.4%)		7 (38.9%)		10 (55.6%)	
Female	14	10 (71.4%)	0.16	8 (57.1%)	0.47	9 (64.3%)	0.72
Number of nodules							
Solitary	13	2 (15.4%)		3 (23.07%)		5 (38.46%)	
Multiple	19	16 (84.2%)	0.001	12 (63.16%)	0.03	14 (73.68%)	0.07
Tumor size							
<5	11	3 (27.27%)		2 (18.18%)		4 (36.36%)	
>5	21	15 (71.43%)	0.02	13 (61.90%)	0.02	15 (71.42%)	0.06

* Chi square test or Fisher's exact test for small sample size (Sig. $p < 0.05$).

Table 5 Comparison of expression of marker genes in blood samples of primary HCC patients.

		AFP			MAGE3		
	n	Negative	Positive	p^*	Negative	Positive	p^*
MAGE1							
Negative	14	9	5		11	3	
Positive	18	4	14	0.02	6	12	0.01
Total	32	13	19		17	15	

* Chi square test (Sig. $p < 0.05$).

Table 6 Serum AFP level in different group.

Serum AFP (ng/μl)	Healthy volunteer	HCC	Cirrhosis	Metastatic	*p*
Mean ± SD	4.86 ± 3.39	768 ± 570	78.3 ± 33.6	426 ± 241	
Median	5.0	510	81.5	436	
Minimum	–	123	34	125	$p < 0.05$
Maximum	9	2225	145	980	
Range	9	2132	111	855	

Table 7 Expression of MAGE-1 and 3 mRNAs and AFP mRNA and serum AFP level in primary HCC patients.

	Serum AFP		X^{2*}	*p*
	<400	>400		
n	13	19		
MAGE-1 mRNA	5 (38.5%)	13 (68.4%)	2.80	0.09
MAGE-3 mRNA	4 (30.8%)	11 (57.9%)	2.28	0.13
AFP mRNA	7 (53.8%)	12 (63.2%)	0.22	0.50

* Chi square test (Sig. $p < 0.05$).

cancers. The disease severely affects the Egyptian population. HCC is a high-grade malignancy showing a rapid infiltrating growth, early stage metastasis, poor therapeutic response and disappointing prognosis even after successful curative resection surgery [13]. The early dissemination of cancer cells to secondary sites is the main cause of mortality of patients with solid tumors [14].

The routine diagnosis of HCC has been largely dependent on the serological levels of AFP. However, its clinical application has been lost significantly due to the fact that liver diseases other than HCC are accompanied by high levels of AFP. In addition, a significant proportion of HCC patients did not have an elevated AFP.

False positive results will be unavoidable if qualitative AFP mRNA determination is used as the single marker for the detection of circulating cancer cells. Subsequently, AFP represents liver cell-specific, not tumor specific markers [3,14]. Using a multi-marker assay combining cancer specific markers and liver specific marker may improve the methods of screening of HCC.

The purpose of this study was to detect circulating HCC cells in blood samples using tumor specific markers, MAGE-1, MAGE-3mRNAs and liver specific marker AFPmRNA by nested PCR which provides 100 fold sensitivities. Moreover, nested RT-PCR has increased the specificity owing to the use of two pairs of specific primers.

In this study, the positive rate of MAGE-1, MAGE-3 and AFPmRNAs was 56.3%, 46.9% and 59.4% respectively in primary HCC patients, 100%; 88.9% and 100% in HCC patients with extrahepatic metastasis. In 24/32 (75%) patients, there is at least one positive marker. 10/32 (31.3%) patients were positive for the three markers which strongly indicate the presence of malignant hepatocytes in the circulatory system. In contrast, none of the healthy clinical control samples show detectable

levels of MAGE-1 and MAGE-3and AFPmRNAs in their peripheral blood.

Our results indicated that MAGE-1 and MAGE-3 mRNAs are cancer specific, and could be detected in samples from patients with both primary and metastatic cancers in liver and the detection of AFPmRNA in peripheral blood is associated not only with primary hepatocellular carcinoma but also with cirrhosis. AFPmRNA represents a liver specific marker not a tumor specific marker and AFP transcript is valuable in the diagnosis of HCC but not a specific marker to the diagnosis of HCC; thus cancer-specific MAGE-1 and MAGE-3 genes are a powerful complement to the hepatocyte specific AFP gene for the detection of circulating HCC cells.

Recently, several different multimarker assays have been developed for the detection of tumor cells in the peripheral blood of patients with variable malignancies. Several reports [15] [16] have revealed that; MAGE gene transcripts were highly expressed in HCC tissues even in alpha fetoprotein negative HCC, but not in non-tumor liver tissues.

