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Cyclooxygenase-2 gene and MAGE Gene m-RNA in hepatocellular carcinoma

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ABSTRACT

Tumor markers in the early detection of tumors are promising tools that could improve the control and treatment of tumors. While alpha-fetoprotein (AFP) is a commonly used tumor marker in the detection of hepatocellular carcinoma (HCC), its sensitivity and specificity are insufficient to detect HCC in all patient samples. This study aimed to evaluate MAGE-1 m-RNA expression in blood, to identify functional single nucleotide polymorphisms in the cyclooxygenase-2 gene promoter and to evaluate the level of serum alpha-L-fucosidase (AFU) and alpha-fetoprotein which evaluated in a trial to assess their possible use as a diagnostic tool for HCV infection complications like cirrhosis and HCC. This study was carried out on the following groups: group I: 25 healthy individuals served as control, group II: 50 HCV infection patients without any complications, group III: 50 HCV infected patients complicated with cirrhosis and group IV: 75 HCV infected patients complicated with (45 localized and 30 metastatic) HCC. MAGE-1 m-RNA expression in blood, identification of SNP in COX-2 gene promoter, serum levels of AFU, detection of alpha-fetoprotein using an enzyme-linked immunosorbent assay (ELISA) and some routine liver function (AST, ALT, T.B, Alb and PT) were determined and the results were statistically analyzed revealing the following: MAGE-1 m-RNA determination in blood by RT-PCR assay revealed 46.1 % (31 out of 75 HCC patient) were found to be positive for MAGE-1. We found -1195A allele carriers had a higher risk of HCC with HCV infection. As regard the obtained results of serum AFU, a significant increase was detected in HCC as compared with cirrhosis, hepatitis and healthy control groups ($p < 0.001$). Concerning the obtained results of serum AFP, when HCC group was compared with cirrhosis, hepatitis and healthy controls, a significant increase was observed ($P < 0.001$), In conclusion: detection of MAGE-1 m-RNA in blood, identification of SNP in COX-2 gene promoter and evaluation of serum AFU and AFP have different significances and might be used as markers in screening individuals at high risk of HCC and give a red light in early detection of HCC which may be reduce its fatal incidence.

KEYWORDS

Hepatocellular carcinoma;
MAGE-1 m-RNA gene;
SNP in COX-2 gene promoter;
Alpha-L-fucosidase;
Alpha-fetoprotein.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and lethal cancers in the world[1]. More than one million cases of HCC occur in the world each year[2]. Although many treatment modalities for HCC are available (including hepatic resection, liver transplantation, radiofrequency ablation, transarterial chemombolization, etc.) at present, the prognosis of HCC patients remains dismal because it is detected at an advanced, non-resectable stage. Early diagnosis of HCC can improve the prognosis of HCC patients. So far, alpha-fetoprotein (AFP) is the generally accepted serological marker. Serum AFP alone contributes to the diagnosis of HCC if its level is markedly elevated (over 400 μg/L as a threshold value), which occurs in less than 50% of cases at the time of diagnosis[3]. Moreover, serum AFP level is negative or slightly elevated in 20%-40% patients, which can significantly reduce the sensitivity of an assay based on over-expression of AFP. The serum AFP level in patients with acute or chronic hepatitis or liver cirrhosis but without malignant disease is often elevated. Since detection of serum AFP level in blood samples appears to be nonspecific[4]. Therefore, the diagnostic sensitivity and specificity of AFP are unsatisfactory and questionable. It is thus necessary to select other specific methods for the diagnosis of HCC.

Transcripts of tumor-specific genes can be amplified and detected by reverse transcripts-polymerase chain reaction (RT-PCR). In[5], Smith et al first successfully adopted RT-PCR technique to assess tyrosinase messenger RNA (mRNA) as a tumor marker in detecting circulating melanoma cells. Since then, this technique has been applied to the detection of CTC in solid tumors[6]. Melanoma antigen-1 (MAGE-1) has been designated as cancer-testis antigens (CTA)[7]. It has been reported that MAGE-1 mRNA is expressed with a high percentage and specificity in HCC[8]. Multiple gene alterations such as allelic deletion, mutation, and altered methylamines are marked in HCC. Studies also indicated hat aberrations in certain genes were responsible for certain clinical features of HCC[9].

