

MAGE-3 And -4 Genes as Possible Markers for Early Detection of Metastases in HCV Egyptian Patients Complicated By HCC

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ABSTRACT

The dissemination of hepatocellular carcinoma (HCC) cells into the circulation plays a critical role in postoperative recurrence and metastasis. Early detection of metastatic tumor cells is critical to identify HCC patients at high risk of relapse. MAGE-3 and -4 genes were evaluated by reverse transcription polymerase chain reaction for the possibility of using them as new markers for early detection of metastases in 160 HCV Egyptian patients, 115 of them were complicated with HCC. The expression of MAGE-3 and MAGE-4 mRNA in peripheral blood of patients with metastatic HCC, were 36 % and 52%, respectively. While the expression of MAGE-3 and MAGE-4 mRNA in peripheral blood of patients with localized HCC, were 12 % and 16%, respectively. Moreover at least one type of MAGE-3 or MAGE-4 mRNA was found in the peripheral blood of 68% of the metastatic HCC patients and in 20% of the localized HCC patients. While neither the controls nor the cirrhotic patients show expression of MAGE-4 mRNA in their peripheral blood. MAGE-3 and MAGE-4 may be a promising diagnostic tool for monitoring the prognosis of HCC patients and early detection of occult hematogenous metastasis of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies. Though regular abdominal ultrasonography (US) examination and serum alpha-fetoprotein (AFP) can detect small HCC at an early stage and there are many modalities of treatment, the recurrence and metastasis are frequent and the prognosis remains unsatisfactory. The high recurrence rate is probably attributed to the dissemination of HCC cells into blood

circulation and that they proliferate in an immunosuppressive environment during and before therapy. The dissemination of HCC cells into the circulation plays a critical role in postoperative recurrence and metastasis⁽¹⁾.

Hepatocellular carcinoma (HCC) accounts for 80% of all primary liver cancers and ranks globally as the fourth leading cause of cancer-related death. Partial hepatectomy remains the best treatment option for selected patients with HCC without cirrhosis. Liver transplantation is well

established as the gold standard for patients with HCC and cirrhosis in the absence of extrahepatic spread and macro-vascular invasion. Local regional therapy is indicated in selected patients who are not surgical candidates⁽²⁾.

Early detection of metastatic tumor cells is critical to identify HCC patients at high risk of relapse and for the prescriptive therapy. However, it is difficult to detect such dissemination of HCC cells through blood route with conventional techniques⁽³⁾.

With respect to HCC, although the serum level of AFP is a good biomarker for HCC, it does not specifically reflect metastasis from HCC; previously both albumin and AFP mRNA are widely used as tumor markers for HCC cells in circulation. However, the reliability of using them as tumor markers have been challenged because both albumin and AFP mRNA are abundantly expressed in normal liver cells, they are released in the peripheral blood by either surgical injury of the liver or by diseases other than HCC such as hepatitis virus infection⁽⁴⁾.

The MAGE gene family encodes tumor-specific antigens recognized by autologous cytotoxic T lymphocytes⁽⁵⁾ and composed of more than 25 genes in humans and are classified as type I MAGE genes (including MAGE-A, MAGE-B, and MAGE-C genes) and type II MAGE genes, which include those that reside outside the MAGE-A, MAGE-B, and MAGE-C genomic clusters⁽⁶⁾. The MAGE-A subfamily comprises 12 genes (MAGE-A1 to MAGE-A12), and is expressed in various types of

tumors, but not in normal adult tissues, except for testis and placenta. The MAGE-A antigens are of particular interest for antitumor immunotherapy because they are strictly tumor specific and are shared by many tumors. Despite the isolation of growing numbers of MAGE genes, their function in normal tissues remains mostly unknown⁽⁷⁾.

MAGE-3 belongs to MAGE-A gene family, and is expressed in a significant proportion of tumors of various histological malignancies, and is silent in normal somatic cells except male germ line cells⁽⁸⁾. MAGE-3 protein also exists in cytoplasm showing homogeneous, focal or scattered pattern of expression, and the expression undergoes a substantial change in distribution with increase in tumor size and invasiveness⁽⁹⁾.

How to discriminate well differentiated HCCs from non-cancerous lesions, and how to suppress the recurrences that frequently occur after treatment are problems which should be solved. Detection of MAGE transcripts in peripheral blood mononuclear cells (PBMC) may be helpful in detecting circulating tumor cells which also may help in the early prediction of the relapse and prognosis of the HCC patients.

In the present study, we have carried out the reverse transcription polymerase chain reaction (RT-PCR) for MAGE-3 and -4 genes to evaluate the possibility of using these genes as new markers for early detection of metastases in hepatitis C virus (HCV) infected Egyptian patients complicated by HCC.

