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Original Article

Sperm protein 17, MAGE-C1 and NY-ESO-1 in hepatocellular carcinoma: expression frequency and their correlation with clinical parameters

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Abstract: This study is dedicated to investigate the expression patterns of sperm protein 17 (Sp17), melanoma-specific antigen (MAGE)-C1 and New York esophageal squamous cell carcinoma-1 (NY-ESO-1), to explore the correlation between these cancer-testis antigens and clinical parameters, and to evaluate their values in diagnosis and differentiation of hepatocellular carcinoma. Methods: Immunohistochemical staining was performed in 45 paraffin-embedded hepatocellular carcinoma specimens. 45 normal peripheral hepatic tissues collected from adjacent non-cancerous areas were used as controls. Results: Positive results of immunohistostaining were obtained in 16 (35.6%), 7 (15.6%) and 36 (80.0%) samples using MAGE-C1, NY-ESO-1 and Sp17 antibodies, respectively. The immunoreactivity of Sp17 was also found in 7 (14.0%) control samples. A statistical correlation between the frequency of Sp17 expression and tumor differentiation grade in hepatocellular carcinoma was confirmed. Conclusions: Sp17 is highly expressed in hepatocellular carcinoma cells. The frequency of Sp17 expression is closely related to the pathologic differentiation in hepatocellular carcinoma.

Keywords: Sp17, MAGE-C1, NY-ESO-1, cancer-testis antigens, hepatocellular carcinoma

Introduction

As a common malignancy representing the fifth most common cancer worldwide [1-4], hepatocellular carcinoma (HCC) becomes one of the major public problems with an increasing incidence all over the world [4, 5].

Despite the advances in cellular, molecular and pathologic knowledge, there is still limited understanding in the pathogenesis of hepatocellular carcinoma [6]. Also, there are still few options with definite clinical benefits for early diagnosis, accurate staging, immunotherapy and progress surveillance in hepatocellular carcinoma.

As the first biomarker of HCC described by Abelev in 1960s [7], alpha fetoprotein (AFP) has been used as a serum marker for HCC in humans for several decades. Despite a sensitivity of 39%–65%, a specificity of 76%–94% and a positive predictive value of 9%–50% [8], AFP is still disputed in many previous studies because of its high false-positive and high-negative rates, leading to a limited utility in differentiating benign hepatic disorders from HCC [9, 10].

Cancer-testis (CT) antigens which represent a novel category of biomarker in the field of oncology has been proposed, investigated and discussed in recent several years. CT antigens are confirmed to be primarily expressed in male germ cells but not in adult somatic tissues [11]. Interestingly, many reports also declared a high frequency of CT antigens expression in various human tumor tissues, such as ovarian cancer [12], endometrial and cervical cancer [13], esophageal cancer [14] and breast cancer [15].

