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Multimarker Detection of MAGE-1, MAGE-3 and AFP mRNAs by a Real-time Quantitative PCR Assay: a Possible Predictor of Hematogenous Micrometastasis of Hepatocellular Carcinoma

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OBJECTIVE To explore the relationship between multimarker detection of MAGE-1, MAGE-3 and AFP mRNAs in the peripheral blood of patients with hepatocellular carcinoma and micrometastasis using a real-time quantitative-PCR (real-time Q-PCR) assay.

METHODS Peripheral blood samples were obtained from control subjects and 86 patients with hepatocellular carcinoma (HCC). Real-time Q-PCR was used to detect MAGE-1, MAGE-3, and AFP mRNAs in the blood cells.

RESULTS In 86 tumor specimens, the positivity for MAGE-1, MAGE-3, and AFP genes was respectively 34.9% (30/86), 60.5% (52/86) and 69.8% (60/86). All specimens expressed at least one marker. MAGE-1, MAGE-3, and AFP transcripts were detected respectively in 12 (14.0%), 18 (20.1%) and 29 (33.7%) of the 86 blood specimens from hepatocellular carcinoma patients, 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. The positive rate correlated with the TNM clinical stages, extrahepatic metastasis and portal vein carcinothrombosis ($P < 0.05$). No correlation was found between tumor size, tumor number, differentiation, serum a-fetoprotein (AFP) and the positive rate.

CONCLUSION Our results indicate that a multimarker real-time Q-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 with better sensitivity and specificity.

KEY WORDS: real-time Q-PCR, micrometastasis, MAGE-1, MAGE-3, AFP, hepatocellular carcinoma.

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Introduction

Hepatocellular carcinoma (HCC), one of the most intractable malignancies, ranks 6th in frequency but 3rd in mortality worldwide[1]. About 200,000 patients die from HCC in China every year, only next in deaths from gastric cancer in the countryside and lung cancer in the cities. Although routine sonographic examination and serum alpha-fetoprotein (AFP) can detect small HCCs, there are many other modalities of medical and surgical treatment. However the recurrences and metastases are frequent and the prognosis is still unsatisfactory[2-5]. Studies have shown that the 5-year postoperative recurrence rate for HCC is 61.5%, and 43.5% for small HCC[6]. Clinical studies show that HCC cells are disseminated in the systemic 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[7,8]. To improve the prognosis of patients with HCC, therefore, it is important to be able to detect such blood-borne HCC cells or micrometastasis at an early period.

Although the serum level of alpha-fetoprotein (AFP) is a good biomarker for HCC, it does not specificly reflect metastasis from HCC. Previously, albumin mRNA and AFP mRNA were both widely used as tumor markers for HCC cells in the peripheral blood[9,10]. However, because both albumin mRNA and AFP mRNA are abundantly expressed in normal liver cells, these mRNAs may be released into the circulation with surgical injury[11,12].

The melanoma antigen (MAGE) gene family consists of at least 12 members, which are activated in spermatozoa, are silent in normal somatic cells, but reactivated in various histological types of cancers[13]. In recent years, MAGE-1 and MAGE-3 have been used as tumor markers to detect circulating HCC cells[14-16]. Since cancer cells are heterogeneous in gene expression, different cancers express different markers, and even cells from the same tumor may not be isologous[17,18]. Therefore use of single marker reverse transcription-PCR (RT-PCR)-based assays is limited in sensitivity and specificity. To overcome this problem, in recent years, multimarker RT-PCR assays have been developed for the detection of tumor cells in the peripheral blood of patients with various malignancies[15, 19, 20]. RT-PCR can detect a few tumor cells among millions of peripheral blood mononuclear cells (PBMCs)[21], but there are limitations on the specificity and reliability of assays based on gel electrophoresis systems[22,23]. Recently, real-time quantitative-PCR(real-time Q-PCR) assays have offered a more powerful, accurate, and less labor-intensive approach that allows rapid and reproducible quantitative analysis for detection of a few HCC cells in blood[22, 24].