Cyclooxygenases also known as prostaglandin endoperoxide H synthases or prostaglandin G/H synthases) are key enzymes in mediating the conversion of free archidonic acid into prostaglandin H2. These active products are important regulators of many biologic processes such as inflammation, immune function, cell proliferation, and angiogenesis, which are all relevant to cancer development and progression)[10]. There are two isoforms of cyclooxygenase (COX-1 and COX-2). COX-1 is expressed in many tissues and COX-2 is responsible for prostaglandins produced in sites of inflammation and is induced by various carcinogens[11]. Several studies have indicated that Cyclooxygenase-2 (COX-2) gene is up-regulated in HCC[12]. Alpha-L-fucosidase (AFU) enzyme is a lysosomal enzyme present in all mammalian cells and its activity was significantly increased ($p < 0.001$) in patients suffering from hepatocellular carcinoma[13]. Serial determinations of serum AFU activity were found to be useful in the early detection of HCC[14].

Both AFP and AFU are good markers for HCC and their simultaneous determination may improve the detection of HCC[15].

In this study, we evaluated the diagnostic significance of a highly sensitive nested RT-PCR assay for the MAGE-1 in peripheral blood and identified single nucleotide polymorphisms (SNPs) in the COX-2 gene promoter and performed genotype and to investigate the clinical usefulness of AFU with AFP in our population, we assayed this markers in patients with HCC, cirrhosis, chronic hepatitis and controls.

PATIENTS & METHODS

Samples

The present study was carried out on 200 individuals (85 females and 115 males) with age ranged from (19 to 69 years). They were selected from Clinical Oncology and Internal Medicine Outpatient clinics, Faculty of Medicine, Zagazig University Hospital. They were divided into the following groups: Group I: consists of 25 healthy individuals (13 males and 12 females) aged from 19-49 years served as control. Group II: consists of 50 HCV infected patients without complications (33 males and 17 females) aged from 22 to 61 years. Group III: consists of 50 HCV infected patients with Cirrhosis (32 males and 18 females) aged from 42-68 years. Group IV: consists of 75 HCV infected Patients complicated with cirrhosis and HCC (45 localized and 30 metastatic) (37 males and 38 females)

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aged from 43-69 years.

Each patient was subjected to the following:

- Liver function profile (serum bilirubin, AST, ALT, albumin and Prothrombin time).
- ELISA for the detection of Alpha-fetoprotein in serum according to[16].
- Serum activity of α-L-fucosidase enzyme was assayed by a modification of the method of[17].

Preparation of mRNA

mRNA was extracted with a Micro prep mRNA extraction kit (Pharmacia Biotech, Uppsala, Sweden). Extracted mRNA was dissolved in 40µl of distilled water.

Complementary DNA synthesis

Each mRNA sample was incubated with 1µl of random hexamers (Takara Biomedicals, Ohtsu, Japan), 4 µl of 5*reverse-transcriptase buffer (Life Technologies, Gaithersburg, MD), 2 µL of 0.1 mol/L dithiothreitol (Life Technologies), 1 µl of dNTP mixture (2.5 mmol/L of each deoxyribonucleoside triphosphate) (Takara Biomedicals), and 200 units of Molony murine leukemia virus RT (Life Technologies) (final volume of 20 µl). After incubation at 37ºC for 60 minutes, the RT products were used for PCR amplification with 421 bp (MAGE-1).

Detection of β-actin complementary DNA

5 µl of complementary DNA sample was used for PCR amplification of β-actin primer (Stratagene, La Jolla, CA), dNTP mixture, PCR buffer, and 1 unit of *Taq* polymerase (Takara Biomedicals) constituted the reaction mixture (final volume of 25 µl). Amplification was performed for 35 cycles (1.5 minutes at 72ºC, 45 seconds at 92ºC, and 45 seconds at 60ºC). The PCR product were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and observed with UV lamp.