SUBJECTS & METHODS

We studied 165 Egyptian patients with HCV (50 patients with HCV complicated with cirrhosis, 40 patients with localized HCC and 75 patients with metastatic HCC)

The 50 patient presented by post chronic HCV hepatitis cirrhosis were selected according to the clinical and laboratory examination (18 child B & 32 child C according to child-pugh classification). Hepatic malignancies in those patients were excluded by both abdominal US and AFP.

The 40 HCV patients who presented by localized HCC were selected by abdominal US, all patients with single focal lesions with typical features of HCC, the HCC confirmed by Triphasic computed tomography (CT) and AFP. Fine needle aspiration biopsy (FNAB) was done in selected cases (focal lesion < 2cm with normal AFP). Patients with multiple focal lesion and/or portal vein thrombosis were excluded. Distant metastases had been excluded by chest and abdominal CT, isotopic bone scan and brain CT if there is a neurological manifestation.

Patients with HCC with distant and /or macro-vascular metastasis (n=75) were selected from patients who presented by metastases during selection of the previous group.

75 healthy control Egyptian subjects age- and sex-matched with the patients, were randomly recruited.

Biochemical measurements

Blood samples were drawn from all subjects after an overnight fast. Sera were separated immediately and stored at -20°C.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), Albumin, urea and creatinine were measured in serum by routine enzymatic methods (spinreact), serum AFP concentration was measured by ELISA (kit provided by Biosource Europe S.A, Belgium), HCV antibodies by ELISA.

RNA Extraction

Total RNA was extracted using the protocol provided by the manufacturer. The cDNA was reverse transcribed in a 10 µl mixture containing 6 µl of total RNA, 0.5 µl random primers (Promega, Madison, WI), 2 µl of 5× reverse-transcriptase buffer (Life Technology, Gaithersburg, MD), 1 µl of deoxyribonucleoside triphosphate (dNTP) mixtures (10Mm), 0.5 µl of AMV reverse transcriptase (5U/ µl). The mixture was incubated at 37°C for 10 min, 52°C for 45 min, 95°C for 5 min and ice path for 5 min.

RT-PCR and Electrophoresis

Detection of MAGE-3 and MAGE-4 genes were done according to (10). The amplification reaction (50 µl) contained 2.5 µl cDNA, 0.5 µl each of 5 µM primers, 0.5 µl of Taq polymerase (GIBCO BRL), 0.5 µl of deoxyribonucleoside triphosphate (dNTP) mixtures (10Mm) in buffer solution. The sequences of primers for PCR amplification were as follows:

MAGE-3; sense, 5-TGGAGGACCAGAGCCCCC-3, and antisense, 5-GGACGATTATCAGGAGGCCTGC-3

MAGE-4; sense, 5-ACCAAGGAGAAGATCTGCCAGTGGGTCTC-3, and antisense, 5-

GTCGCCCTCCATTGCATTGTGC-
3

PCR was performed at 96°C for 5 minute followed by 35 cycles of 94°C for 1 min, 68°C for 2 min and 72°C for 4 min, this was followed by 15 min of final extension at 72°C for MAGE-3, and 94°C for 30 sec, 58°C for 30 sec and 74°C for 30 sec, this was followed by 15 min of final extension for MAGE-4

Reverse- transcription RT- PCR for β -actin was done for each sample to ensure no degradation of RNA. Using forward primer-5'-GGC ATC GTG ATG GAC TCC G-3' and reverse-5'-GCT GGA AGG TGG ACA GCG A-3', PCR was performed at 96°C for 5 minute followed by 28 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 45 sec .

The PCR products are separated in 2% agarose gel and visualized with ethidium bromide staining. The expected sizes of PCR products are 725 bp for MAGE-3, 726 bp for MAGE-4, and 613 bp for β -actin.

Statistical analysis

The results for continuous variables are expressed as means \pm SD. The means of the three genotype groups were compared in a one-way analysis of variance. The statistical significances of differences in frequencies of variants between the groups were tested using the chi-square (χ^2) test. A difference was considered significant at $P < 0.05$. All data were evaluated using SPSS version 10.0 for windows.