These findings noted above indicate that a multimarker real-time Q-PCR assay with cancer-specific MAGE markers and a hepatocyte-specific AFP marker may be a promising diagnostic tool to detect circulating HCC cells with better sensitivity and specificity. In our study we developed a sensitive assay that uses MAGE-1, MAGE-3 and AFP gene transcripts as multimarkers for detecting minimal cancer cells in peripheral blood of HCC patients and HCC-free controls. The objective of this study was mainly to evaluate the efficacy of this innovative diagnotic technique of a multimarker real-time Q-PCR assay for detecting HCC cells, and in turn to improve the efficacy of therapies used to manage this malignancy.

Patients and Methods

Patients

Samples of HCC tissues and preoperative peripheral blood were collected from 86 HCC patients (51 male, 35 female; mean age 49 years, range 26~74 years) who underwent hepatectomy in the Department of Hepatobiliary Surgery, Tianjin Medical University Cancer Institute and Hospital from November 2003 to June 2005. All patients enrolled in the study had documented physical and medical histories, and their TNM stage of disease was determined and recorded at the time of the blood draw. The blood was taken from the HCC patients (21 with Stage I, 33 with Stage II, 20 with Stage III, and 12 with Stage IV disease) immediately before they received any treatment. The diagnosis of all of the HCC patients was made based on ultrasound, computed tomography, serum AFP and final pathologic diagnosis. The following subjects as a contol group provided informed consent and their peripheral blood: 25 patients with chronic HBV or HCV and cirrhosis; 28 healthy volunteers with no signs of hepatitis or liver functional abnormalities. The PBMCs of the HCC patients and HCC-free controls were used for RNA extraction.

Primer design and synthesis

Oligo 6 Software was utilized to design the sequences of AFP mRNA, MAGE-1 mRNA and MAGE-3 mRNA primers by the TaKaRa Co. The sequences of primers and size of PCR products were as follows: MAGE-1 (299 bp) 5’-ACA GAG GAG CAC CAA GGA GAA G-3', 5'-AGT TGA TGG TAG TGG GAA AGG C-3'; MAGE-3 (370 bp), 5'-CGG AGG AGC ACT GAA GGA GAA G-3', 5'-CCT CC T CTT CTT GGT TGC TGG-3'; AFP (193 bp) 5'-GTT CCA GAA CCT GTC ACA AG-3', 5'- CTT TGT TTG GAA GCA TTC AAC TGC-3'.

RNA preparation

The SMMC-7721 HCC cell line (stored in our department) was cultured in RPMT-1640 medium containing 10% FBS (GIBCO Co.) in a humidified 5% CO_2 incubator at 37℃. Doubling dilutions of 20,000 SMMC-7721 cells in 500 μl of PBS was performed for further use. One, 10, 100, 1000, 10,000, 100,000 SMMC-7721 cells were mixed with 106 PBMCs from healthy donors and centrifugated. The supernatant was then removed and the precipitate used for RNA extraction.

After centrifugation at the rate of 2,500 rpm for 10 min, 5 ml of S-ACK lysate was added into the nucleated cell layer. After 15 min in the ice bath the transparent solution, was centrifugated at the rate of 2,500 rpm for 10 min, after which the supernatant was removed and the RNA was extracted from the precipitate.

Reverse transcription

The RT-PCR reaction was performed by a one-step procedure for total RNA isolation according to instructions related to molecular cloning. Reverse transcription was carried out in volume of 20 μl containing 1 μl (0.1 μg/μl) of oligonucleotide primer, 2 μg of total RNA, 4 μl of

5 × reverse transcription buffer, 2.2 μl (9 U/μl) of AMV reverse transcriptase, 0.6 μl (40 U/μl) of RNA enzyme inhibitor, 1μl (10 mmol/L) of dNTPs and deionized water. PCR was performed in a volume of 50 μl including 10 μl of cDNA, 1 μl of downstream primer respectively, 10 μl of 5 × buffer, 1 μl (10 mmol/L) of dNTPs, 5 μl (15 mmol/L) of $MgCl_2$, 0.5 μl (4 U/μl) of *Taq* enzyme and water. The cycling conditions were as follows: 94℃ for 30 s, 55℃ for 1 min, 72℃ for 1 min, 27 cycles; 72℃ for 10 min, one cycle. PCR products were electrophoresed on agarose gel and analyzed.