Genotyping

Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood lymphocytes using the TIANamp blood DNA kit (Tiangen Biotech [Beijing] Co., Ltd., China). For SNP screening, PCR was then performed in a 50 µl reaction system containing 200 ng genomic DNA, 400 pmol of each primer (Shanghai Sangon Company, China), 200 pmol of each deoxynucleotide triphosphate (dNTP), 5 µl 10 x PCR buffer (500 mmol KCl, 100 mmol Tris-HCl, and 15 mmol $MgCl_2$), and 1.25 U Taq polymerase (Takara Biotechnology, Dalian, China). The cycling conditions were: 95ºC for 45 s, 60ºC for 45 s and 72ºC for 30 s and a final extension at 72ºC for 10 min. The 304-base pair (bp) product was amplified, and then digested with *pvu* II (New England Biolabs, UK) for 4h in a 37ºC water bath.

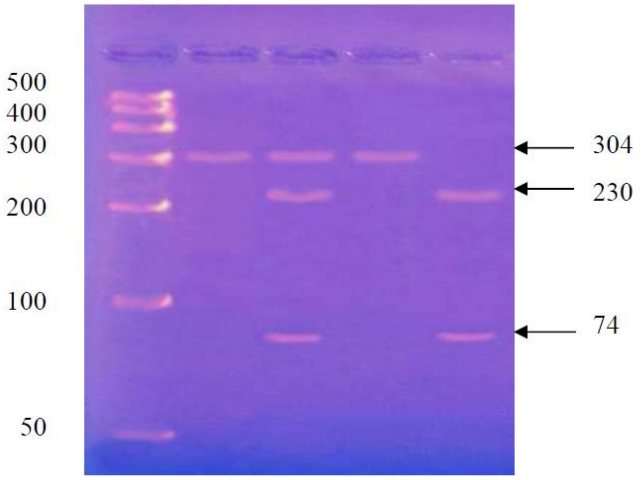

Figure 1 : Agarose gel electrophoresis of polymerase chain reaction (PCR) products digested with pvu II. Lan 1: 50-500 bp DNA marker; lan 2: PCR product (304 bp); lan 3: GA heterozygote (304 bp + 230 bp + 74 bp); lan 4: AA homozygote (304 bp); lan 5: GG homozygote (230 bp + 74 bp).

The digested product was separated on a 2% agarose gel electrophoresis at a 120 V constant voltage for 0.5 h and then visualized under ultraviolet light. The variant G allele has a *pvu* II restriction site while the A allele lacks the *pvu* II restriction site, so the GG homozygote results in two bands (230 bp and 74 bp), the GA heterozygote produces three bands (304 bp + 230 bp + 74 bp) and the AA homozygote produces a single 304 bp band.

Statistical analysis

All statistical analyses were done by a statistical for social science package "SPSS" 10.0 for Microsoft Windows, SPSS Inc.).

RESULTS

MAGE-1 m-RNA expression in blood and serum levels of AFU and AFP and COX-2 gene -1195 G/A genotyping were determined and the results were sta-

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tistically analyzed revealing the following:

MAGE-1 m-RNA determination in blood by RT-PCR assay revealed 46.1 % (31 out of 75 HCC patients) were found to be positive for MAGE-1 in the peripheral blood samples as the 421-bp band. This 421-bp band was analyzed by gel electrophoresis.

