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*Original Article*

# Combined cancer testis antigens enhanced prediction accuracy for prognosis of patients with hepatocellular carcinoma

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**Abstract:** Cancer testis antigens (CTAs) are selectively expressed in malignant cells and can serve as ideal targets for immunotherapy. We investigated the expressions of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 to determine if combinatorial expressions of CTAs might be as potential prognostic markers for patients with hepatocellular carcinoma (HCC). In tumor tissues of 142 HCC patients, the mRNA expressions of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 were 78.9%, 33.8%, 74.6% and 14.1% respectively. Furthermore, the expressions of MAGE-A3, MAGE-A4 and combination of MAGE-A3, MAGE-A4 and NY-ESO-1 (CTAs-A3/A4/NY) showed positive correlations with serum AFP, tumor stages and Ki-67 ($P < 0.05$). In addition, mRNA expressions of CTAs were significantly consistent with protein expressions of CTAs by immunohistochemistry ($P > 0.05$). Receiver operating characteristic curves (ROC) analysis showed that CTAs-A3/A4/NY had larger areas under ROC curve (0.768), specificity (99.1%), Youden's index (44.6), positive predictive value (90.9%) and negative predictive value (89.9%) for predicting HCC recurrence than other CTAs. Moreover, the combinatorial expression of CTAs-A3/A4/NY was significantly associated with HCC recurrence by Kaplan-Meier analysis (HR = 69.36, $P < 0.01$) and multivariate Cox analysis (RR = 17.11, $P < 0.01$). The combinatorial expression of CTAs-A3/A4/NY mRNA promotes the predictive accuracy of HCC recurrence and itself may be a potential target for immunotherapy of HCC as well.

**Keywords:** Hepatocellular carcinoma, cancer testis antigen, prognosis, marker

## Introduction

Identification of highly immunogenic and tumor-specific antigens is urgently needed for immunotherapy because these antigens can serve as important tools for diagnostic, therapeutic and prognostic approaches. Cancer testis antigens (CTAs) are selectively expressed in malignant cells, but not in normal tissues except for human testicular germ cells and can serve as ideal targets for immunotherapy [1, 2]. So far, more than 100 CTAs have been identified [3]. The expression of CTAs has been found in various tumors including lymphoma, melanoma, lung cancer, breast cancer and liver cancer [1, 4, 5]. Based on their immunogenicity and frequencies of expression, CTAs have been targeted in multiple cancer vaccine trials, as well as, adoptive T cell transfer trials for immunotherapy of cancer using either CTL or TCR gene modified T cells [6, 7].

Recent evidence has suggested that CTAs are considered as important factors in carcinogenesis, immortality, invasiveness, immune evasion and metastatic capacity of tumor cells [8]. Down-regulation of CTAs can alter tumor cell morphology, adherence, proliferation, invasion and migration [9, 10]. Furthermore, many studies suggested that CTAs expressions are strong-

ly correlated with tumor clinicopathological parameters. The increased expressions of CTAs are related with advanced tumor stages, serum alpha fetoprotein (AFP), tumor recurrence, metastasis and poor clinical outcome [11-13]. Thus, the expressions of CTAs can be as ideal predictors for prognosis of cancer.

Since 1991, MAGE-1 (melanoma associated antigen) gene has been cloned from melanoma cells [14], dozens of MAGE genes have been identified [15]. In cancers, these genes are reactivated and their encoding proteins are frequently expressed in various histological types of cancers. Because of this distribution characteristic, the MAGE proteins are termed as CTAs [16]. Many studies reported that MAGE-A3, MAGE-A4 and MAGE-C2 transcripts are highly expressed in HCC tissues, but not in non-HCC liver tissues, nor in non-HCC liver diseases such as HBV/HCV infection and cirrhosis [17-20]. Moreover, the expressions of those MAGE genes are strongly associated with metastasis, recurrence and negative prognosis of HCC patients [18, 19, 21]. The NY-ESO-1 antigen originally found in esophageal cancer belongs to the CTAs family as well [22]. The NY-ESO-1 gene is also expressed in non-small cell lung cancer, colorectal cancer and HCC [23-25]. Furthermore, NY-ESO-1 is closely associated with advanced and metastasis of HCC [25, 26]. Current researches reported that the combinatorial expressions of CTAs have been frequently observed in lung cancer, head and neck cancer, breast cancer, bladder carcinoma and HCC [12, 27, 28]. The combinatorial expressions of CTAs could enhance specificity of tumor diagnosis and accuracy of immunotherapy [29]. However, few researches discuss that whether the specific combinatorial expressions of CTAs such as MAGE genes and NY-ESO-1 gene can be as tumor-specific targets and enhance the predictive accuracy of HCC prognosis during early stages of therapy.

In this study, we investigated the expressions of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 by RT-PCR and immunohistochemistry in cancer tissues and paired adjacent non-cancerous tissues from 142 HCC patients respectively. Then, we explored the correlations between the expressions of CTAs and the clinicopathological parameters of patients. Furthermore, we compared various combinatorial expressions of CTAs to screen the potential predictive markers of recurrence of HCC patients after hepatic resection.

## Materials and methods

### *Cell lines and human individuals*

HCC cell lines (HepG2, Hep3B, Huh7 and SMMC-7721) were obtained from American Type Cell Culturing (ATCC HB-8065) and immortalized normal liver cell line LO2 were obtained from Bioye Biotechnology Co. in China. All cell lines were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), at 37°C in a humidified incubator containing 5% $CO_2$.

A total of 142 HCC patients were recruited in Department of Hepatobiliary Surgery at First Affiliated Hospital of Zhengzhou University (Zhengzhou, Henan, China) from January 2012 to January 2013. Fresh HCC tumor tissues and paired adjacent non-cancerous tissues (>5 cm from tumor edge) were collected from the 142 HCC patients. Three testis tissues were obtained from patients with testicular trauma. Twenty non-tumor tissues from liver were collected from patients undergoing resection of hepatic hemangioma. Ethical approval was obtained from the Human Research Ethics Committee (the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China). Written informed consent was obtained from each participant.

### *Clinicopathological parameters*

Tumors were classified according to the WHO Classification of the Digestive System (2010) [30]. All patients were treated with surgical resection or liver transplantation initially. The stages of tumor performed according to the TNM classification of the International Union Against Cancer (UICC) [31]. The clinical diagnosis was confirmed by pathological examination. Clinicopathological parameters investigated in this study included patient age, gender, alanine aminotransferase (ALT), human leukocyte antigen A*02 (HLA-A*02), serum alpha fetoprotein (AFP), cancer antigen 125 (CA-125), cancer antigen 199 (CA-199), infection of hepatitis B and/or C viral, carcinoembryonic antigen (CEA), tumor size, tumor stages, tumor metastasis, proliferation rate (Ki-67) and recurrence-free survival time.

### *Tissue and blood specimen*

All the tissues used in this study were immediately stored in liquid nitrogen after resection

until RNA extraction. Whole blood samples were drawn one week before surgery. Approximately 10 ml of peripheral venous blood were collected from each patient. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation. Liver function testing was performed on Beckman DXC800 automatic biochemical analyzer (Beckman Coulter, CA, USA).

*Serum CA-125, CA-199, CEA and AFP detection*

The concentrations of serum CA-125, CA-199, CEA and AFP were determined one week before surgery by electrochemiluminescence immunoassay (Cobas, Roche Diagnostics, Germany). Whole procedure performed according to the manufacturer's instruction. The normal reference values were as follows: CA-125 ≤ 35 U/ml, CA-199 ≤ 39 U/ml and CEA ≤ 10 μg/L. According to serum AFP levels, the patients were divided into two groups: Group A: AFP < 400 ng/ml (n = 88); Group B: AFP ≥ 400 ng/ml (n = 54).