Real-time Q-PCR

The target genes were amplified by real-time Q-PCR using a LightCycle-DNA Amplicaton Kit, SYBR Green I (Roche) and LightCycler-control kit DNA (Roche). The transcription reaction was the same as previously described. Real-time Q-PCR amplification: first, standard human genomic DNA was diluted by 1:1, 1:10, 1:100, 1:1000, 1:10000, and initial copy numbers were adjusted to 10^5, 10^4, 10^3, 10^2 and 10^1, respectively. The PCR amplification system included H_2O, 14.8 μl of PCR grade, 0.4 μl (final concentration 1.5 mmol/L) of $MgCl_2$ stock solution, 0.4 μl (concentration 20 μmol/L) of primer respectively, 2 μl of template DNA (DNA awaiting measurement and standard DNA) and 2 μl of LightCycler FastStart DNA Master SYBR Green I. A PE 5700 type PCR instrument was used for amplification. Reaction conditions were as follows: 94℃ 10 min→ (94 ℃ 30 s→ 60℃ 30 s→ 72℃ 40 s) × 30→72℃ 10 min. Correlative Ct values were recorded and standard curve was drawn. Numbers of DNA templates were calculated and agarose-gel electrophoretic results were analyzed.

Standard curve

A standard curve was calculated using linear regression analysis. This standard curve displayed a linear relationship between Ct values and the logarithm of the initial number of positive cells. The amount of product in a particular sample was determined by interpolation from a standard curve of Ct values generated from the positive-cell dilution series (Fig.1).

Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences Version 13.0 software (SPSS). The χ^2 test was used to assess the experimental results, and the level of significance was set at $P < 0.05$.

Results

Expression in HCC tissues

Positive rates of AFP, MAGE-1, and MAGE-3 mRNAs in 86 HCC tissues were respectively 69.8% (60/86), 34.9% (30/86) and 60.5% (52/86). There was at least one mRNA expression in all HCC tissues. Positive results are shown in Fig. 2.

Sensitivity detection

Different numbers of HCC cells obtained, using a doubling dilution method, were mixed with 10^6 of PBMCs from healthy donors, then the total RNA was extracted for RT-PCR amplification, to show if there is one hepatic carcinoma cell in 10^6 of the PBMCs, AFP, MAGE-1 and MAGE-3 mRNAs can be detected (Fig.3).

Preoperative expression in PBMCs in patients with HCC

The positive rates of AFP, MAGE-1 and MAGE-3 mRNAs were respectively 33.7% (29/86), 14.0% (12/86) and 20.1% (18/86). At least one kind of mRNA was found in peripheral blood of 52.3% (45/86) of the patients, which is markedly higher than 33.7% of the patients positive only by AFP mRNA detection. With progression of the HCC, the positive rate was increased. The positive rate in TNM Stages I and II was 45.0% (27/60), and in Stages III and IV was 69.2% (18/26). The difference showed a statistical significance ($P <$ 0.05, Table 1). The expression of MAGE-1, MAGE-3 and AFP mRNAs in the peripheral blood of a partial HCC patients is shown in Fig.4.

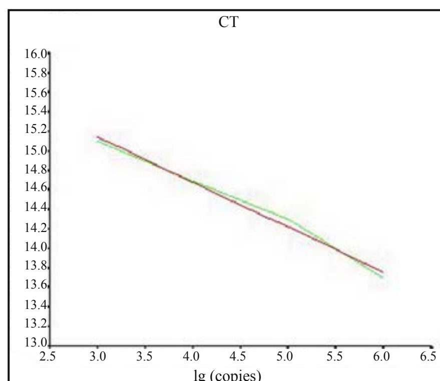

Fig.1. Standard curve for quantitative real-time RT-PCR. Ct = 16.52 - 0.46 × lg (copies) Ct values were recorded automatically after amplification, and DNA copy numbers were calculated by the instrument according to the standard curve to determine positive or negative.