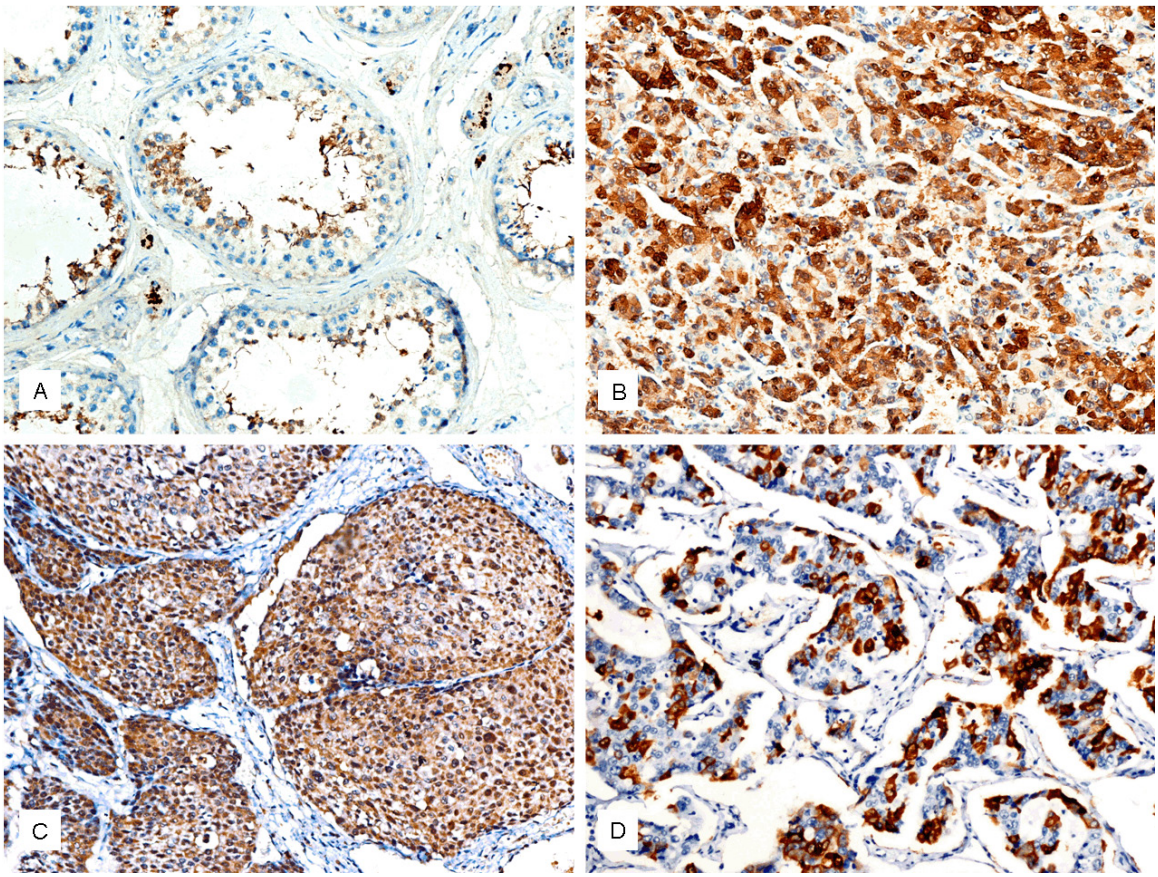

Figure 1. A: Immunohistochemical distribution of our Sp17 antibody in normal testis from a patient with prostatic cancer and in hepatocellular cancer. Sp17 is predominantly expressed in spermatozoa. Hepatocellular cancer tissue display cytoplasmic staining for Sp17(B), MAGE-C1 (C), and NY-ESO-1 (D). original magnification ×100 (B, C), ×200 (A, D).

Because of its restricted expression pattern, CT antigens are widely explored as promising targets for tumor diagnosis, differentiation and immunotherapy.

As a member of CT antigen family, Sp17 is a highly conserved mammalian protein in the testis and spermatozoa of humans and animals [16-18]. The expression of Sp17 in malignant cells was first discovered in 1997 [19], followed by various studies confirming the aberrant expression of Sp17 in various cancers, including multiple myeloma [20], ovarian cancer [21] and nervous system tumors [22]. Simultaneously, Sp17 is believed correlates with chemosensitivity [12, 23] and tumor metastasis [12, 24], making it as an attractive molecule for diagnosis, treatment and surveillance in associated cancers.

Until now, a number of CT antigens, including SSX-1 [25], SSX-2 [26], MAGE-A3 [27], MAGE-C1 and NY-ESO-1 [28-30] have been demonstrated expressed a prolific and specific profile in HCC, providing a possibility of early detection, antigen-specific immunotherapy and polyvalent vaccination. However, to our knowledge, Sp17 has not been studies and compared with other CT antigens in HCC in the literature. Therefore, current study is designed to investigate and compare the expression patterns of Sp17, MAGE-C1 and NY-ESO-1, to explore the possible correlation between these CT antigens and clinical parameters, and to evaluate their values in diagnosis and differentiation of HCC.

Materials and methods

Patient specimens

A total of 45 specimens of HCC were retrieved from the archival resource of the Department of Pathology, Jinling Hospital, from 2007 to 2011. The diagnosis of HCC was based on the

Table 1. Expression of CT antigens and clinical parameters

	n	MAGE-C1		NY-ESO-1		Sp17		CTA	
		+	-	+	-	+	-	+*	-
Total number	45	16	29	7	38	36	9	38	7
Gender									
Male	36	13	23	5	31	28	8	30	6
Female	9	3	6	2	7	8	1	8	1
Tumor Diameter									
≤5 cm	20	8	12	2	18	16	4	17	3
>5 cm	25	8	17	5	20	20	5	21	4
TNM Stage									
I or II	20	7	13	3	17	17	3	17	3
III or IV	25	9	16	4	21	19	6	21	4
HBV Infection									
HBV (+)	29	5	24	5	24	22	7	24	5
HBV (-)	16	11	5	2	14	14	2	14	2
Differentiation									
Low	15	7	8	4	11	15	0	15	0
Moderate	23	6	17	2	21	20	3	20	3
High	7	3	4	1	6	1	6	3	4

*At least one of the three CT antigens was positive (+).

pathological examination. 45 control samples were collected from the adjacent non-cancerous areas (>5 cm from the tumor). The absence of pathologic cells or tissues in all control samples were subsequently confirmed by two experienced pathologists (more than 10-years clinical experience) under optical microscopes.

Clinical data of all enrolled patients, including age, gender and detection of hepatitis B surface antigen (HBsAg), were obtained from the Clinical Electronic System in Jinling Hospital. Tumor-related data, including tumor diameter, histological grade and vascular invasion were collected from pathological records. The TNM stage was determined according to the criteria issued by the International Union against Cancer [31].