*HLA-A02 expression detection*

The PBMC ($1\times10^5$ cells) was stained with anti-CD3-FITC, anti-CD8-APC and anti-HLA-A02-PE antibodies (BD Bioscience, CA, USA) for 15 minutes at room temperature. Control cells were stained with isotype-matched mono-antibodies (mAbs) (BD Bioscience, CA, USA). After washing with FACS buffer, the cells were re-suspended in 300 μl FACS buffer and analyzed on a FACS Cano II (BD Bioscience, CA, USA).

*Ki-67 immunohistochemistry staining*

Immunohistochemistry on paraffin embedded sections with Ki-67 was performed using a fully automated system (Ventana Benchmark, CA, USA). Briefly, heat retrieval was standard for 60 minutes. Sections were incubated with Ki-67 antibody (M7240, Dako, Denmark). After staining, only distinct nuclear staining of carcinoma cells was used for scoring via the light microscope. Assessment was carried out on the entire tumor represented in the section.

*CTAs Immunohistochemistry staining*

Sections (4-μm-thick) of formalin-fixed paraffin-embedded tissues mounted on glass slides were processed for immunohistochemistry. All slides were dewaxed in xylene and dehydrated in an alcohol gradient; endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min. Antigen retrieval was obtained by heating the slides covered with citrate buffer (pH 6.0) at 95°C for 10 min. Subsequently, 10% goat serum albumin was used to block non-specific binding by incubating the sections for 1 h at room temperature. Gently tilting without washing, the sections were then incubated with anti-human MAGE-A3 mAb, MAGE-A4 mAb, MAGE-C2 mAb and NY-ESO-1 mAb (Abcam, England) at the concentration of 1:150, 1:200, 1:400 and 1:200 respectively in a dark box at 4°C overnight. The sections were then incubated with the secondary antibody at room temperature for 1 h and rinsed in phosphate-buffered saline (PBS). Diaminobenzidine (DAB) was used as the chromogen and the sections were counterstained with hematoxylin. Brown particles present in the cytoplasm and/or nuclei were considered positive. Complete absence of staining was considered negative for each CTA tested. Detection of CTAs expression in testis tissue was used as a positive control, but in the negative control, absence of primary antibody was performed. Imaging analysis was conducted under a microscope (Leica, Tokyo, Japan).

*RNA Extraction and reverse transcription polymerase chain reaction (RT-PCR)*

Total RNA was extracted by the Trizol RNA extraction method (Gibco Co. USA) and cDNA was synthesized by SuperScript III reverse transcriptase Kit (Invitrogen Co. USA). Whole procedure performed according to the manufacturer's instruction. The CTAs primers were designed using the Primer Express v 2.0 software (Applied Biosystems Co. USA). Primer sequences were as follows: MAGE-A3 forward: 5'-AGTCCGAGTTCCAAGCAG-3' and reverse: 5'-GCAGGTGGCAAAGATGTA-3'; MAGE-A4 forward: 5'-TGCCTTACCCACTACCATC-3' and reverse: 5'-TGCTCCAGGACTTTCACATA-3'; MAGE-C2 forward: 5'-TGAGTTAGAAGACTGGGTAGATGC-3' and reverse: 5'-ATGCTCTCGGTAAGATTTGGTATC-3'; NY-ESO-1 forward: 5'-AGTTCTACCTCGCCATGCCT-3' and reverse: 5'-TCCTCCTCCAGCGACAAACAA-3'. The housekeeping gene GAPDH (forward: 5'-ACCACAGTCCATG CCATCAC-3' and reverse: 5'-TCCACCACCCTGTTGCTGTA-3') was used to control the amount of cDNA template.

The amplification was performed for 33 cycles (30 seconds at 94°C and 30 seconds at 60°C and 30 seconds at 72°C for MACE-A3 and MACE-C2) or for 35 cycles (30 seconds at 94°C and 30 seconds at 61°C and 30 seconds at 72°C for MAGE-A4 and NY-ESO-1) followed by a

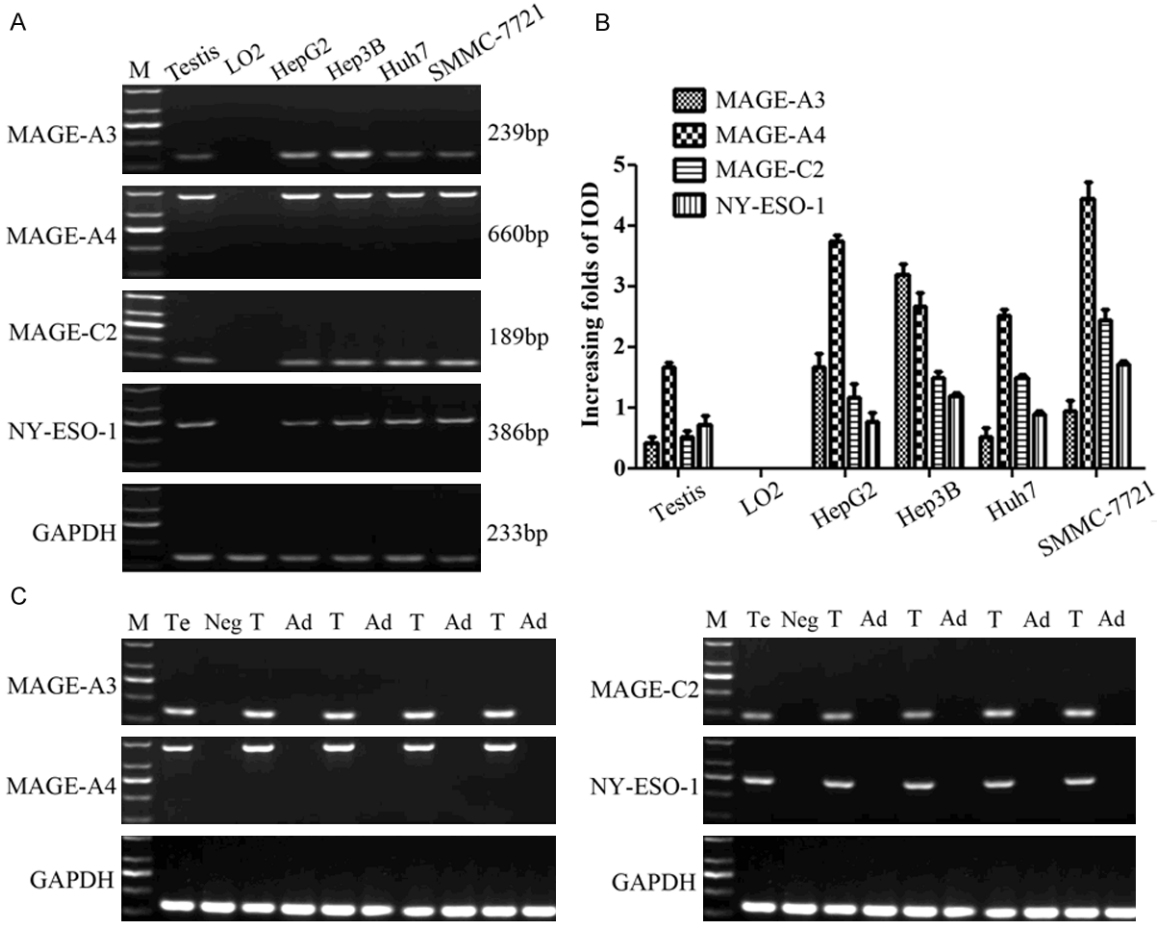


**Figure 1.** mRNA expressions of CTAs were evaluated in HCC tissues and cell lines. A. The mRNA expressions of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 were detected in testis tissue, LO2 cell line and 4 cultured liver cancer cell lines as 239-bp, 660-bp, 189-bp and 386-bp PCR product respectively. B. The mRNA expressions of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 in HCC cell lines by densitometry analysis with Image J software. C. Expressions of CTAs were positive in tumor tissues but negative in paired adjacent noncancerous tissues. RT-PCR for GAPDH was used to monitor the quality of the RNA sample with a 233-bp PCR product. Abbreviations: M: marker; IOD: integrated option density; Te: testis tissue; Ne: negative control, normal liver tissues; T: tumor tissues; Ad: adjacent noncancerous tissues.

final extension for 10 minutes at 72°C. The products were electrophoresed in 1% agarose gel and visualized by ethidium bromide staining.