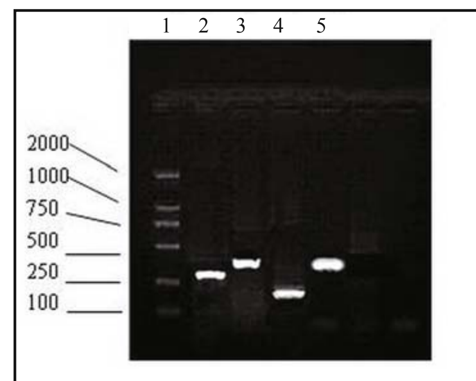

Fig.2. Positive electrophoresis results of MAGE-1, MAGE-3 and AFP mRNAs expressed in HCC tissue. Lane 1, marker (DL-2000); Lane 2, MAGE-1 (299 bp); Lane 3, MAGE-3(370 bp); Lane 4: AFP (193 bp); Lane 5, β-actin (340 bp).

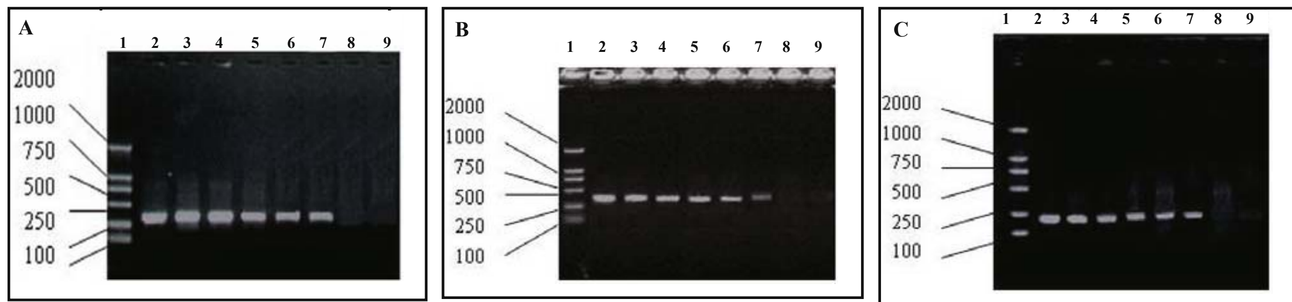

Fig.3. The doubling dilution method was used to check the sensitivity of the real-time Q-PCR assay. This method can detect only one HCC cell in 106 PBMC from a healthy donor. A, Gene magnification fragment of MAGE-1; B, Gene magnification fragment of MAGE-3; C, Gene magnification fragment of AFP. Lane 1, DL-2000; Lane 2, HCC cell/ PBMC as 1/10; Lane 3, HCC cell/ PBMC as $1/10^2$; Lane 4, HCC cell/ PBMC as $1/10^3$; Lane 5, HCC cell/ PBMC as $1/10^4$; Lane 6, HCC cell/ PBMC as $1/10^5$; Lane 7, HCC cell/ PBMC as $1/10^6$; Lane 8, 9, HCC cell/ PBMC as $1/10^7$.

Fig.4. Expression of MAGE-1, MAGE-3, AFP mRNA in peripheral blood of a portion of the HCC patients. A: Lane 1, Marker; Lane 2, 7, positive expression of MAGE-1 in peripheral blood, the others were negative; B: Lane 1, Marker; Lane 2, 3, 7, positive expression of MAGE-3 in peripheral blood, the others were negative; C: Lane 1, Marker; Lane 2, 3, 7, 9, positive expression of AFP mRNA in peripheral blood, the others were negtive.

Table 1. The relationship among results of combined detection with multimarkers in peripheral blood of HCC and clinical data.

Characteristic	*n*	Positive (*n*)	%	χ^2	*P* value
TNM stage					
I~II	60	27	45.0		
III~IV	26	18	69.2	4.269	0.039
Tumor diameter					
≥ 5 cm	48	28	58.3		
< 5 cm	38	17	44.3	1.572	0.210
Differentiation grade					
Well	29	15	51.7		
Moderate and poor	57	30	52.6	0.006	0.937
Portal vein thrombosis					
Present	30	21	70.0		
Absent	56	24	42.9	5.769	0.016
Extrahepatic metastasis					
Present	11	11	100.0		
Absent	75	34	45.3	13.945	0.000
Tumor number					
1	76	39	51.3		
≥ 2	10	6	60.0	0.267	0.605
Serum AFP					
< 20 μg/L	27	15	55.6		
≥ 20 μg/L	59	30	50.8	0.165	0.685

The relation between detection results and clinical data

The positive rate of combined detection in peripheral blood was not correlated with the tumor size, tumor number, differentiation and serum AFP, but was associated with the TNM clinical stage, portal vein carcinothrombosis and extrahepatic metastasis (with significant differences, $P < 0.05$, Table 1) .