MAGE gene transcripts have been regarded as tumor specific markers and found to be highly expressed in a variety of histological types of cancer; the positive rate was 46–80% of MAGE-1 transcript and 42–68% of MAGE-3 transcript in HCC samples. A proportion of 74–86% of HCC tissue samples were positive for at least one MAGE gene transcript, while no expression of MAGE transcript was detected in surrounding non-cancerous tissues, nor in liver cirrhosis [17–19].

Miyamoto et al. [8] reported that MAGE-1, MAGE-3 and AFP transcripts were detected in 9 (12.7%), 3(4.8%), and 10(15.9%) of 71 blood specimens from HCC patients, respectively. In contrast, MAGE-1mRNA and AFPmRNA were detected in 27(41.5%) and 35(53.8%) of 65 blood samples from the HCC patients respectively as reported by Yang et al. [9] which agree with our study. The difference in result is due to the method used; nested RT-PCR.

Zhao et al. investigated MAGE-1 gene expression in HCC, cirrhosis and normal liver tissues. They found that MAGE-1 was detectable in 72.2% of HCC samples, while none of the cirrhotic or normal liver tissues expressed this mRNA [20].

Zhang et al. reported that, in 86 tumor specimens, the positivity for MAGE-1, MAGE-3, and AFP genes was 34.9% (30/86), 60.5% (52/86) and 69.8%(60/86) respectively, and all specimens expressed at least one marker. MAGE-1, MAGE-3, and AFP transcripts were detected in 12 (14.0%), 18 (20.1%) and 29 (33.7%) of 86 blood specimens from hepatocellular carcinoma patients, respectively, while 45 specimens (52.3%) were positive for at least one marker. In addition, MAGE-1, MAGE-3 and AFP gene transcripts were not detected in any peripheral blood specimens from 25 chronic liver disease patients and 28 normal healthy volunteers [21].

El Aggan et al. also reported that, in HCC patients, the positive rate of MAGE-1 and MAGE-3 mRNA expression was 53.3% and 33.3% in peripheral blood samples respectively, while the positive rate was 53.3% and 40% in HCC tissue samples, respectively. By contrast, MAGE-1 and MAGE-3 mRNA were not detected in the adjacent non-neoplastic liver tissues or in the peripheral blood samples of cirrhotic patients without HCC and healthy subjects [22].

In this study the frequencies of the positive rate MAGE-1 and MAGE-3mRNAs in blood were associated with the progression of HCC as indicated by clinical parameters such as TNM tumor stage, tumor number and tumor size ($p < 0.05$) but the correlation of AFP mRNA molecular marker with the main HCC clinico-pathological parameters remains unclear; the positive rate of AFP mRNA when correlated with TNM clinical stages, no correlation was found between tumor number or tumor size.

Zhang et al., (2009) reported that the expression of MAGE-1 mRNA and/or AFP mRNA in peripheral blood was closely correlated with the pathological stage [23].

In contrast; Chen et al. [18] and Lupo et al. [24] found that MAGE-1 genes are expressed in most HCC samples, but no correlation was found with tumor size or stage of the disease; the reason for this discrepancy may be due to the small number of the tested patients.

The present study also demonstrated that although HCC patients with a high level of serum AFP exhibited detectable concentrations of MAGE-1, MAGE-3 and AFP mRNAs more often than those with a low serum AFP level, there is no significant difference. The reason for the discrepancy among various studies may be multifactorial. The limited number of tested patients and in the method of isolating circulating carcinoma cells may be contributory factors.

Circulating hepatocytes positive for AFP mRNA have rarely been reported in healthy persons [25–27]. In agreement with the majority of other investigators [28–30], none of the healthy controls included in the current study were positive for circulating or MAGE-1, MAGE-3 and AFP mRNAs.

RT-PCR is a very sensitive technique for amplifying nucleic acids [31,32], which could often be used to detect tiny amounts of mRNA copies. Comparison of the sensitivities of RT-PCR and nested RT-PCR revealed that nested RT-PCR provides 100 fold sensitivities. Moreover, the nested RT-PCR has increased the specificity owing to the use of two pairs of specific primers. Hence, nested RT-PCR is superior to RT-PCR with respect to specificity and sensitivity for the detection of micrometastatic tumor cells.

Multimarker assay with cancer-specific MAGE-1 and MAGE-3 mRNAs and liver specific AFP mRNA appears to be a readily available and highly sensitive and specific method for the detection of circulating HCC cells. Since nested PCR utilizes a couple of internal primers to reamplify the specific PCR product, it exhibits higher sensitivity, stronger specificity and lower false-positive occurrence as compared to single RT. Long-term follow-up of RT-PCR positive patients will be required to determine its clinical relevance. If validated as a predictor of early dissemination of cancer cells (metastatic potential), this method would provide a powerful complement to routine histopathologic analysis of HCC.

Conflicts of interest

The authors indicate that they do not have any conflict of interest.

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