*Sequence analysis of PCR products*

Sequence analysis of PCR products verified that the nucleotide sequences of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 fragments were identical to those in the database of the GenBank, NM_005362, NM_001011548, NM_016249, HSU87459 respectively.

*Follow-up*

All 142 patients were followed up and each patient followed for 12 months. The tumor recurrence was assessed at the 1$^{th}$, 3$^{th}$, 6$^{th}$, 9$^{th}$, and 12$^{th}$ month after operation. All the patients were checked with ultrasonography and computed tomography (CT) to examine any metastasis and/or recurrence. The endpoint of follow-up was set at the HCC recurrence of the patient.

*Statistical analysis*

Statistical analysis was performed using the SPSS software (version 16.0; SPSS Inc, Chicago, USA) and GraphPad Prism software (version 5.0; San Diego, CA, USA). The correlations between CTAs expressions and clinicopathological characteristics were analyzed by the Chi-square test or Fisher's exact tests. McNemar test and Kappa test were used to analyze the consistence of paired mRNA ex-

**Table 1.** A. The mRNA expressions of CTAs genes in testis, liver cancer cell lines and immortalized normal liver cell line; B. mRNA expression of CTAs in HCC, adjacent non-cancerous and hepatic hemangioma tissues; C. Expressions of CTAs proteins in HCC by RT-PCR and immunohistochemistry

**A.**

| Category | MAGE-A3 | MAGE-A4 | MAGE-C2 | NY-ESO-1 |
|---|---|---|---|---|
| Testis | + | + | + | + |
| HepG2 | + | + | + | + |
| SK-Hep 1 | + | + | + | + |
| Huh7 | + | + | + | + |
| SMMC-7721 | + | + | + | + |
| LO2 | - | - | - | - |

**B.**

| Diseases | No. of patients | MAGE-A3 n (%) | MAGE-A4 n (%) | MAGE-C2 n (%) | NY-ESO-1 n (%) |
|---|---|---|---|---|---|
| HCC | 142 | 112 (78.9) | 48 (33.8) | 106 (74.6) | 20 (14.1) |
| AC | 142 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| HH | 20 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

**C.**

| Methods | | Immunohistochemistry | | *Kappa* | *McNemar P* value |
|---|---|---|---|---|---|
| RT-PCR | | Positive | Negative | | |
| MAGE-A3 | Positive | 104 | 8 | 0.76 | 0.39 |
| | Negative | 4 | 26 | | |
| MAGE-A4 | Positive | 41 | 7 | 0.84 | 0.34 |
| | Negative | 3 | 91 | | |
| MAGE-C2 | Positive | 92 | 14 | 0.63 | 0.19 |
| | Negative | 7 | 29 | | |
| NY-ESO-1 | Positive | 18 | 2 | 0.91 | 1.00 |
| | Negative | 1 | 121 | | |

HCC: hepatocellular carcinoma tissue; AC: adjacent non-cancerous tissue; HH: hepatic hemangioma tissue. Kappa test and McNemar test were used to analyze consistence of the results of paired mRNA expressions and protein expressions of CTAs. All *P* values were two-sided. *P* value of less than 0.05 was considered as statistically significant.

pressions and protein expressions of CTAs. Overall recurrence rate was measured from the time of surgery. The Kaplan-Meier method and Cox proportional hazard model were used to identify the predictive factors of HCC recurrence. Receiver operating characteristic curve (ROC) was adopted to screen the combinatorial expressions of CTAs for predicting the recurrence of HCC. All *P* values were two-sided. *P* value of less than 0.05 was considered as statistically significant in all cases.

## Results

*CTAs were preferentially expressed in HCC cell lines but not in immortalized normal liver cell line*

We investigated the mRNA expressions of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 in HepG2, Hep3B, Huh7 and SMMC-7721 HCC cell lines and immortalized normal liver cell line LO2 by RT-PCR method. All four CTAs transcripts were simultaneously expressed in each HCC cell line, but immortalized normal liver cell line LO2 did not express any CTAs (**Figure 1A, 1B** and **Table 1A**).

*CTAs were only expressed in tumor tissues but not in paired adjacent non-cancerous tissues and normal liver tissues*

To determine the clinical relevance of high CTAs expression observed in liver cancer cell lines, RT-PCR and immunohistochemistry analysis were performed to evaluate the CTAs expressions in tumor tissues and paired adjacent non-cancerous tissues and normal liver tissue (**Figures 1**, **2**). In tumor tissues from 142 HCC patients, 78.9% expressed MAGE-A3 mRNA, 33.8% expressed MAGE-A4 mRNA, 74.6% expressed MAGE-C2 mRNA and only 14.1% expressed NY-ESO-1 mRNA. Furthermore, 87.3% expressed at least one type of CTAs

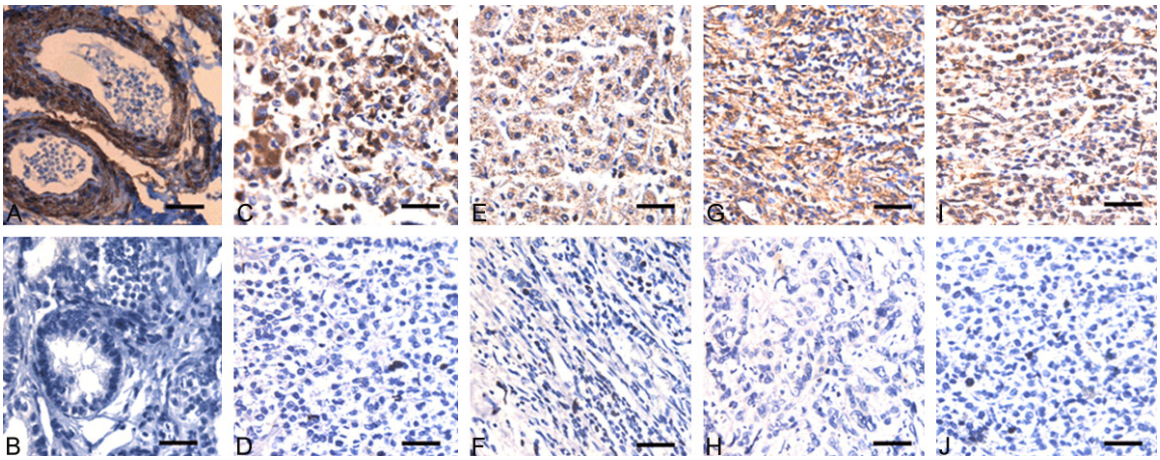


**Figure 2.** MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 were widely expressed in tumor tissues of HCC patients by immunohistochemistry. A. Normal testis tissue subjected to immunohistochemical staining with MAGE-A3 mAb was positive control. B. Normal testis tissue absence of primary antibody performed was negative control. C, E, G, I. Tumor tissues were stained with MAGE-A3 mAb, MAGE-A4 mAb, MAGE-C2 mAb and NY-ESO-1 mAb respectively. D, F, H, J. Paired adjacent non-cancerous tissues were stained with MAGE-A3 mAb, MAGE-A4 mAb, MAGE-C2 mAb and NY-ESO-1 mAb respectively. Brown particles present in the cytoplasm and/or nuclei were considered positive. Complete absence of staining was considered negative for each CTA tested. Bar represents 50 μm. Imaging analysis was conducted under a microscope (Original magnification x 200). These experiments were performed independently at least twice with similar results.

mRNA (CTAs-multiple), 73.2% expressed two types of CTAs mRNA, 32.4% expressed three types of CTAs mRNA, and only 5.6% expressed all four types of CTAs mRNA in tumor tissues. Three testis tissues expressed four types of CTAs mRNA simultaneously. However, the paired adjacent non-cancerous tissues and normal liver tissues did not express any CTAs mRNA (**Figure 1C**). In addition, McNemar test and Kappa test demonstrated that there was significant consistence between the mRNA expressions and protein expressions of CTAs in tumor tissues ($P > 0.05$; **Table 1C**; **Figure 3**). So we highlighted the correlations between mRNA expressions of CTAs and the prognosis of HCC patients after surgery. Those results demonstrated that the HCC tumor tissues can simultaneously express several CTAs which provides a possibility of polyvalent vaccinations for HCC.