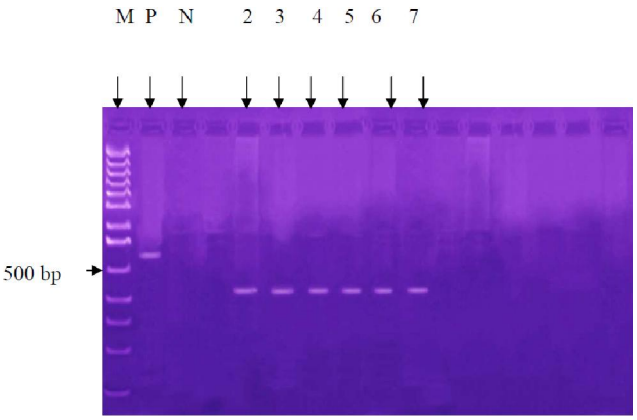

Figure 2 : Electrophoresis of PCR products of gene transcripts in PBMCs samples from Metastatic HCC patients where M: molecular marker, N: negative control, P: positive control and patients 2, 3, 4,5,6,7 showing MAGE-1 transcript positive.

Detection of MAGE-1 transcript in PBMCs is closely correlated to the pathological stages of HCC. The more advanced stages of HCC, the higher rate of micro-metastasis of cancer cells detectable in peripheral blood and the higher frequency of positivity of MAGE-1 transcript among the group IV (b) (Metastatic HCC) (70 %, 21 out of 30), while in the group IV (a) (Localized HCC), the positivity was (22.2 %, 10 out of 45) (with significant increase in group IV (b) than group IV (a); $p < 0.05$). Whereas, all samples from healthy volunteers and patients with hepatitis and cirrhosis were negative. These results indicate that MAGE-1 m-RNA is a cancer specific and could be detected in samples from patients with HCC (Specificity 100%).

There were no significant differences in the characteristics of subjects among the three groups tested for the -1195 G/A polymorphism, the GG, GA, AA genotype frequencies were 12 (16%), 49 (65.3%), 14(18.6) in HCC patients (group IV), respectively 8(16 %), 5 (10%), 37 (74%) in group III, respectively 25 (50%), 12 (24%), 13 (26%) in group II, respectively; and 13 (52%), 5 (20%), 7 (28%) in healthy control group I, respectively ($p < 0.000$).

As regard the obtained results of the mean value of serum AFU, a significant increase was detected in HCC group (35.099 ± 19.07) than that found in patients with cirrhosis (16.54 ± 7.83) or with chronic hepatitis (4.04 ± 0.98) and in control subjects (4.91 ± 1.41), ($p < 0.000$). No significant differences were found between controls and patients with chronic hepatitis.

TABLE 1 : COX-2 genotypes distribution in four groups n, % .

SNP	HCC	Cirrhosis	Hepatitis	Control
Genotype - 1195G/A				
GG	12 (16)	8 (16)	25 (50)	7 (28)
G/A	49 (65.3)	5 (10)	12 (24)	5 (20)
AA	14 (18.6)	37 (74)	13 (26)	13 (52)

SNP: single nucleotide polymorphism; HCC: hepatocellular carcinoma.

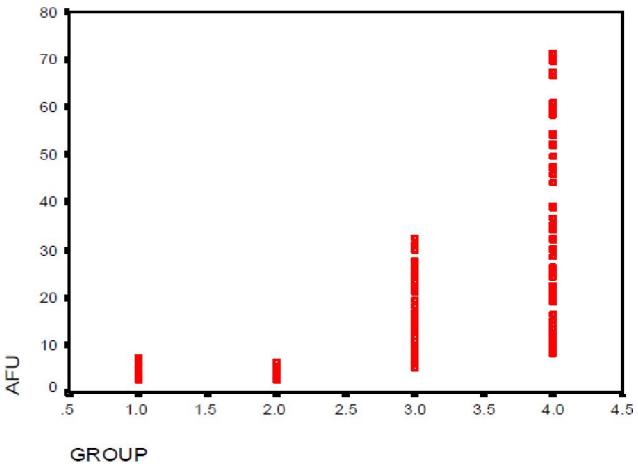

Figure 3 : Dot diagram of α-L-fucosidase enzyme activity in patients with HCC, cirrhosis and hepatitis.

Concerning the obtained results of serum AFP, when localized and metastatic HCC group was compared with cirrhosis, hepatitis and healthy controls ones, a significant increase was observed ($p < 0.000$). Also, a non significant differences was detected between cirrhosis and hepatitis groups but when they were compared with healthy control one, a significant increase of serum AFP was detected ($p < 0.000$).