Expression in patients with hepatitis or cirrhosis and healthy donors

Positive results of AFP, MAGE-1 or MAGE-3 mRNAs were not found in peripheral blood sample from 25 patients with chronic liver diseases and 28 healthy donors. There was a statistical significance compared with the HCC group ($P < 0.05$, Table 2).

Table 2. Results of combine detection with multimarkers in peripheral blood of HCC, hepatitis and cirrhosis patients and healthy donors.

Sample source	*n*	Positive (*n*)	%	χ^2	*P* value
HCC group	86	45	52.3		
Hepatitis and cirrhosis group	25	0	0	22.00*	0.000
Healthy donors	28	0	0	24.21*	0.000

*, all compared with the HCC group.

Discussion

Surgical excision is the chief means of treating liver cancer. However, since a small number of cancer cells enter the blood circulation before operation in a portion of the patients, relapse may occur after an operation even though the HCC is of a small size[25]. Blood dissemination is the main metastatic route for HCC[26], so it is important to identify and dynamically monitor blood cancer cells in guiding treatment and estimating a prognosis.

Amplification of RNA from peripheral blood cells using RT-PCR to confirm the presence of tumor cells has become a well-developed technique in recent years[27]. The detection of AFP mRNA in peripheral blood of HCC patients has become a more common method to find circulating hepatic cancer cells[28] as reported by others in China and abroad[28-31]. However, there has still been a problem of a low positive rate of detection. Multimarker-combined detection may make up for negative shortage of AFP mRNA in patients with HCC, and is likely to become a sensitive method to detect HCC hematogenous micrometastases.

The MAGE-1 and MAGE-3 genes are members of the CT (cancer/testis) antigen family used as cancer-specific markers. It has been reported that MAGE-1 and MAGE-3 mRNAs are positive in about 85% of HCC tissue[16, 32, 33], but there is no MAGE expression in pericancerous tissue, hepatitis, cirrhosis or normal liver tissue. Our goal is to combine these markers with AFP mRNA to detect HCC cells in the peripheral blood in order to elevate sensitivity.

Miyamoto et al.[15] reported that positive rates of MAGE-1, MAGE-3 and AFP mRNA expression in HCC tissue and 71 PBMCs samples were respectively 71%, 67%, 88% and 12.7%, 4.8%, 15.9%. Combined detection of the cancer-specific MAGE marker and hepatocyte-specific AFP marker was considered as a promising method which possesses high sensitivity and specificity in monitoring patients with HCC. Mou et al.[16] reported that the detection of MAGE mRNA in PBMCs was considered to be a convenient and reliable method to forecast relapse and prognosis for HCC patients.

Although use of RT-PCR in detecting AFP mRNA in blood was reported[15, 27], false-positive results were inevitable when the sensitivity of those methods was increased. A real-time Q-PCR technique was used in our study. Only one HCC cell among 10^6 PBMCs from healthy donors could be detected in our study, indicating that real-time Q-PCR technique is a more sensitive method to detect HCC cells in the peripheral blood. Moreover, the new assay system can offer several advantages over conventional RT-PCR techniques: first, this method is a one-step assay that can be completed in a closed-tube system requiring less labor and sample handling; thus it can reduce the risk of false positives resulting from cross-contamination among samples; second, without post-PCR processing, the assay can be carried out within 3 h; last, this assay has high intra-assay precision and reproducibility with a wide dynamic range of quantification. Therefore, this new method may be applied to clinical fields for the purpose of quantitatively examining micrometastasis from HCC.