*Association between CTAs expression and clinicopathological parameters in HCC*

No correlations were found between the expressions of CTAs and the clinicopathological parameters such as patients' gender, age, ALT, HLA-A02, CA-125, CA-199, CEA, tumor size, HBV and/or HCV infection ($P > 0.05$) (**Table 2**). However, the expression of MAGE-A3 was associated with higher serum AFP, advanced tumor stage, metastasis and higher Ki-67 ($P < 0.05$); MAGE-A4 was associated with higher serum AFP, advanced tumor stages and higher Ki-67 ($P < 0.05$); MAGE-C2 was associated with advanced tumor stages and higher Ki-67 ($P < 0.05$); NY-ESO-1 was associated with higher serum AFP ($P < 0.05$) (**Table 2**; **Figure 3**). These results indicated that CATs might be associated with the prognosis of HCC.

We further investigated the combinatorial expressions of various types of CTAs in tumor tissues. We found that frequencies of three types of CTAs in stage III or IV were significantly higher than that in stage I or II tumor ($P < 0.05$; **Figure 4**). Moreover, combinatorial expression of MAGE-A3, MAGE-A4 and NY-ESO-1 (CTAs-A3/A4/NY) was associated with higher serum AFP, advanced tumor stage and higher Ki-67 ($P < 0.05$; **Figure 2**).

*CTAs-A3/A4/NY as a biomarker for HCC recurrence*

A total of 130 HCC patients (91.5%) completed a 12-months follow-up and 16.9% patients (22/130) experienced tumor recurrence during the follow-up. In order to detect whether the combinatorial expressions of CTAs could enhance the predictive precision for HCC recurrence, various combinations of CTAs expressions were evaluated by ROC analysis. As

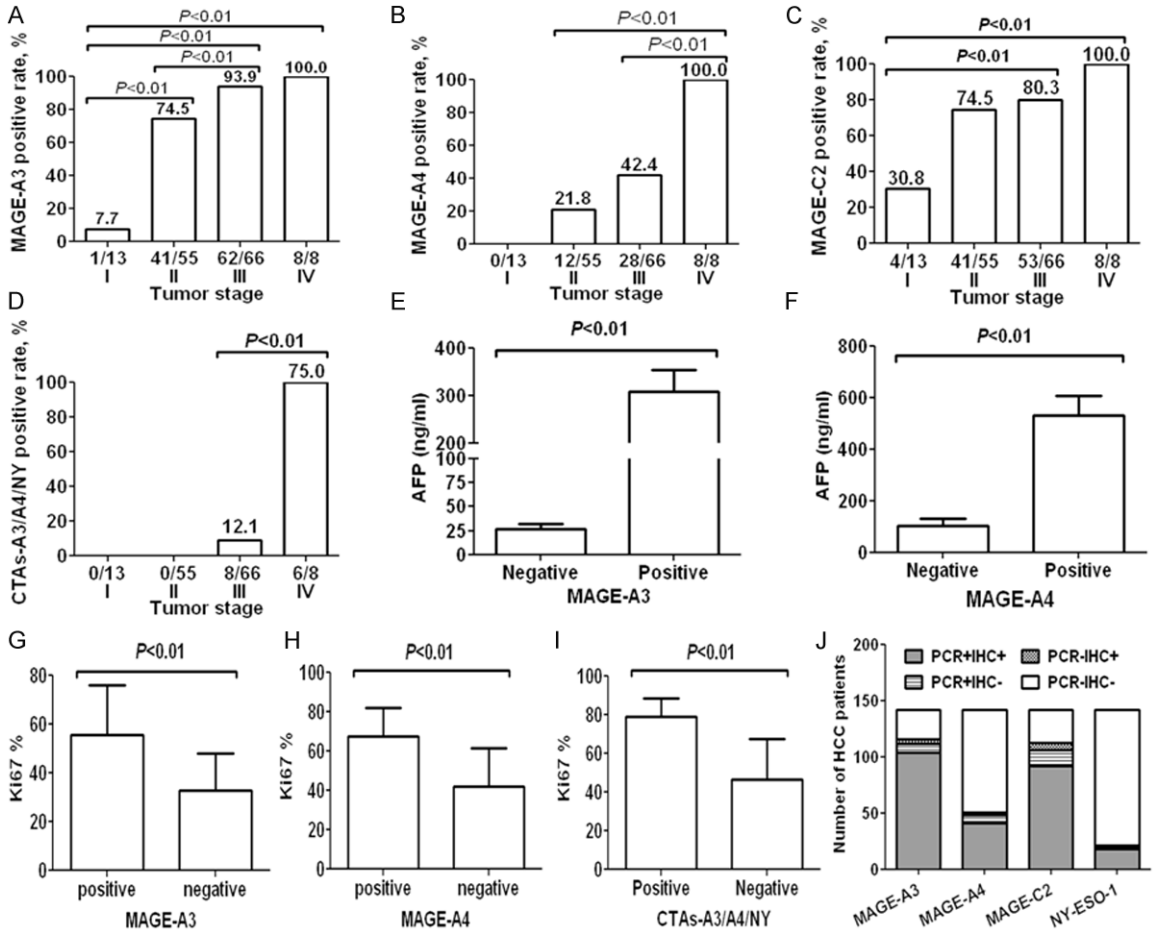


**Figure 3.** The expressions of CTAs were correlated with clinicopathological parameters in 142 HCC patients. (A-D) The comparison of expressions of MAGE-A3 (A), MAGE-A4 (B), MAGE-C2 (C) and CTAs-A3/A4/NY (D) in various tumor stages. (E-F) The expressions of MAGE-A3 (E) and MEGE-A4 (F) were correlated with high serum AFP. (G-I) The expressions of MAGE-A3 (G), MAGE-A4 (H) and CTAs-A3/A4/NY were correlated with Ki-67 ($P < 0.05$). (J) The consistence analysis of the mRNA expressions and protein expressions of CTAs tested by RT-PCR and immunohistochemistry respectively in tumor tissues of HCC patients. Chi-square test or Fisher's exact tests was used for qualitative data. All *P* values were two-sided. *P* value of less than 0.05 was considered statistically significant in all cases.

showed in **Figure 5** and **Table 3**, the combinatorial expression of CTAs-A3/A4/NY had the largest areas under ROC curve (AUC = 0.768) among various combinations of CTAs. The CTAs-A3/A4/NY had higher specificity (99.1%), positive predictive value (PPV, 90.9%), negative predictive value (NPV, 89.9%) and Youden's index (44.6%) than other combinations of CTAs (**Table 4**). The results suggested that combinatorial expression of CTAs-A3/A4/NY enhanced the accuracy of HCC recurrence prediction.

*CTAs-A3/A4/NY correlated with prognosis of HCC as an independent predictor*

To screen the possible predictive factors of HCC recurrence, we analyzed all the clinicopathological parameters as above. In Kaplan-Meier analysis, the indexes such as serum AFP, CA-125, tumor stages, Ki-67, MAGE-A3, MAGE-A4, NY-ESO-1 and CTAs-A3/A4/NY were significantly correlated with HCC recurrence ($P < 0.05$, **Table5**; **Figure 6**). Then those significant variables in the univariate analysis were put into the multivariate Cox model to identify the independent predictive factors of HCC recurrence. In the end, the combinatorial expression of CTAs-A3/A4/NY (RR = 17.11; $P < 0.01$), tumor stage (RR = 10.81; $P < 0.01$) and serum AFP (RR = 6.68; $P < 0.01$) were independent predictors of HCC recurrence (**Table 6**; **Figure 6**). The results indicated that special combinatorial expression of CTAs-A3/A4/NY could be a useful marker for predicting the recurrence of HCC even better than the tumor stage and the serum AFP.