Monoclonal antibodies of cancer-testis antigens

Commercial MAGE-C1 mAb (Clone, No. CT7-33) and NY-ESO-1 mAb (Clone, No. E978) [32, 33] were purchased for immunohistochemical staining. Recombinant Sp17 and its mAb were produced according to the protocols described in our previous studies [13, 34]. Briefly, the nucleotide sequence encoding Sp17 was amplified from human testicular RNA and cloned in pET-28a (+) (Novagen) containing an N-terminal 6-histidine fusion tag. Proteins were expressed in Escherichia coli BL21 (DE3). After lysis of the bacteria Sp17 was purified via nickel affinity chromatography. The recombinant Sp17 protein was used to immunize BALB/c mice for generating monoclonal antibody-producing hybridomas. The hybridoma supernatants were screened by ELISA for the presence of antibodies and the hybridoma cell lines producing antibodies against the recombinant Sp17 protein were cloned. The specificity of these antibodies was then confirmed by immunohistochemistry of natural Sp17 from the human testis and ejaculated spermatozoa [13] (**Figure 1A**). Monoclonal antibodies were purified from hybridoma ascites using a HiTrap Protein G HP affinity column (Amersham Biosciences) [13].

Immunohistochemistry

For Sp17 antibody staining, tissue sections (3 μm) were placed on glass slides, heated, (60°C, 20 min), and deparaffinized with xylene and ethanol. For antigen retrieval, tumor specimens mounted on glass slides were immersed in preheated antigen retrieval solution (DAKO high pH solution; DAKO) for 20 min and cooled for another 20 min at room temperature. After the inactivation of endogenous peroxidase, 2 μg/ml Sp17 mAb were added and incubated overnight at 4°C. The primary antibody was detected with HRP-anti-mouse IgG (DAKO). Diaminobenzidine (DAB) substrate was added for 7 min followed by washing with deionized water, and hematoxylin was applied for 1 min to counterstain the tissue sections. The tissue sections were dehydrated with graded ethanol followed by xylene and the coverslips were attached with Permount. The immunohistochemical reaction was evaluated by the two experienced pathologists under the light microscope at 40× and 100× objective magnifications.

For MAGE-C1 (CT7-33, Santa Cruz Biotechnology, Heidelberg, Germany) and NY-ESO-1 (E978, Santa Cruz Biotechnology, Heidelberg, Germany), similar immunohistochemical staining processes were performed as described above. Final concentrations (2 μg/ml for MAGE-C1, 1 μg/ml for NY-ESO-1) of mAbs were determined by the manufacturer's instructions.

Statistical analysis

All statistical tests were performed with SPSS software (SPSS for Windows, version 13.0, SPSS, Chicago, IL). All analysis were two-tailed and differences were considered statistically significant when *P*-values <0.05. Continuous variables were described as mean and SEM (standard error of mean). The correlation between CT antigens and clinical parameters was evaluated by chi-squared test (Fisher's exact test).

Ethical considerations

This study was approved by the Ethics Committee of Jinling Hospital.

Results

Immunohistostaining of CT antigen proteins in HCC specimens

Totally, 45 archival hepatocellular carcinoma specimens were investigated by immunohistochemical staining with MAGE-C1, NY-ESO-1 and Sp17 mAbs. Positive results were obtained in 16 (35.6%), 7 (15.6%) and 36 (80.0%) samples using the three antibodies, respectively (**Figure 1B-D**). **Table 1** demonstrated the distribution of each CT antigen and each clinical parameter in all enrolled subjects.

For MAGE-C1 and NY-ESO-1, the expression of protein was not found in the adjacent non-cancerous areas in all specimens. We also observed that MAGE-C1 and NY-ESO-1 were mainly localized in cytoplasm of tumor cells, with only one sample displaying immunoreactivity of MAGE-C1 in nuclear of HCC cells.

For Sp17, the high frequency of positive staining (80.0%) in HCC cells was quite inspiring. Nevertheless, the expression pattern seemed heterogeneous. While Sp17 protein was primarily appeared in cytoplasm of tumor cells, we detected that it was also distributed in cytoplasm of adjacent non-cancerous cells in 7 (14.0%) control samples.

Highly frequent co-expression of multiple members of CT antigens was observed in HCC samples. The proportion of synchronous expression of at least one, two or three proteins was 84.4% (38/45), 42.2% (19/45) and 4.4% (2/45), respectively. We also analyzed the 9 specimens with negative Sp17 staining results to inspect targets for immunotherapy other than Sp17. Among them, 7 cases displayed negative expression of both MAGE-C1 and NY-ESO-1, and the remaining two cases displayed MAGE-C1 without NY-ESO-1 expression.