We found that there was at least one kind of mRNA expression among MAGE-1, MAGE-3 and AFP in the 86 HCC tissues, so that combining detection of MAGE-1, MAGE-3 and AFP mRNAs in PBMCs is a feasible method. The positive rates of AFP, MAGE-1 and MAGE-3 mRNA detection in the peripheral blood of patients with HCC were respectively 33.7%, 14.0% and 20.1%. At least one type of mRNA was found in the peripheral blood of 52.3% of the patients which is markedly higher than the 33.7% of the patients who were positive only by AFP mRNA detection. With progression of HCC, the positive rate was increased from 45.0% in Stages I and II, and to 69.2% in Stages III and IV, showing that in later HCC stages, the occurrence of blood metastasis is greater. These results are consistent with the conclusion that the positive rate is markedly increased in peripheral blood of patients with portal vein carcinothrombosis and extrahepatic metastasis. However, the positive rate of combined detection in peripheral blood is not correlated to the tumor size, differentiation, tumor number or serum AFP. Early metastasis may occur in small and well-differentiated hepatic cancer, and is chiefly associated with tumor cells that invade into the blood circulation.

We also found that there was no MAGE-1, MAGE-3 and AFP mRNA expression in peripheral blood from 25 patients with hepatitis and cirrhosis or 28 healthy donors, showing that the method possesses good specificity.

Our study indicates that the combined detection of MAGE-1, MAGE-3 and AFP mRNAs in the PBMCs of patients with HCC increases the positive rate, showing that this method possesses high sensitivity and specificity, and is practical in detecting tumor cells in the blood. Using a relatively large sample size, the clinical application of the method was further confirmed, indicating that this technique is helpful in diagnosing HCC, in monitoring early relapse and metastases, estimating patient's prognosis and providing a guide for clinical treatment.