**Table 2.** Correlations between the expression of CTAs and clinicopathological parameters of patients with HCC

| Category | No. | MAGE-A3 | | MAGE-A4 | | MAGE-C2 | | NY-ESO-1 | | CTAs-A3/A4/NY | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | n (%) | *P* | n (%) | *P* | n (%) | *P* | n (%) | *P* | n (%) | *P* |
| Gender | | | | | | | | | | | |
| Male | 108 | 86 (79.6) | 0.69 | 32 (29.6) | 0.06 | 80 (74.1) | 0.78 | 12 (11.1) | 0.07 | 12 (11.1) | 0.52* |
| Female | 34 | 26 (76.5) | | 16 (47.1) | | 26 (76.5) | | 8 (23.5) | | 2 (5.9) | |
| Age (year) | | | | | | | | | | | |
| < 60 | 94 | 70 (74.5) | 0.07 | 36 (38.3) | 0.11 | 72 (76.6) | 0.46 | 16 (17.0) | 0.16* | 10 (10.6) | 0.66* |
| ≥ 60 | 48 | 42 (87.5) | | 12 (25.0) | | 34 (70.8) | | 4 ( 8.3) | | 4 ( 8.3) | |
| ALT (U/L) | | | | | | | | | | | |
| < 80 | 122 | 94 (77.0) | 0.19 | 38 (31.1) | 0.10 | 88 (72.1) | 0.09 | 16 (13.1) | 0.41* | 10 (8.2) | 0.11* |
| ≥ 80 | 20 | 18 (90.0) | | 10 (50.0) | | 18 (90.0) | | 4 (20.0) | | 4 (20.0) | |
| HLA-A02 | | | | | | | | | | | |
| Positive | 82 | 68 (82.9) | 0.17 | 28 (34.1) | 0.92 | 60 (73.2) | 0.64 | 8 (9.8) | 0.08 | 5 (6.1) | 0.08* |
| Negative | 60 | 44 (73.3) | | 20 (33.3) | | 46 (76.7) | | 12 (20.0) | | 9 (15.0) | |
| Etiology | | | | | | | | | | | |
| HCV | 12 | 12 (100.0) | 0.15 | 4 (33.3) | 0.62 | 11 (91.7) | 0.50 | 2 (16.7) | 0.91* | 0 (0.0) | 0.83* |
| HBV | 90 | 70 (77.8) | | 32 (35.6) | | 67 (74.4) | | 14 (15.6) | | 10 (11.1) | |
| HBV/HCV | 4 | 4 (100.0) | | 2 (50.0) | | 3 (75.0) | | 0 (0.0) | | 0 (0.0) | |
| No | 36 | 26 (72.2) | | 10 (27.8) | | 25 (69.4) | | 4 (26.7) | | 4 (11.1) | |
| AFP (ng/ml) | | | | | | | | | | | |
| < 400 | 88 | 62 (70.5) | < 0.01 | 20 (22.7) | < 0.01 | 64 (72.7) | 0.15 | 6 (6.8) | < 0.01 | 2 (2.3) | < 0.01* |
| ≥ 400 | 54 | 50 (92.6) | | 28 (51.9) | | 42 (77.8) | | 14 (25.9) | | 12 (22.2) | |
| CA125 (U/ml) | | | | | | | | | | | |
| < 35 | 102 | 78 (76.5) | 0.26 | 32 (31.4) | 0.33 | 73 (71.6) | 0.18 | 13 (12.7) | 0.46 | 8 (7.8) | 0.20 |
| ≥ 35 | 40 | 34 (85.0) | | 16 (40.0) | | 33 (82.5) | | 7 (17.5) | | 6 (15.0) | |
| CA199 (U/ml) | | | | | | | | | | | |
| < 37 | 84 | 64 (76.2) | 0.35 | 28 (33.3) | 0.89 | 58 (69.0) | 0.07 | 12 (14.3) | 0.93 | 6 (7.1) | 0.19 |
| ≥ 37 | 58 | 48 (82.8) | | 20 (34.5) | | 48 (82.8) | | 8 (13.8) | | 8 (13.8) | |
| CEA (ng/ml) | | | | | | | | | | | |
| < 5 | 112 | 85 (75.9) | 0.09 | 36 (32.1) | 0.42 | 80 (71.4) | 0.09 | 13 (11.6) | 0.14 | 8 (7.1) | 0.08 |
| ≥ 5 | 30 | 27 (90.0) | | 12 (40.0) | | 26 (86.7) | | 7 (23.3) | | 6 (20.0) | |
| Tumor size (cm) | | | | | | | | | | | |
| < 5 | 87 | 72 (82.8) | 0.15 | 29 (33.3) | 0.88 | 68 (78.2) | 0.23 | 14 (16.1) | 0.39 | 8 (9.2) | 0.74 |
| ≥5 | 55 | 40 (72.7) | | 19 (34.5) | | 38 (69.1) | | 6 (10.9) | | 6 (10.9) | |
| Tumor stage | | | | | | | | | | | |
| I or II | 68 | 42 (61.8) | < 0.01 | 12 (17.6) | < 0.01 | 45 (66.2) | 0.03 | 6 (8.8) | 0.08 | 0 (0.0) | < 0.01* |
| III or IV | 74 | 70 (94.6) | | 36 (48.6) | | 61 (82.4) | | 14 (18.9) | | 14 (18.9) | |
| Metastasis | | | | | | | | | | | |
| Yes | 70 | 64 (91.4) | < 0.01 | 29 (41.4) | 0.06 | 54 (77.1) | 0.50 | 9 (12.9) | 0.68 | 9 (12.9) | 0.24 |
| No | 72 | 48 (66.7) | | 19 (26.4) | | 52 (72.2) | | 11 (15.3) | | 5 (6.9) | |
| Ki67 (%) | | | | | | | | | | | |
| < 40 | 60 | 37 (61.7) | < 0.01 | 5 (8.3) | < 0.01 | 36 (60.0) | < 0.01 | 5 (8.3) | 0.09 | 1 (1.7) | < 0.01* |
| ≥ 40 | 82 | 75 (91.5) | | 43 (52.4) | | 70 (85.4) | | 15 (18.3) | | 13 (15.9) | |

*Fisher's exact tests was used; No.: number of patients; CTAs-A3/A4/NY: combinatorial expression of MAGE-A3, MAGE-A4 and NY-ESO-1.Chi-square test was used to analyze the correlations between CTAs expression and clinicopathological characteristics. All *P* values were two-sided. *P* value of less than 0.05 was considered as statistically significant.