Correlation between CT antigens and clinical parameters

The relationships between CT antigen expression and clinical variables, including gender, tumor diameter, TNM stage, HBV infection and pathologic differentiation were investigated in current study.

Among the 45 HCC samples, statistical analysis revealed a positive correlation between MAGE-C1 expression and non-HBV infection (p=0.001), indicating that the existence of MAGE-C1 protein suggested a possible negative HBV infection.

Notably, the positive correlation between Sp17 expression and HCC differentiation might be more significant (p=0.0009). **Table 1** illustrated that the frequency of Sp17 expression in low-differentiated HCC was 100% (15/15), while the frequency in moderate and high-differentiated HCC were 86.9% (20/23) and 14.3% (1/7), respectively.

Discussion

In current study, we demonstrated the positive rates of immunohistostaining of three CT antigens, MAGE-C1, NY-ESO-1 and Sp17 in hepatocellular carcinoma cells and adjacent non-cancerous areas. We also analyzed the relationships between CT antigen expression and several clinical parameters.

We found that all the three CT antigen proteins were mainly localized in the cytoplasm of tumor cells, despite the immunoreactivity of MAGE-C1 in nuclear membrane in only one specimen.

The frequency of Sp17 expression (80.0%) in HCC cells was highest compared with MAGE-C1 (35.6%) and NY-ESO-1 (15.6%). The present study demonstrated the presence of Sp17 at protein level in a high proportion of HCC. The result of our study displayed a probable immunologic superiority of Sp17 in HCC, promising it as a valuable marker for detection and differentiation of HCC.

Notably, the positive immunohistostaining result of Sp17 in adjacent non-cancerous tissues (14.0%) revealed a heterogeneous expression profile of Sp17 in HCC. Interestingly, previous studies also generated controversies on the normal tissue expression of Sp17. Wen et al. [35] reported that Sp17 was present in a panel of mouse somatic tissues, such as ovary, lung, skeletal muscle, liver and kidney. Lacy et al. [36] further confirmed the Sp17 expression in normal and malignant lymphocytes. However, extensive studies by Zhang et al. [37] clearly verified a very low level of Sp17 expression in normal somatic tissues, including liver. Nevertheless, our previous work [13] discovered that the distribution of Sp17 in gynecological epithelial cancers was positive but with different intensity and density among adenous, adenosquamous, and squamous cancers. It was even positive in a few hyperplastic glands in normal peripheral tissues. In brief, further studies are expected to investigate the expression pattern of Sp17 in normal hepatic tissues.

For MAGE-C1 and NY-ESO-1, their expression profiles in HCC in our study were in accordance with previous findings. Peng et al. [27] reported an expression frequency of 48.0% and 42.5% for MAGE-C1 and NY-ESO-1 in HCC, respectively. Zhang et al. [38] found that NY-ESO-1 was expressed in 13.8% cases of HCC, and expressed mainly in the cytoplasm of tumor cells. Neither of them detected CT antigen mRNA expression in adjacent tissues.

In present study, we discovered a close correlation ($p=0.0009$) between Sp17 expression frequency and pathologic differentiation grades in HCC. There was an increased expression of Sp17 from 14.3%, 1/7 in high-differentiated HCC, 86.9%, 20/23 in moderate-differentiated HCC and 100%, 15/15 in low-differentiated HCC. This finding may suggest a pivotal role of Sp17 in the tumor progression. Similarly, previous studies [12, 24] proposed a possible role of Sp17 in mediating cell adhesion and interaction, therefore involving in the migration of malignant cells. Our results, together with previous findings, confirmed that Sp17 was a suitable biomarker to monitor tumor progression in HCC.

We are aware of limitations in our study. First, the information of clinical treatment and follow-up results were unavailable in this retrospective study. Therefore, it is unable to investigate the utility of Sp17 in predicting treatment response and prognosis. Second, the expression of CT antigens in HCC at the level of gene expression was not measured. However, the frequency of mRNA expression do not necessarily directly correlate with protein expression frequencies, and discrepancies between gene and protein expression frequencies may reflect variations in tissue sampling, tumor heterogeneity or different level of sensitivity of detection.

To summarize, our study discovered a remarkable immunologic superiority of Sp17 in HCC, displayed the immunoreactivity pattern of each selected CT antigen, and confirmed an intense correlation between Sp17 expression frequency and pathologic differentiation grade in HCC.

Acknowledgements

Authors' contributions: QYX conceived and designed the study and drafted the manuscript. SL analysis the data and was involved in drafting the article. XJZ and FQL coordinated the study and contributed to the acquisition. WBH and LNS selected the archived samples and performed the experiment. XJZ and QYX also scored the immunohistochemistry staining. All the authors have read and approved the final manuscript.

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

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