The overall positive rate of AFP with a high concentration in serum and MAGE-1 m-RNA in PBMC samples was 62.5 %. However, the serum AFP was negative (≤ 10 ng/ml) in 75 samples but it was positive with a low concentration (10 ng/ml < AFP ≤ 100 ng/ml) in 36 HCC patients. 39 samples had the serum AFP level higher than 100 ng/ml and 8 samples had the se-

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rum AFP level higher than 1000 ng/ml.

Regarding the correlation a positive correlation between AFU and AFP in all studied individuals ($p < 0.001$) whereas non significant correlations were detected in healthy control, hepatitis and cirrhosis groups.

In conclusion, detection of MAGE-1 m-RNA in blood and serum AFU and AFP and COX-2 genotyping promoter has different significances; As regard MAGE-1 m-RNA, it seems to be a parameter to detect HCC earlier than any other means, so detection of MAGE-1 transcript in blood, especially with follow up, may help to prefigure HCC metastasis and monitor the response to the therapy.

TABLE 2 : General characteristics of patients in this study.

Characteristics	Group I	Group II	Group III	Group IV
Female	48 %	34 %	36 %	50.7 %
Male	52 %	66 %	64 %	49.3 %
Age	19 - 49	22 - 61	42-68	43 - 69
AFP ng/ml	(1.5- 9.3)	(3.3 - 36.8)	(4.8-40)	(14 - 6050)
AFU U/L	(2.54-7.21)	(2.89-6.48)	(6.22-32.54)	(8.11-71.34)
MAGE-1	0 %	0 %	0 %	46.1 %
COX-2 genotype				
GG	28 %	50 %	16 %	16 %
AA	52 %	26 %	74 %	18.7 %
GA	20 %	24 %	10 %	65.3 %

DISCUSSION

The integration of molecular and immunological techniques has led to the identification of a new category of tumor-specific antigens, also known as cancer-testis antigens, such as melanoma antigen (MAGE). The CTAs are a distinct and unique class of differentiation antigens. Attributing genes to this gene group is based on their characteristics, including mRNA expression in normal tissues of testis, Fetal ovary, and placenta, and mRNA expression in different cancers. Until now, at least 70 families of cancer-testis gene with 140 members have been attributed to this group and their expression has been studied in different types of tumors[19,20].

In the present study, MAGE-1 was expressed with a high percentage and specificity in HCC. The positive rate was as high as 46.1 %. Conversely, no expression was detected in the PBMC samples from healthy donors and cirrhotic patients.

Our results verified that exponential amplification of target cDNA converted from mRNA could allow to detect a single malignant cell within millions of normal blood cells and hence, to sensitively detect the Metastatic tumor cells in peripheral blood. The sensitivity of this assay is within the range of other published reports[21,22] which is much more sensitive than antibody-based serology[23,24].

The positive rate of nested RT-PCR was as high as 46.1 %. In addition, detecting MAGE-1 gene in PBMC samples from HCC patients would directly represent the presence of tumor cells in peripheral blood, suggesting that this method has a higher specificity than serum AFP and is thus able to improve the diagnosis of HCC.

Molecular biology technology contributes to the early diagnosis of HCC. However, its disadvantages are also obvious, including its cost and availability. PCR assay, a commonly used molecular biology technology, is more expensive and troublesome than serological tests, and is thus not the first choice in early detection of HCC. However, it plays a supplementary role in the diagnosis of HCC. Hopefully in the not so distant future, this technology will become increasingly popular and automatic with its cost decreased.

At present, the serum AFP level is still the gold standard for diagnosis of liver cancer. The AFP level is normal in 0 % of HCC Patients at the ime of diagnosis and usually remains low even in patients wih advanced HCC[4,25]. AFP > 400 Mg/L is considered diagnostic for HCC, although fewer than 50 % of HCC patients may meet this standard[3,26]. The specificity of AFP is close to 100 % at the cost of its sensitivity fallen to less than 45 %[4,27].