## Discussion

It is widely recognized that early diagnosis and treatment are critical for a better clinical outcome of HCC patients [32, 33]. Using biomarkers to identify patients with a risk of poor prognosis may thus reduce mortality and medical costs [34]. Recent studies suggested that

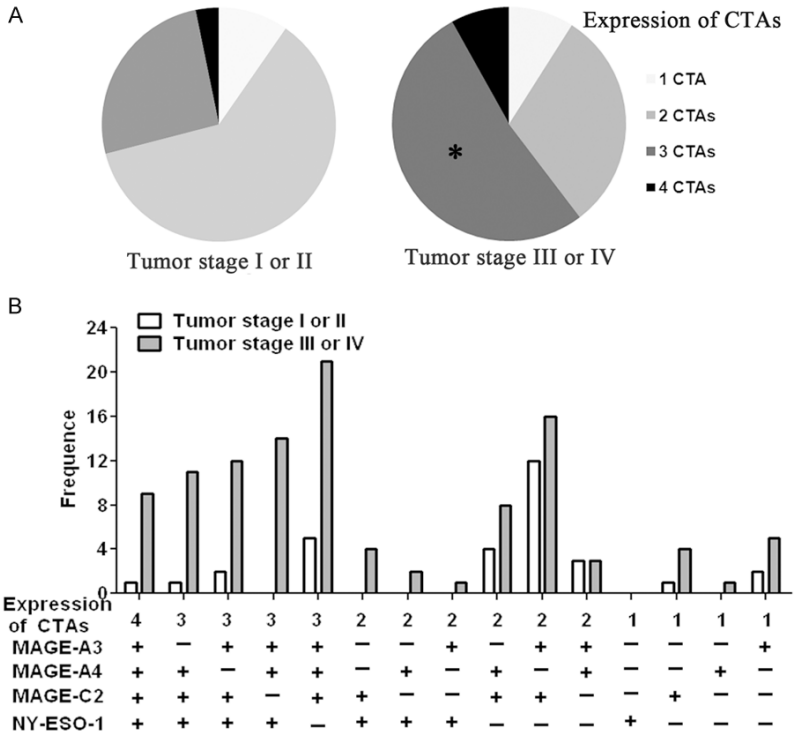


**Figure 4.** There were significant differences of the combinatorial expressions of CTAs in different tumor stages. A. The combinatorial expression of the MAGE-A3, MAGE-A4, MAGE-C2, NY-ESO-1 in two groups of tumor stages. The colors in the pie charts represent the proportions of mono CTA expression (light gray), two CTAs expression (gray), three CTAs expression (dark gray) and four CTAs expression (black) in the tumor tissues of HCC patients, as determined by RT-PCR. B. The bar chart shows the frequency of HCC tissues in each expression category, with blank bars corresponding to tumor stage I or II and gray bars corresponding to tumor stage III or IV. The frequency of combinatorial expression of three CTAs in stage III or IV tumors was higher than that of stage I or II tumors by Chi-square test ($P$ = 0.02). All $P$ values were two-sided. $P$ value of less than 0.05 was considered statistically significant.

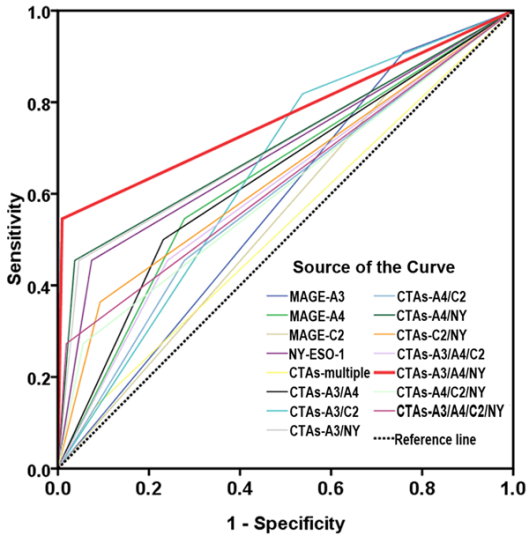


**Figure 5.** ROC plots for recurrence of HCC prediction model. The ROC AUC value of CTAs-A3/A4/NY (0.768) was the biggest in all combinations of CTAs. Abbreviations: CTAs-multiple, expression of at least one type of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1; A3: MAGE-A3; A4: MAGE-A4; C2: MAGE-C2; NY: NY-ESO-1. ROC was adopted to screen the combinatorial expression of CTAs for predicting the recurrence of HCC. All $P$ values were two-sided. $P$ value of less than 0.05 was considered as statistically significant.

expressions of CTAs are related with carcinogenesis, proliferation, invasiveness and metastatic capacity of tumor cells [8]. SSX as one of the CTAs is essential for the entry of tumor cells into S-phase of the cell cycle and, consequently, tumor cells that express SSX sustain cell proliferation and long-term survival [35]. Down-regulation of CTAs such as CAGE alters tumor cell morphology, invasion and migration [10]. As a sequence, the expressions of CTAs may be as ideal biomarkers for diagnosis, therapy and prognosis of malignances.

The frequency of CTAs expressions varies greatly in different tumor types [36]. For instance, mRNA expression of NY-ESO-1 has been observed in 52% of melanoma [37], 27% of non-small-cell lung carcinoma [38], but none of the renal cell carcinoma [22]. The expressions of CTAs also show a significant discrepancy in the same tumor type. Such as the frequencies of MAGE-A3 expressions in HCC were 83.3% and 39.0% respectively in two different studies [39, 40]. In this study, the frequencies of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 expressions were 78.9%, 33.8%, 74.6% and 14.1% respectively, and the results were

**Table 3.** Correlations between CTAs expressions and HCC recurrence by ROC analysis

| Category | AUC | SE | *P* value | AUC CI.95% | |
|---|---|---|---|---|---|
| MAGE-A3 | 0.575 | 0.062 | 0.269 | 0.453 | 0.676 |
| MAGE-A4 | 0.634 | 0.067 | 0.048 | 0.502 | 0.776 |
| MAGE-C2 | 0.548 | 0.065 | 0.479 | 0.420 | 0.658 |
| NY-ESO-1 | 0.690 | 0.071 | 0.005 | 0.551 | 0.742 |
| CTAs-multiple | 0.527 | 0.070 | 0.696 | 0.390 | 0.759 |
| CTAs-A3/A4 | 0.634 | 0.069 | 0.048 | 0.500 | 0.806 |
| CTAs-A3/C2 | 0.641 | 0.060 | 0.038 | 0.523 | 0.723 |
| CTAs-A3/NY | 0.704 | 0.071 | 0.003 | 0.565 | 0.751 |
| CTAs-A4/C2 | 0.588 | 0.069 | 0.192 | 0.453 | 0.776 |
| CTAs-A4/NY | 0.704 | 0.071 | 0.002 | 0.569 | 0.742 |
| CTAs-C2/NY | 0.636 | 0.072 | 0.046 | 0.495 | 0.898 |
| CTAs-A3/A4/C2 | 0.607 | 0.069 | 0.115 | 0.471 | 0.751 |
| CTAs-A3/A4/NY | 0.768 | 0.069 | 0.001 | 0.634 | 0.903 |
| CTAs-A3/C2/NY | 0.627 | 0.072 | 0.062 | 0.485 | 0.769 |
| CTAs-A4/C2/NY | 0.609 | 0.073 | 0.109 | 0.466 | 0.751 |
| CTAs-A3/A4/C2/NY | 0.627 | 0.073 | 0.061 | 0.483 | 0.771 |

ROC: receiver operating characteristic curve; AUC: area under the receiver operating characteristic curve; SE: standard error; CI: confidence interval; CTAs: cancer testis antigens; A3: MAGE-A3; A4: MAGE-A4; C2: MAGE-C2; NY: NY-ESO-1; CTAs-multiple: expression of at least one type of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1. ROC was adopted to screen the combinatorial expression of CTAs for predicting the recurrence of HCC. All *P* values were two-sided. *P* value of less than 0.05 was considered as statistically significant.