RESULTS

Table 1. Characteristic of the studied group

	Control Group (n=75)	Cirrhosis Group (n=50)	Localized HCC group (n=40)	Metastatic HCC group (n=75)
Age	51.6 \pm 7.5	50.7 \pm 5.5	54.2 \pm 6.5	56.3 \pm 8.1
ALT	27.2 \pm 1.9	64.2 \pm 30.8*	63.2 \pm 30.8*	59.2 \pm 19.2*
AST	23.4 \pm 6.8	64.5 \pm 36*	62.5 \pm 36*	82.3 \pm 34.3*
AFP (ng/ml)	3.2 \pm 1.2	127.5 \pm 40*	151.5 \pm 70*	671 \pm 328.4*§

* Significant difference from control group, § significant difference from cirrhosis group

Table 2. mRNA expression of MAGE-3 and -4 genes in all studied groups

	Control Group (n=75)	Cirrhosis Group (n=50)	Localized HCC group (n=40)	Metastatic HCC group (n=75)
MAGE3 +	0	0	3 (12%)*	27 (36%)*§
MAGE4+	0	0	4 (16%)*	39(52%)*§
Combined +	0	0	5 (20%)*	51 (68%)*§

* Significant difference from control group, § significant difference from localized HCC group

Expression of MAGE-3 and MAGE-4

MAGE-3 and MAGE-4 mRNAs were positively expressed in peripheral blood of localized HCC patients 12% (3/25) and 16% (4/25) respectively; moreover when combining the expression of MAGE-3 and MAGE-4 mRNAs in the peripheral blood of patients with localized HCC, the rate of expression was 20% (5/25).

MAGE-3 and MAGE-4 mRNAs were positively expressed in peripheral blood of metastatic HCC patients 36% (27/75) and 52% (39/75) respectively; moreover when combining the expression of MAGE-3 and MAGE-4 mRNAs in the peripheral blood of patients with metastatic HCC, the rate of expression were 68% (51/75). On the contrary none of the healthy subjects and cirrhotic patients showed expression of MAGE-3 and MAGE-4 mRNAs in their peripheral blood

DISCUSSION

The early dissemination of malignant cells to secondary sites is the main cause of mortality of patients with malignant solid tumors. Metastatic spread through vessels is the most important factor affecting the prognosis of HCC patients. If the metastatic HCC cells via hematogenous route can be sensitively and specifically determined at early stage, more beneficial therapeutic methods could be manipulated⁽¹¹⁾.

Conventional diagnostic procedures usually fail to detect circulating tumor cells, while reverse transcriptase-polymerase chain

reaction (RT-PCR) amplification of specific DNA sequences using synthesized oligonucleotide primers that flank the target DNA fragment of interest is increasingly applied in the detection of micrometastasis of cancer cells⁽¹¹⁾. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of genes expressed by the tumor in a tissue-specific manner is the method with the highest diagnostic sensitivity allowing the identification of one tumor cell in up to 10^7 normal cells in various sources such as blood, bone marrow, lymph nodes, urine or stool^(12&13).

Detection of micrometastasis in HCC is still a controversial issue. As the two frequently used mRNA markers in detection of HCC, albumin and mRNA AFP are also shown to be expressed in blood of patients with hepatitis, liver cirrhosis and the liver infectious diseases such as HBV/HCV infection⁽¹⁴⁾. Furthermore, AFP transcripts was detected even in the blood samples from patients without HCC after the surgical injury of the liver, which results in the shedding of liver cells into circulation under surgical operation⁽¹⁵⁾. Thus, AFP represents an indicator of ongoing liver damage without absolute specificity for hepatocellular carcinoma (Peck-Radosavljevic et al., 1998)^(16,17&18).

MAGE gene transcripts have been regarded as tumor-specific markers and were found to be highly expressed in variety of histological types of cancers⁽³⁾. When overexpressed in the cytosol of cancer cells, MAGE proteins are proteolytically processed, transported to the endoplasmic reticulum, and

then presented on the cell surface as antigenic major histocompatibility complex-associated peptides⁽⁶⁾. Thus, attention has been focused on the potential of MAGE as a target for cancer immunotherapy.

The MAGE-3 and MAGE-4 genes are members of the CT (cancer/testis) antigen family used as cancer specific markers. It has been reported that MAGE-3 and MAGE-4 mRNAs are positive in HCC tissue, but there is no MAGE expression in pericancerous tissue, hepatitis, cirrhosis or normal liver tissue^(3,11&19).

Our goal is to detect occult hematogenous metastasis of HCC cells by RT – PCR of MAGE-3 and/or MAGE-4 transcripts and to combine these markers with each other to detect HCC cells in the peripheral blood in order to elevate their sensitivity and specificity.

The important implication for the detection of occult hematogenous metastasis of HCC cells by RT – PCR of MAGE-3 and/or MAGE-4 transcripts, is its value in the prediction of recurrence after treatment and prognosis of the disease.