The current study was designed to evaluate the expression of MAGE-1 mRNA and the levels of AFP in peripheral blood as potential biochemical markers for diagnosis and prognosis of HCC.

Determination of MAGE-1 mRNA in PBMCs by RT-PCR assay revealed that, 46.1 % (31 out of 50 HCC) patients were found to be positive for MAGE-1 mRNA: Detection of MAGE-1 transcript in PBMCs is closely correlated o the pathological stages of HCC, he higher frequency of positivity of MAGE-1 transcript among group 4(a) (Metastatic HCC) (70 %, 21 out of

30), while in group 4(b) (Localized HCC), the positivity was 22.2 % (10 out of 45). All samples from healthy volunteers and patients with hepatitis and cirrhosis were negative. These results indicate that MAGE-1 mRNA is cancer-specific and could be detected in samples from patients with HCC (Specificity = 100%). These results agree with (Kobayashi et al (15), Peng et al (18), Mou et al (9), Yang et al (19), and Yang e al (10)).

Concerning the obtained results of serum AFP, when localized and Metastatic HCC groups were compared with cirrhosis, hepatitis and healthy controls ones, a significant increase was observed ($P < 0.001$), whereas a non significant difference was observed when they were compared with each other ($P > 0.05$). Also, a non significant difference was detected between cirrhosis and hepatitis groups ($P > 0.05$) but when they were compared with healthy control one, a significant increase of serum AFP was detected ($P < 0.001$). The current results agree with those of Kreczko et al (15); Mou et al (9); Peng et al (26); Ali et al (27); He et al (28) and Di Biscegli et al (29)).

The conflicting findings between the results of the current study and the others may be explained by Di Bisceglie et al (29) who postulated that AFP levels are affected by the severity of chronic hepatitis C. Also, Chu et al (30) reported that, elevated serum AFP levels are significantly correlated with advanced fibrosis/cirrhosis.

Serum AFP was detected, by the use of ELISA technique, in all 75 HCC patients (100%) which is more better than the previously reported results (60%-70%) obtained by Arrieta et al.[38]. Nikolic[39], reported that increases in serum AFP are found in illnesses accompanied by damage to hepatocytes in the liver. Certain differences in the structure of the oligosaccharide portion of the molecule have been shown between AFP synthesized by benign or by malignant cells and between AFP synthesized by hepatocytes or by cells of endodermal origin. These differences have been used as an aid in the diagnosis of liver diseases where serum AFP is elevated also, Chen[40], reported that variations of serum AFP levels in HCC patients is likely due to the differential activity of enhancer/silencer elements that control AFP. The AFP level in the control group was found to be 3.9± 2.0 ng/ml; our results are in agreement with Herberman, 1979 who reported that the normal value 0.3- 14.6 ng/ml and AFP level in HCC group was a statistically highly significant elevated 511.8±1195.1 ng/ml when compared with the control.

In the present study it was found that, 13.5 % (18 out of 75 patients of group 4) with serum AFP levels being lower than 40 ng/ml (the cut off value suggested by Mou et al, (9) to diagnose HCC patients). Also, the present study showed that, the elevation of AFP is not correlated with the progression of the HCC.

Regarding the non- significant correlation between the expression of MAGE-1 mRNA and AFP, it was explained by Yang et al (20) who stated ha, the level of serum AFP in patients with HCC is not necessarily consistent with the expression of MAGE-1 mRNA. The level of secreted serum AFP may be not proportional with the presence of MAGE-1 mRNA producing HCC cells in the circulatory system.

In conclusion, detection of MAGE-1 mRNA in blood and serum AFP levels has different significances. So, detection of MAGE-1 transcript in blood, especially with follow up, may help to prefigure HCC metastasis and monitor the response to the therapy. Moreover, in case of AFP, that parameter may not represent a tumor-specific marker and the present results suggest using it as a supplementary marker which may help diagnosis of HCC. Therefore, combination of multiple markers may be more valuable in the diagnosis and prognosis of HCC.

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