**Table 4.** Predictive value of CTAs expression for recurrence of HCC

| Expression of CTAs | Positive % (No.) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Recu (22) | Recu-f (108) | Sens | Spec | YI | PPV | NPV |
| MAGE-A3 | 90.9 (20/22) | 75.9 (82/108) | 90.9 | 24.1 | 15.0 | 19.6 | 92.9 |
| MAGE-A4 | 54.5 (12/22)* | 27.8 (30/108) | 54.5 | 72.2 | 26.8 | 28.6 | 88.6 |
| MAGE-C2 | 81.8 (18/22) | 72.2 (78/108) | 81.8 | 27.8 | 9.6 | 18.8 | 88.2 |
| NY-ESO-1 | 45.5 (10/22)* | 7.4 (8/108) | 45.5 | 92.6 | 38.1 | 55.6 | 89.3 |
| CTAs-multiple | 86.4 (19/22) | 91.7 (99/108) | 86.4 | 8.3 | -5.3 | 16.1 | 75.0 |
| CTAs-A3/A4 | 50.0 (11/22)* | 23.1 (25/108) | 50.0 | 76.9 | 26.9 | 30.6 | 88.3 |
| CTAs-A3/C2 | 81.8 (18/22)* | 53.7 (58/108) | 81.8 | 46.3 | 28.1 | 23.7 | 92.6 |
| CTAs-A3/NY | 45.5 (10/22)* | 4.6 (5/108) | 45.5 | 95.4 | 40.9 | 66.7 | 89.6 |
| CTAs-A4/C2 | 45.5 (10/22) | 27.8 (30/108) | 45.5 | 72.2 | 17.7 | 25.0 | 86.7 |
| CTAs-A4/NY | 45.5 (10/22)* | 3.7 (4/108) | 45.5 | 96.3 | 41.8 | 71.4 | 89.7 |
| CTAs-C2/NY | 36.4 (8/22)* | 9.3 (10/108) | 36.4 | 90.7 | 27.1 | 44.4 | 87.5 |
| CTAs-A3/A4/C2 | 36.4 (8/22)* | 25.9 (28/108) | 36.4 | 74.1 | 10.5 | 22.2 | 85.1 |
| CTAs-A3/A4/NY | 45.5 (10/22)* | 0.9 (1/108) | 45.5 | 99.1 | 44.6 | 90.9 | 89.9 |
| CTAs-A3/C2/NY | 31.8 (7/22)* | 6.5 (7/108) | 31.8 | 93.5 | 25.3 | 50.0 | 87.1 |
| CTAs-A4/C2/NY | 27.3 (6/22)* | 5.6 (6/108) | 27.3 | 94.4 | 21.7 | 50.0 | 86.4 |
| CTAs-A3/A4/C2/NY | 27.3 (6/22)* | 1.9 (2/108) | 27.3 | 98.1 | 25.4 | 75.0 | 86.9 |

**P*-values relative to recurrence-free patients with HCC, $P < 0.05$; Recu: recurrence; Recu-f: recurrence-free; Sens: sensitivity (Sens = expression of CTAs/Recu x 100%); Spec: specificity (Spec = 100 - expression of CTAs/Recu-f x 100%); CTAs: cancer testis antigens; A3: MAGE-A3; A4: MAGE-A4; C2: MAGE-C2; NY: NY-ESO-1; CTAs-multiple: expression of at least one type of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1; YI: Youden's index (YI = Sens + Spec - 100); PPV: positive predictive value (PPV = expression of CTAs in Recu/(expression of CTAs in Recu + expression of CTAs in Recu-f) x 100%); NPV: negative predictive value (NPV = (Recu-f - expression of CTAs in Recu-f) / ((Recu - expression of CTAs in Recu) + (Recu-f - expression of CTAs in Recu-f)) x 100%).

**Table 5.** Analysis of possible predictors for HCC recurrence by Kaplan-Meier analysis

| Category | Sub-category | No. | Recurrence | | HR. (CI.95%) | *P* value |
|---|---|---|---|---|---|---|
| | | | Yes | % | | |
| Gender | Male | 100 | 17 | 17.0 | 2.98 (0.89-9.97) | 0.08 |
| | Female | 30 | 5 | 16.7 | | |
| Age (year) | < 60 | 84 | 14 | 16.7 | 0.39 (0.11-1.36) | 0.14 |
| | ≥ 60 | 46 | 8 | 17.4 | | |
| HLA-A02 | Positive | 72 | 10 | 13.9 | 0.66 (0.22-1.99) | 0.46 |
| | Negative | 58 | 12 | 20.7 | | |
| Etiology | HBV/HCV | 96 | 18 | 18.8 | 1.27 (0.33-4.89) | 0.12 |
| | No | 34 | 4 | 11.8 | | |
| AFP (ng/ml) | < 400 | 80 | 6 | 7.5 | 0.08 (0.03-0.20) | < 0.01 |
| | ≥ 400 | 50 | 16 | 32.0 | | |
| CA125 (U/ml) | < 35 | 92 | 10 | 10.9 | 0.23(0.09-0.59) | 0.01 |
| | ≥ 35 | 38 | 12 | 31.6 | | |
| CA199 (U/ml) | < 37 | 76 | 12 | 15.8 | 0.90 (0.38-2.15) | 0.81 |
| | ≥ 37 | 54 | 10 | 18.5 | | |
| CEA (ng/ml) | < 5 | 102 | 16 | 15.7 | 0.72 (0.25-2.06) | 0.54 |
| | ≥ 5 | 28 | 6 | 21.4 | | |
| Tumor size (cm) | < 5 | 76 | 12 | 15.8 | 0.53 (0.21-1.34) | 0.18 |
| | ≥ 5 | 54 | 10 | 18.5 | | |
| Tumor stage | I or II | 62 | 2 | 3.2 | 0.22 (0.09-0.52) | < 0.01 |
| | III or IV | 68 | 20 | 29.4 | | |
| Tumor metastases | Yes | 64 | 14 | 21.9 | 1.89 (0.53-6.69) | 0.32 |
| | No | 66 | 8 | 12.1 | | |
| Ki67 (%) | < 40 | 52 | 2 | 3.8 | 0.22 (0.09-0.52) | < 0.01 |
| | ≥ 40 | 78 | 20 | 25.6 | | |
| MAGE-A3 | Positive | 102 | 20 | 19.6 | 0.25 (0.10-0.64) | < 0.01 |
| | Negative | 28 | 2 | 7.1 | | |
| MAGE-A4 | Positive | 42 | 12 | 28.6 | 4.36 (1.66-11.46) | < 0.01 |
| | Negative | 88 | 10 | 11.4 | | |
| MAGE-C2 | Positive | 96 | 18 | 18.8 | 2.95 (0.79-11.07) | 0.11 |
| | Negative | 34 | 4 | 11.8 | | |
| NY-ESO-1 | Positive | 18 | 10 | 55.6 | 9.53 (2.63-34.57) | < 0.01 |
| | Negative | 112 | 12 | 10.7 | | |
| CTAs-A3/A4/NY | Positive | 11 | 10 | 90.9 | 69.36 (7.56-94.32) | < 0.01 |
| | Negative | 119 | 12 | 10.1 | | |
| CTAs-multiple | Positive | 118 | 19 | 16.1 | 2.95 (0.63-13.77) | 0.17 |
| | Negative | 12 | 3 | 25.0 | | |

No.: number of patients; CI: confidence interval; HR: hazard ratio; CTAs-A3/A4/NY: combinatorial expression of MAGE-A3 and MAGE-A4 and NY-ESO-1; CTAs-multiple: expression of at least one type of MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1. Kaplan-Meier method was used to identify the predictive factors of HCC recurrence. All *P* values were two-sided. *P* value of less than 0.05 was considered as statistically significant.

according with early studies [19, 41, 42]. The discrepancy of CTAs expressions in tumor tissues may due to the intratumoral heterogeneity, post-transcriptional regulation of CTAs expressions or different RT-PCR conditions [43].