In our study, the expression of MAGE-3 and MAGE-4 mRNA in peripheral blood of patients with metastatic HCC, were 36 % and 52%, respectively. While the expression of MAGE-3 and MAGE-4 mRNA in peripheral blood of patients with localized HCC, were 12 % and 16%, respectively. This is in accordance with⁽³⁾ who reported that MAGE-3 was expressed in 33.3% in peripheral blood of HCC patients and 53.3% in HCC tissues while Zhang et al. (2008)⁽²⁰⁾ reported that MAGE-3 was

expressed in 20.1% in peripheral blood of HCC patients and 60.5% in HCC tissues.

Up to our knowledge our study is the first study to detect MAGE-4 mRNA expression in peripheral blood of patients with HCC. MAGE-4 mRNA was expressed in peripheral blood of 52% of patients with HCC, while neither the controls nor the cirrhotic patients show expression of MAGE-4 mRNA in their peripheral blood. Previous studies about MAGE-4 and HCC is minimal. ⁽¹¹⁾ reported that MAGE-4 mRNA was expressed in 18% HCC tissues while⁽²¹⁾ found that MAGE-4 protein was not only significantly increased in HCC, but also in HCV-related cirrhotic liver patients and these cirrhotic patients are already at high risk to develop HCC.

At least one type of mRNA was found in the peripheral blood of 68% of the metastatic HCC patients, which is markedly higher than the 36% and 52% of the patients who were positive only by MAGE-3 or MAGE-4 mRNA detection, we also found at least one type of mRNA was found in the peripheral blood of 20% of localized HCC patients, which is markedly higher than the 12 % and 16% of the patients who were positive only by MAGE-3 or MAGE-4 mRNA detection. So combining detection of MAGE-3 and MAGE-4 mRNAs in PBMCs is a feasible method as this method possesses high sensitivity and specificity, and is practical in detecting tumor cells in the blood.

Interestingly our results showed that the positive rate of expression MAGE-3 and MAGE-4 mRNA is markedly increased in peripheral

blood of metastatic HCC patients than in peripheral blood of localized HCC patients, while patients with hepatitis, cirrhosis or the 75 healthy subjects showed no expression of MAGE-3 or MAGE-4 mRNA so using MAGE-3 or MAGE-4 mRNA expression in peripheral blood of in patients with localized HCC may be helpful in early detection of circulating cancer cells and consequently early identification of distant metastasis. As blood dissemination is the main metastatic route for HCC⁽²²⁾, identifying and dynamically monitoring blood cancer cells may be helpful in estimating prognosis of HCC, guiding treatment and avoiding recurrence since if a small number of cancer cells enter the blood circulation before surgery, relapse may occur after surgery even though the HCC is of a small size⁽²³⁾

Conclusion

A cancer specific multi-marker assay such as MAGE-3 and MAGE-4 may be a promising diagnostic tool for monitoring HCC in HCV infected Egyptian patients and early detection of occult hematogenous metastasis of HCC with better sensitivity and specificity and this may be valuable in the prediction of the prognosis of the disease and construction of the guidelines for treatment.

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المخلص العربى

يلعب أنتشار خلايا سرطان الكبد بالدم دورا حرجا فى ارتجاع الورم او انتشاره بعد الجراحة، لذا فأن الاكتشاف المبكر للخلايا المنتشرة من سرطان الكبد يلعب دورا حيويا فى التعرف على المرضى ذو الاحتمالات العاليه للأرتجاع.

لقد تم تقييم كل من جينات ماج-3 و 4 بواسطة التفاعل المتسلسل العكسى وذلك لامكانية استخدامهما كدلائل جديدة للأكتشاف المبكر للثانويات فى 160 مريضا مصريا مصابين بالالتهاب الكبدى الفيروسى سى منهم 115 مريضا مصابين بسرطان الكبد.

وجد ان التعبير عن ماج 3 و ماج 4 ار ان ايه الرسولوى فى الدم للمرضى المصابين بسرطان الكبد المنتشر 36% و 52% على التوالى فى حين انه وجد فى 12% و 16% فى دم المرضى المصابين بسرطان الكبد المحدود والغير منتشر، فى حين وجد ان كل من ماج 3 و ماج 4 موجودين فى دم 68% من المرضى المصابين بسرطان الكبد المنتشر و 20% من المرضى المصابين بسرطان الكبد المحدد الغير المنتشر فى حين لم يتم اكتشافهما فى الدم لكل من المجموعه الضابطه والمرضى المصابين بتليف الكبد.

من هذه الدراسه يمكن استخلاص ان كل من ماج 3 و ماج 4 يعتبران من الأدوات التشخيصية الواعده لمتابعة والاكتشاف المبكر لوجود ثانويات سرطان الكبد فى الدم.