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References

1 Parkin DM, Bray F, Ferlay J, et al. Global Cancer Statistics. CA Cancer J Clin 2005; 55: 74-108.
2 Llovet JM, Fuster J, Bruix J. Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. Hepatology 1999; 30: 1434-1440.
3 Lam CM, Lo CM, Yuen WK, et al. Prolonged survival in selected patients following surgical resection for pulmonary metastasis from hepatocellular carcinoma. Br J Surg 1998; 85: 1198-1200.
4 Schlitt HJ, Neipp M, Weimann A, et al. Recurrence patterns of hepatocellular and fibrolamellar carcinoma after liver transplantation. J Clin Oncol 1999; 17: 324-331.
5 Yamamoto J, Kosuge T, Takayama T, et al. Recurrence of hepatocellular carcinoma after surgery. Br J Surg 1996; 83: 1219-1222.
6 Tang ZY, Zhou XD, Lin ZY, et al. Surgical treatment of hepatocellular carcinoma and related basic research with special reference to recurrence and metastasis. Chin Med J 1999; 112: 887 -891.
7 Arvelo F, Poupon MF. Molecular and cell aspects of the cancer metastasis. Acta Cient Venez 2001; 52: 304-312.
8 Sakon M, Nagano H, Nakamori S, et al. Intrahepatic recurrences of hepatocellula carcinoma after hepatectomy:analysis based on tumor hemodynamics. Arch Surg 2002; 137: 94-99.
9 Hillaire S, Barbu V, Boucher E, et al. Albumin messenger RNA as a marker of circulating hepatocytes in hepatocelular carcinoma. Gastroenterology 1994; 106: 239-242.
10 Ijichi M, Takayama T, Matsumura M, et al. Alphatetoprotein mRNA in the circulation as a predictor of postsurgical recurrence of hepatocellular carcinoma: a prospective study. Hepatology 2002; 35: 853-860.
11 Chou HC, Sheu JC, Huang GT, et al. Albumin messenger RNA is not specific for circulating hepatoma cells. Gastroenterology 1994; 107: 630-631.
12 Cillo U, Naraglia F, Vitale A, et al. Clinical significance of alpha-fetoprotein mRNA in blood of patients with hepatocellual carcinoma. Clin Chim Acta 2004; 347: 129-138.
13 De-Plaen E, Arden K, Traversari C, et al. Structure, chromosomal localization, and expression of 12 genes of the MAGE family. Immunogenetics 1994; 40: 360-369.
14 Yang SZ, Dong JH, Li K, et al. Detection of AFPmRNA and melanoma antigen gene-1mRNA as markers of disseminated hepatocellularcarcinoma cells in blood. Hepatobiliary Pancreat Dis Int 2005; 4: 227-233.
15 Miyamoto A, Fujiwara Y, Sakon M, et al. Development of a multiple-marker RT-PCR assay for detection of micrometastases of hepatocellular carcinoma. Dig Dis Sci 2000; 45: 1376-1382.
16 Mou DC, Cai SL, Peng JR, et al. Evaluation of MAGE-1 and MAGE-3 as tumour-specific markers to detect blood dissemination of hepatocellular carcinoma cells. Br J Cancer 2002; 86: 110-116.
17 Pantel K, Schlimok G, Kutter D, et al. Frequent downregulation of major histocompatibility class I antigen expression on individual micrometastatic carcinoma cells. Cancer Res 1991; 51: 4712-4715.
18 Pelosi G, Pastorino U, Pasini F, et al. Independent prognostic value of fascin immunoreactivity in stage I nonsmall cell lung cancer. Br J Cancer 2003; 88: 537-547.
19 Kufer P, Zippelius A, Lutterbuse R, et al. Heterogeneous expression of MAGE-A genes in occult disseminated tumor cells: a novel multimarker reverse transcription-polymerase chain reaction for diagnosis of micrometastatic disease. Cancer Res 2002; 62: 251-261.
20 Sher YP, Shih JY, Yang PC, et al. Prognosis of non-small cell lung cancer patients by detecting circulating cancer cells in the peripheral blood with multiple marker genes. Clin Cancer Res 2005; 11: 173-179.
21 Jeng KS, Sheen IS, Tsai YC. Does the presence of circulating hepatocellular carcinoma cells indicate a risk of recurrence after resection? Am J Gastroenterol 2004; 99 1503-1509.
22 Morimoto O, Nagano H, Miyamoto A, et al. Association between recurrence of hepatocellular carcinoma and alpha-fetoprotein messenger RNA levels in peripheral blood. Surg Today 2005; 35: 1033-1041.
23 Miyashiro I, Kuo C, Huynh K, et al. Molecular strategy for detecting metastatic cancers with use of multiple tumor-specific MAGE-A genes. Clin Chem 2001; 47: 505-512.
24 Wang YL, Li G, Wu D, et al. Analysis of alpha-fetoprotein mRNA level on the tumor cell hematogenous spread of patients with hepatocellular carcinoma undergoing orthotopic liver transplantation. Transplant Proc 2007; 39: 166-168.
25 Yanaga K. Current status of hepatic resection for hepatocellular carcinoma. J Gastroenterol 2004; 39: 919-926.
26 Imamura H, Matsuyama Y, Tanaka E, et al. Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. J Hepatol 2003; 38: 200-207.
27 Pfleiderer C, Zoubek A, Gruber B, et al. Detection of tumor cells in peripheral blood and bone marrow from Ewing tumor patients by RT-PCR. Int J Cancer 1995; 64: 135-139.
28 Matsumura M, Niwa Y, Kato N, et al. Detection of alpha-fetoprotein mRNA, an indicator of hematogenous spreading hepatocellar carcinoma, in the circulation: a possible predictor of metastatic hepatocellular carcinoma. Hepatology 1994; 20: 1418-1425.
29 Komeda T, Fukuda Y, Sando T, et al. Sensitive detection of circulating hepatocellular carcinoma cells in peripheral venous blood. Cancer 1995; 75: 2214-2219.
30 Kedda MA, Kew MC, Skelton M, et al. Non-specificity of messenger RNA of alpha- fetoprotein in peripheral blood in detecting early spread of hepatocellular carcinoma in black Africans. J Gastroenterol Hepatol 1998; 13: 885-891.
31 Funaki NO, Tanaka J, Seto SI, et al. Hematogenous spreading of hepatocellular carcinoma cells: possible participation in recurrence in the liver. Hepatology 1997; 25: 564-568.
32 Yuasa T, Okamoto K, Kawakami T et al. Expression patterns of cancer testis antigens in testicular germ cell tumors and adjacent testicular tissue. J Urol 2001; 165: 1790-1794.
33 Tahara K, Mori M, Sadanaga N, et al. Expression of the MAGE gene family in human hepatocellular carcinoma. Cancer 1999; 85:1234-1240.