Previous reports have demonstrated that the combinatorial expressions of CTAs appeared in multiple tumor types, such as in lung cancer, head and neck cancer, breast cancer and bladder carcinoma [12, 27]. In tumors, the expression of one type of CTAs can simultaneously

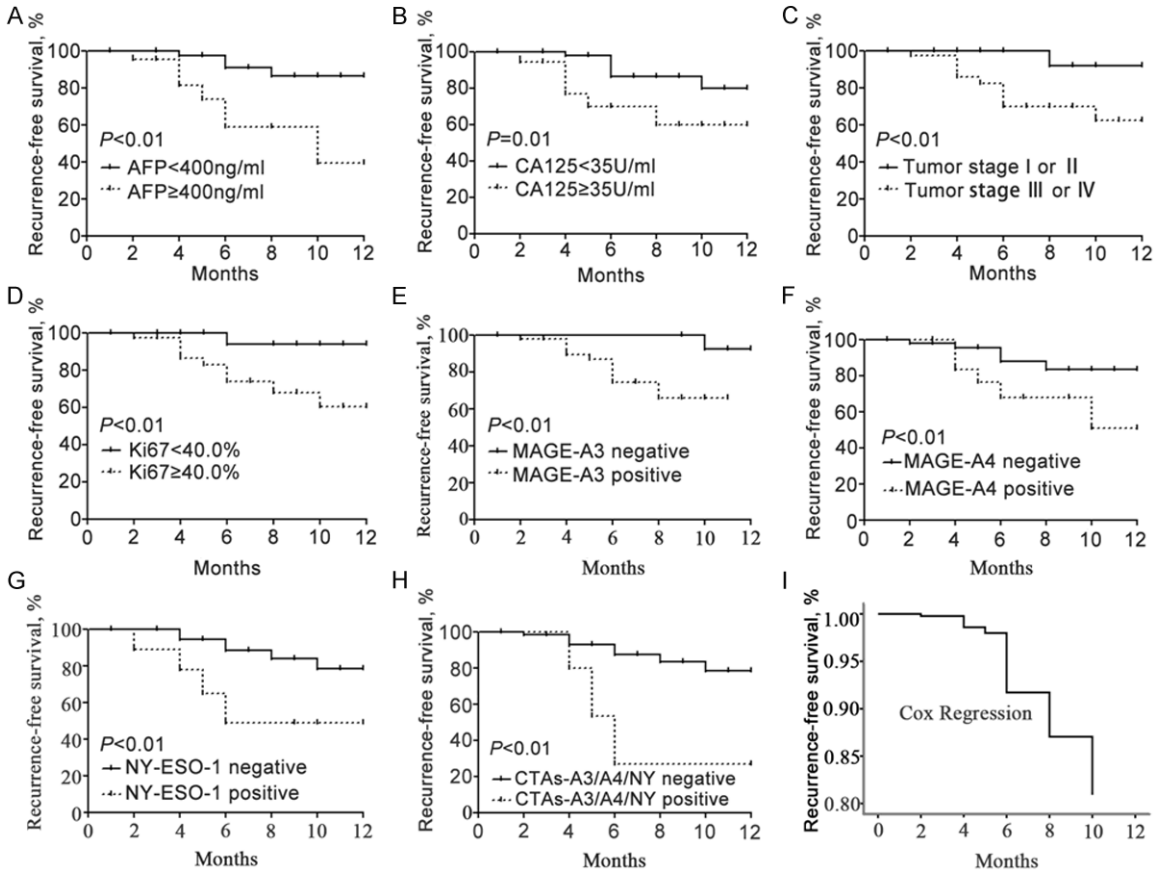


**Figure 6.** Predictive factors of HCC recurrence were analyzed by Kaplan-Meier model. Kaplan-Meier model was used to analyze possible predictors such as (A) serum AFP, (B) CA-125, (C) tumor stage, (D) Ki-67, (E) MAGE-A3, (F) MAGE-A4, (G) NY-ESO-1 and (H) CTAs-A3/A4/NY. Cox model was used to analyze total recurrence-free survival (I). All *P* values were two-sided. *P* value of less than 0.05 was considered as statistically significant.

**Table 6.** Analysis of possible predictors for HCC recurrence by multivariate Cox proportional hazard model

| Category | β | SE | Wald $\chi^2$ | *P* | RR (95% CI) |
|---|---|---|---|---|---|
| CTAs-A3/A4/NY | 2.83 | 0.84 | 11.47 | < 0.01 | 17.11 (3.31-88.48) |
| Tumor stage (III or IV) | 2.38 | 0.63 | 14.30 | < 0.01 | 10.81 (3.15-37.12) |
| AFP (≥ 400 ng/ml) | 1.90 | 0.47 | 16.03 | < 0.01 | 6.68 (2.64-16.94) |

SE: standard error; RR: relative risk; CI: confidence interval; CTAs-A3/A4/NY: combinatorial expression of MAGE-A3, MAGE-A4 and NY-ESO-1. Cox proportional hazard model were used to identify the predictive factors of HCC recurrence. All *P* values were two-sided. *P* value of less than 0.05 was considered as statistically significant.

enhance expressions of other types of CTAs [38]. In this study, we found that HCC cell lines including HepG2, Hep3B, Huh7 and SMMC-7721 simultaneously expressed MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1. Moreover, 73.2% HCC patients expressed two or more types of CTAs in same part of tumor tissues. Those findings increase the possibility of polyvalent vaccines for HCC immunotherapy and suggest the enhanced specificity of combined CTAs for HCC immunotherapy.

The correlations between CTAs expressions and tumor clinicopathological parameters are a hot topic of interest. Many reports have shown correlations between high expressions of MAGE genes and advanced tumor stages [11]. NY-ESO-1 has been found to be expressed in 40% of stage III bladder tumors, but none of stage I tumors [44]. Similarly, we found that stage III or IV tumors had more frequency of MAGE-A3, MAGE-A4, MAGE-C2 and CTAs-A3/A4/NY expressions than that of stage I or II tumors. Since the advanced tumor stages are closely related with the poor outcome of HCC [45], the expressions of CTAs will be useful candidate markers to predict the prognosis of HCC.

Serum AFP remains the most useful tumor marker in screening HCC patients since its discovery 50 years ago [46]. HCC patients with a high AFP concentration (≥ 400 ng/mL) tend to have greater tumor size, massive or diffuse types, portal vein thrombosis and a lower median survival rate [47]. The correlation of serum AFP with increased CTAs expressions was reported in HCC [48]. In our study, the expressions of MAGE-A3, MAGE-A4 and NY-ESO-1 are positively correlated with serum AFP ($P < 0.05$), and serum AFP (≥ 400 ng/mL) is an independent predictor of HCC recurrence ($P < 0.05$) by multivariate Cox model analysis. Thus, the expressions of MAGE-A3, MAGE-A4 and NY-ESO-1 in tumor tissues may be linked with recurrence of HCC and be as an evaluation tool of hepatectomy.

Previous studies found that high expressions of CTAs are correlated with reduced survival rate of patients with malignance [12]. A prognostic significance of MAGE-A3, MAGE-A4 and NY-ESO-1 expressions has also been reported in multiple myeloma, as well as lung, pancreatic and ovarian carcinoma [49, 50]. In this study, the expressions of MAGE-A3, MAGE-A4, NY-ESO-1 and CTAs-A3/A4/NY are associated with reduced recurrence-free survival rate of HCC patients by Kaplan-Meier model analysis. However, the results of multivariate Cox model analysis revealed that the expressions of MAGE-A3, MAGE-A4 or NY-ESO-1 are not independent risk factors of HCC recurrence. The relatively low PPV of one CTA as a predictive marker does not meet the requirements of clinical prediction for the recurrence of HCC. After comparing the PPV and the NPV of various combinations of CTAs, we screened the CTAs-A3/A4/NY which had the highest specificity, PPVs and Youden's index at the same time. The ROC model analysis showed that the AUC of the CTAs-A3/A4/NY (0.768) was the highest in all combinations of CTAs. The CTAs-A3/A4/NY significantly raised the predictive precision for the HCC recurrence. Furthermore, the expression of CTAs-A3/A4/NY was an independent predictive factor of HCC recurrence by Cox model analysis (RR = 17.11, $P < 0.01$). Those results indicated that optimal combination of CTAs could be as a promising biomarker to predict the prognosis of HCC patients after hepatic resection.

In conclusion, our results provided evidence that the mRNA expressions of CTAs in tumor tissues showed significant correlation with clinicopathological parameters such as serum AFP, tumor stages and Ki-67. As an independent predictor of HCC recurrence, the combinatorial expression of MAGE-A3, MAGE-A4 and NY-ESO-1 can promote the prediction accuracy of HCC recurrence and itself may be a potential target for immunotherapy of HCC.

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## Disclosure of conflict of interest

None.

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